Article

Self-assembly Synthesis of Molecularly Imprinted Polymers for the Ultrasensitive Electrochemical Determination of Testosterone

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Abstract: Molecularly imprinted polymers (MIPs) can often bind target molecules with high selectivity and specificity. When used as MIPs, conductive polymers may have unique binding capabilities; they often contain aromatic rings, which have a great tendency to undergo covalent and hydrogen bonding interactions with similarly structured target (or template) molecules. In this work, an electrochemical method was used to optimize the synthetic self-assembly of poly(aniline-*co*-metanilic acid) and testosterone, forming testosterone-imprinted polymers (TIPs) on sensing electrodes. The linear sensing range for testosterone ranged from 0.1 to 100 pg/mL, and the limit of detection was as low as ~pM. Random urine samples were collected and diluted 1000 fold to measure testosterone concentration using the above TIP sensors in comparison with a commercial ARCHITECT ci 8200 system. The testosterone concentrations in the tested samples were in the range of 0.33± 0.09 to 9.13±1.33 ng/mL. The mean accuracy of the TIP-coated sensors was 90.3 ±7.0 %.

Keywords: testosterone; molecular imprinting; electronically conductive polymer; electrochemical sensing; urine.

1. Introduction

For men beyond the age of 30, testosterone levels gradually decline with increasing age [1]. Some possible causes of low testosterone levels are testicular injury or infection [2], dysfunctional hormone excretion, medication, inflammation and chronic illness (such as chronic kidney failure [3], dysthymic disorder [4], alcoholism, hepatic cirrhosis [5] or obesity [6]). A homecare system, monitoring testosterone concentration, may offer important diagnostic benefits.

Molecularly imprinted polymers (MIPs) for measuring the concentration of testosterone have been produced in the last decade and used for *optical sensing* by surface plasmon resonance (SPR) [7-10]. Laboratory-based methods such as gas chromatography (GC) [11,12], liquid chromatography (LC) [13-16] or capillary electrophoresis (EC) linked with mass spectrometry (MS) [17-19] or diode-array detection (DAD) [20,21] of the testosterone in human urine [11,17,20-23] or in goat milk [24] have also been reported. The functional and crosslinking monomers that are utilized in these preparations include acrylamide[15], methacrylic acid (MAA) [7-11,13,16,17,20,21,24-28], trifluoromethacrylic acid (TFMAA) [13], 2-hydroxyethyl methacrylate (HEMA) [9,26], ethylvinylbenzene (EVB) [7], 2-vinylpyridine (2VP) [12], 4-vinylpyridine (4VP) [12,20,26], dopamine

(DA) [14]; divinylbenzene (DVB) [7,13], ethylene glycol dimethacrylate (EGDMA) [8-11,15,17,20-24,26-28], trimethylolpropanetrimethacrylate (TRIM) [11,20,21], pentaerythritol triacrylate (PETRA) [22] and 5⍵-androstane-3⍵, 17⍵-dimethacryloxy ester (AnDMA) [13]. Synthetic functional monomers such as (1) 1-(4-vinylphenyl)-3-(3,5-bis(trifluromethyl)phenyl)urea (FM1) and 1-benzyl-3-vinyl- 2,3-dihydro-1H-imidazolium bromide (FM2) have been used to isolate testosterone glucuronide [22] and (2) the bifunctional cross-linker N,O-bismethacryloylethanolamine (NOBE) have been synthesized to form patterned MIP structures to detect testosterone in buffer, urine and saliva using electrochemical impedance spectroscopy (EIS) [29]. A visible light-activated photo-iniferter agent, 4-cyano-4-[(dodecylsulfanyl- thiocarbonyl) sulfanyl]pentanoic acid (CDTPA), was employed for chain extension with poly(ethylene glycol methacrylate phosphate) brushes by reversible addition–fragmentation chain transfer (RAFT) polymerization [30]. In addition, the imprinting of other steroid hormones (e.g. 17⍵-estradiol) has been used for increasing the retention of testosterone in solid phase extraction [18]. A recent comparison of methods for testosterone determination reported limits of detection (LODs) in the range of 0.08 -20.0 ng/mL [14]. The majority of techniques employed either SPR or chromatographic techniques; electrochemical methods, despite their advantages of low cost and flexibility, have not been reported for testosterone.

The use of conducting polymers in sensor-related technology [31] and electrochemically prepared MIPs have been reviewed [32,33]. Polyaniline (PANI) derivatives can be electrochemically polymerized [34,35], chemically polymerized [36], and even simultaneously self-assembled/polymerized [37,38] in aqueous solutions. PANI derivatives have attracted substantial scientific interest in recent decades owing to their favorable combination of characteristics, including a more diverse structure and better thermal and radiation stability than polypyrrole; lower cost than polythiophene; ease of synthesis; and moderately high conductivity. They have, therefore, been used in a wide range of applications [39], such as micro-electronics, corrosion protection, battery electrodes, and sensors [40].

In this work, an electrochemical method was employed to optimize the synthetic self-assembly of poly(aniline-*co*-metanilic acid) and template molecules by coating on the sensing electrodes, to form testosterone-imprinted polymers (TIPs). The TIPs were characterized by their imprinting effectiveness (⍵), which is the ratio of current densities generated in the sensing of template molecules by imprinted and non-imprinted polymer-coated electrodes, respectively. The surface morphologies and electronic spectra of the TIPs during self-assembly were obtained using a scanning electron microscope (SEM). Finally, random urine samples were collected, and their testosterone concentrations were measured using TIP sensors. The experimental results were compared with results from a commercial ARCHITECT ci 8200 system to confirm the reliability.

2. Materials and Methods

2.1 Reagents

Aniline (ANI, Merck) was distilled under reduced pressure, and metanilic acid or *m*-aminobenzenesulfonic acid (MSAN, Acros) was purified by recrystallization twice from deionized (DI) water. Testosterone (≧ 98.0%), progesterone (≧99.0%), urea (minimum ≧ 98.0%), creatinine (minimum ≧99.0%), and ethanol were purchased from Sigma-Aldrich Co. (St. Louis, MO). 17⍵-Estradiol (≧ 98.0%) was from Alfa-Aesar (Ward Hill, MA). Ammonium peroxydisulfate (APS) used as the initiator was from Wako. The ITO-coated glass substrates (~10 Ωcm^{-2}) were from Merck. Deionized water (18.2 MΩ), produced by a PURELAB Ultra (ELGA, Albania), was used in the preparation of buffers and for rinse solutions. All chemicals were used as received unless otherwise mentioned.

2.*2 Synthesis and Characterization of Testosterone-imprinted Polymer (TIP) films*

Aniline (ANI) and *m*-aminobenzenesulfonic acid (MSAN) in mole ratios from 0.25- 4.0 were dissolved in DI water, keeping the total amino group concentration at 57 mM. Testosterone, employed as the template and target molecule in this study, up to 100 ⍵g/mL and initiator (APS, 0.5 %(w/w)) were then added to the ANI/MSAN mixture. The TIPs were assembled on ITO glass (Merck, 1 x 1 cm^2) by immersion in the monomer mixture at 25°C. The self-assembled films were prepared

with APS as an oxidant performing copolymerization from a mixture of ANI and MSAN aqueous solution.[41] Finally, ethanol solution (5 % v/v) was employed for the removal of target molecules.

The TIP-coated electrode, counter electrode (Pt wire), and Ag/AgCl reference electrode were placed in a mixture solution including 20μL sample(e.g.,testosterone, urea, creatinine, 17◎-estradiol and progesterone) and 20 mL 125 mM KCl, 5mM $K_4Fe(CN)_6$ and 5 mM $K_3Fe(CN)_6$ solution ; the cyclic voltammetry of the electrochemical reactions was assessed by a potentiostat (608-1A, CH Instruments, Inc., Austin, TX). The potential was scanned from -0.3 V to 0.8 V at 0.1 V/s [42,43] and the effects of target and interferent molecules (e.g., urea, creatinine, 17◎-estradiol and progesterone) on the peak currents for the ferri-/ ferrocyanide system were also recorded. TIP films were freeze-dried before examination by a scanning electron microscope (Hitachi S4800, Hitachi High-Technologies Co., Tokyo, Japan), and atomic force microscopy (Solver P47H-PRO, NT-MDT Moscow, Russia) and a golden silicon cantilever (NSG01, NT-MDT).

2.3 The determination of testosterone in human urine samples

Urine samples were collected from colleagues of the authors 4 hours before the test, and diluted 1000-fold with 125 mM KCl, 5mM $K_4Fe(CN)_6$ and 5 mM $K_3Fe(CN)_6$ solution. The urine sample (1 mL) was stored in an Eppendorf microcentrifuge tube at 4 °C and analyzed for testosterone with the ARCHITECT *ci* 8200 system (Abbott Laboratories. Abbott Park, Illinois, U.S.A.). The Abbott Architect ci8200 analyzer was specifically designed to provide clinical chemistry and immunoassay testing, which combines immunoassay and clinical chemistry on one inte-grated platform and runs up to 200 immunoassay tests andup to 1200 clinical chemistry tests an hour. (https://www.corelaboratory.abbott/int/en/offerings/brands/architect/architect-ci8200)

3. Results and Discussion

To assess the performance of TIPs, current density for the ferri-ferrocyanide redox couple was measured in the presence and absence of 10 pg/mL testosterone. The current density difference (with and without testosterone) is plotted in Figure 1, for both imprinted TIPs and non-imprinted polymer-coated electrodes, as a function of composition. As the mole ratio of ANI to MSAN was varied, the largest differences between the testosterone responses for MIPs and NIPs were obtained at 50 mole % aniline, a 1:1 composition. For this composition, the current density differences were 60.70 ± 5.37 and 19.00 ± 3.00 ◎A/cm^2 for TIPs and NIPs, respectively. This corresponds to an imprinting effectiveness of slightly over 3. In the ANI/MSAN copolymers, the sulfonic group is likely to bind to the secondary amine of aniline, and thus to be unavailable to bind testosterone. The effect should be most pronounced at equimolar ratios, and, in agreement with this expectation, the response of non-imprinted polymers to testosterone is minimized at equimolar composition. Interestingly, the response of the imprinted polymers *increases* at equimolar composition, in spite of the potential for reduced hydrogen bonding to the target. This may be caused by increased stiffness of the matrix, improving the binding site shape recognition, and the electrochemical reaction may occur only on the electrode surface. (In addition, of course, hydrogen bonding *in the binding sites* may still occur, as the hydrogen-bonded template will effectively sequester the sulfonic acid and prevent it from binding to ANI.) Figure 1(b) shows that current density differences grow with increased polymerization time. Nonetheless, imprinting effectiveness varies only weakly with polymerization time.

Figure 2 displays scanning electron microscope images of MIPs and NIPs before and after template removal, as well as after they have bonded with target molecules. Many poly(ANI-*co*-MSAN) particles and aggregates were observed on the surface of the sensing electrodes. The sizes of the aggregates were about 60-150 and 30-60 nm on the surface of MIPs and NIPs, respectively. SEM images of MIP and NIP electrodes rinsed in 5% ethanol, shown in Figs. 2(c) and 2(d), reveal that the unbound poly(ANI-*co*-MSAN)s particles on the surfaces had been cleared out. Based on the SEM images, the surface morphologies of the MIP and NIP thin films following readsorption of 10 pg/mL of testosterone are less rough after readsorption (compare Figs 2(e) and 2(f) to 2(c) and 2(d), respectively), when the cavities on the poly(ANI-*co*-MSAN) particle surface were refilled with the

target molecules. The washed, imprinted films show considerable surface roughness, presumably owing to the presence of numerous binding sites or cavities. Further details of the surface morphology of TIPs, determined by AFM, are shown in Fig. 3; the size of polymer particles agreed with SEM images. The average surface roughness increased from 3.3 nm to 4.8 nm when template was removed, and then decreased to 3.4 nm upon rebinding of the target molecules.

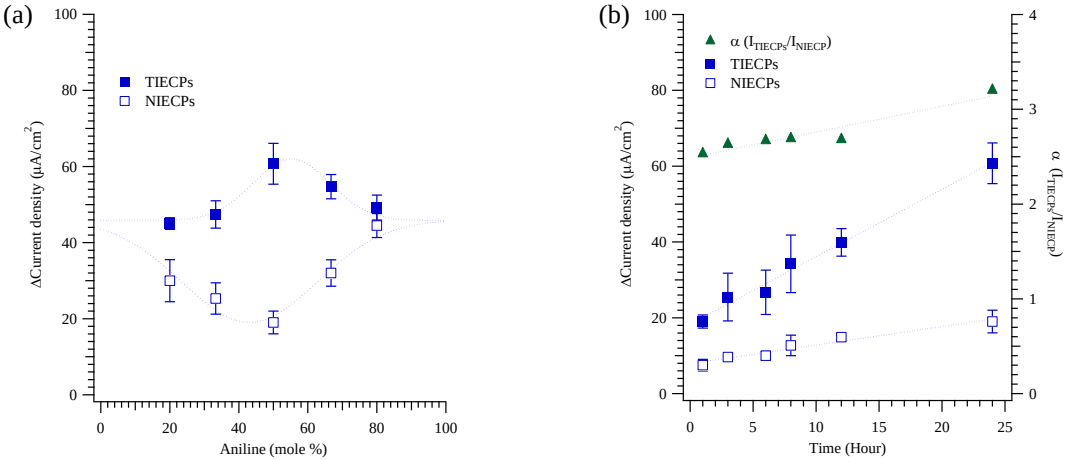
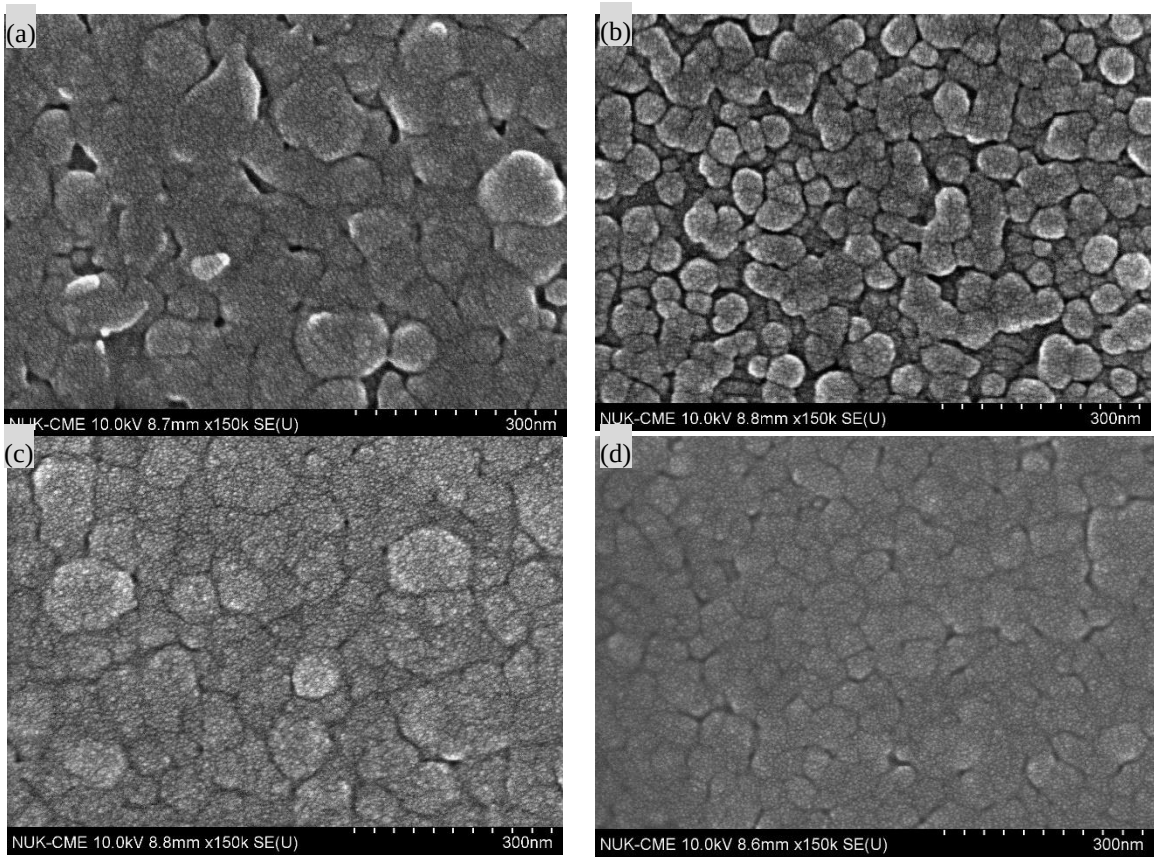


Figure 1. (a) Aniline concentration and current density from cyclic voltammograms (CVs) in the solution of 20 mM potassium ferricyanide ($K_3[Fe(CN)_6]$), 20 mM potassium ferrocyanide ($K_4[Fe(CN)_6]$), and 0.5 M KCl with/without 10 pg mL^{-1} of testosterone on testosterone- and non-imprinted poly(aniline-*co*-metanilic acid)-coated electrodes , **(b)** imprinting effectiveness and current density versus polymerization duration of testosterone-imprinted poly(aniline-*co*-metanilic acid).



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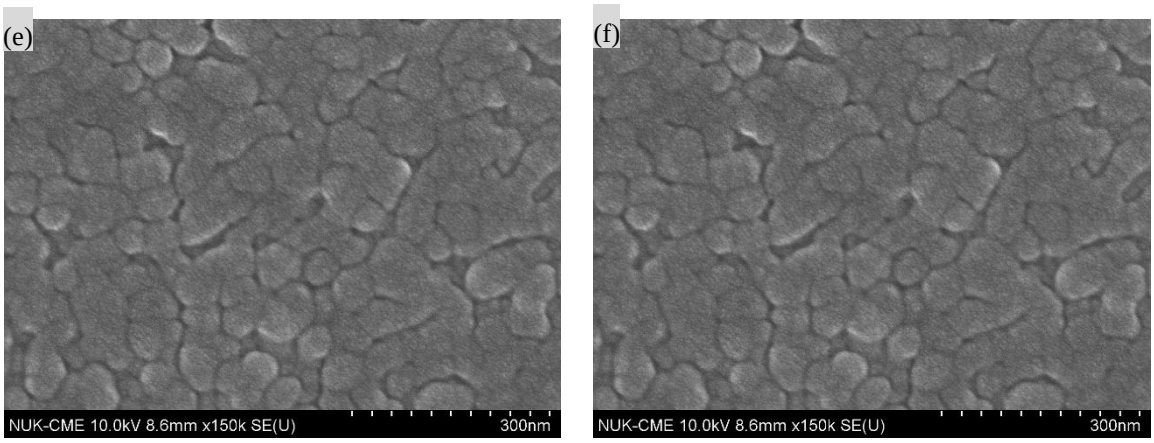

Figure 2. Scanning electronic microscopy images of testosterone-imprinted (left) and non-imprinted (right) poly(aniline-co-metanilic acid) containing 50 mole % of aniline: **(a), (c)**: before washing; **(b)** and **(d)**: after washing; **(e), (f):** after washing (template removal). The washed, imprinted films show considerable surface roughness, presumably owing to the presence of numerous binding sites or cavities.

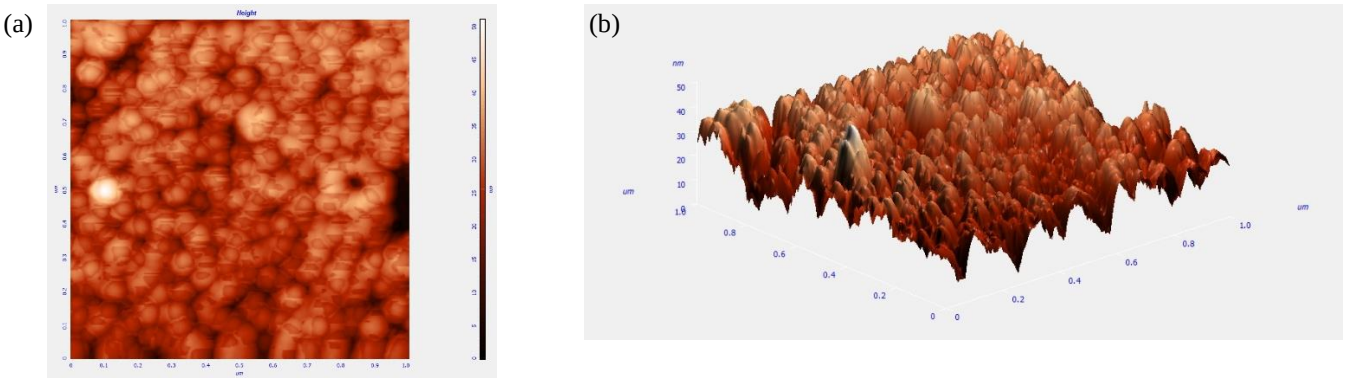

Figure 3. AFM images of a TIPs-coated electrode after template removal. **(a)** Height shown as intensity; **(b)** 3D representation (height exaggerated relative to horizontal scale.)

Cyclic voltammetric sensing of testosterone at concentrations of 0.01 to 5000 pg/mL was conducted using the MIP and NIP-coated electrodes, as presented in Figs. 4(a) and 4(b). The pH value was fixed at different concnetraion of testosterone, because the volume raio of testosterone to buffer kept 1000. The maximum current densities of MIPs and NIPs electrodes were around 1200 and 1000 ⍵A/cm², respectively. The electrochemical current densities were approximately 30-fold higher than our earlier MIPs based on imprinted poly(ethylene-*co*-vinyl alcohol) (EVAL). (EVAL has been successfully used to measure small molecules, such as creatinine and urea [42]).

Figure 5(a) plots the difference between a target-containing solution and a buffer solution. The sensitivity concentration with linear dynamic range is 0.1 to 100 pg/mL for testosterone. Moreover, micro-patterned MIPs on functionalized diamond-coated substrates for testosterone was reported with linearity between a 0.5 to 20 nM [44], and the reflectance spectra of MIPs films showed a shift of the Bragg diffraction peak that correlated with testosterone concentration was in the 5–100 ng/mL[16]. The eletrodes of TIPs were *highly sensitive.* It is possible to interfer the binding of testosterone and TIPs using real sample (urine). There interferent biomolecules (17β-estradiol, progesterone, urea, and creatinine) in urine should be tested to the selectivity of EIPs. In Fig. 5(b), the current densities for interferents (including 17⍵-estradiol, progesterone, urea, and creatinine) were less than 20 ⍵A/cm², close to the background current density. Thus, the selectivity of MIPs that were prepared by polymerization in this study exceeded that achieved in our previous work based on phase inversion [32]; for example, the 5-fold preference for testosterone over other interferents (in this work), vs. about a 2-fold preference for urea over creatinine in [32].

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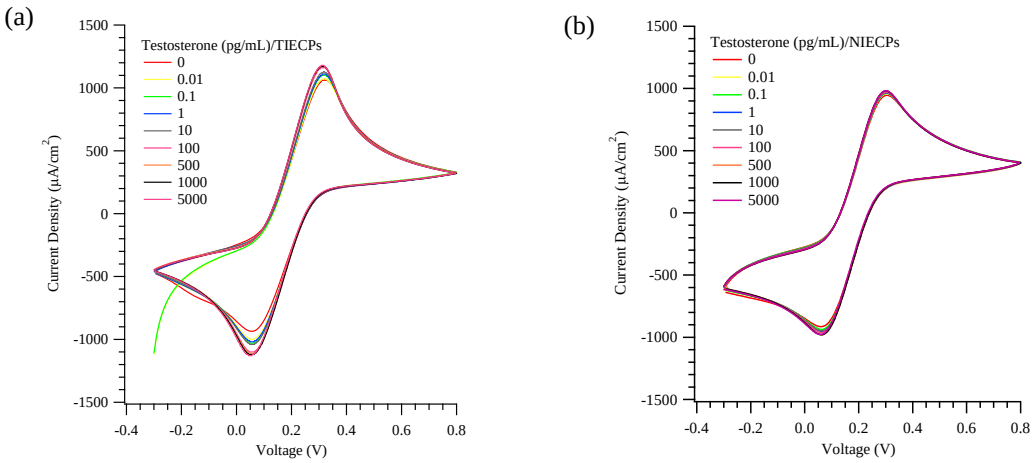

Figure 4. Cyclic voltammograms of various target concentrations on **(a) t**estosterone- and **(b)** non-imprinted poly(aniline-co-metanilic acid) coated electrodes.

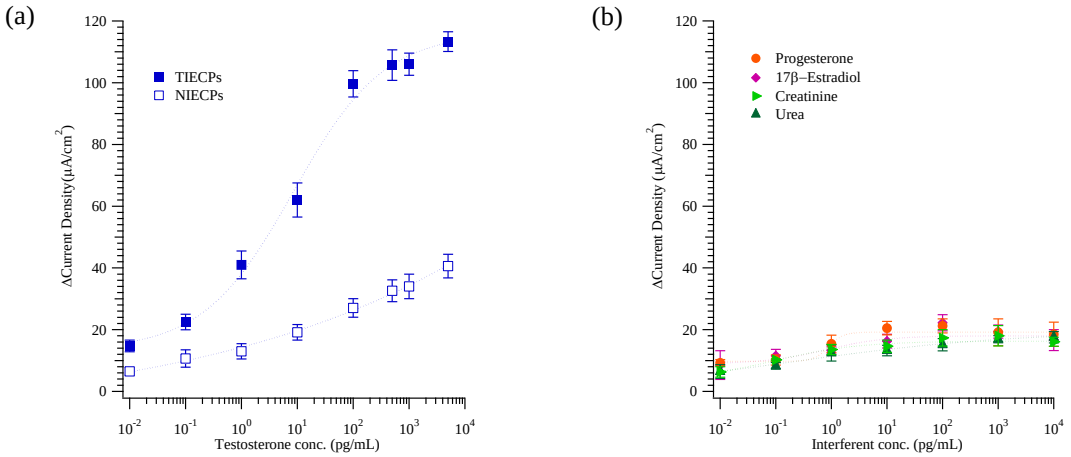

Figure 5. (a) The calibration curve of oxidation-peak current density from CVs against testosterone concentrations on molecularly and non-imprinted poly(aniline-*co*-metanilic acid) based sensors. (b) The effect of the interferents (e.g., 17◎-estradiol (diamonds), progesterone (circles), urea (triangles), and creatinine (right triangles)) on peak current response was tested and is shown (n>3).

Finally, Table 1 summarizes analyses of random urine samples performed by using the ARCHITECT *ci* 8200 system. The concentrations of testosterone in the samples fell in the range of 0.33 ± 0.09 to 9.13 ± 1.33 ng/mL. The current deviations measured by the MIP sensor in at least three urine samples ranged from 27.35± 1.15 to 65.15± 2.95 ◎A/cm² corresponding to concentrations of 0.28 ± 0.07 to 8.99 ± 2.68 ng/mL (standard deviations of at least three individual measurements). The mean accuracy of TIPs-coated sensors was 90.3 ± 7.0 %. Note that the accuracy was slightly lower, approximately 80-85%, when the testosterone concentration in urine was less than about 2.0 ng/mL.

Table 1. Comparison of real sample measurement by ARCHITECT ci 8200 system and the TIP sensors.

Sample No.	ARCHITECT *ci* 8200 system Testosterone (ng/mL)	TIP sensors		Accuracy (%)
		ΔCurrent (μA/cm²)	Avg. conc. (ng/mL)	
1	0.79±0.02	33.85± 0.25	0.64±0.03	81.0
2	1.51±0.08	40.65± 0.75	1.28±0.13	84.8
3	2.32±0.01	47.10± 1.90	2.27±0.50	97.8
4	0.33±0.09	27.35± 1.15	0.28±0.07	84.8
5	3.04±0.18	50.05± 1.95	2.88±0.62	94.7
6	9.13±1.33	65.15± 2.95	8.99±2.68	98.5

4. Conclusions

Monomers bearing an aromatic ring may have a greater tendency than other aliphatic structures to exhibit hydrogen bonding interactions with target (or template) molecules[32], making them attractive for use in molecularly imprinted polymer applications. Our experiments showed these materials are especially suitable for the preparation of molecularly imprinted polymers for steroid hormones, by demonstrating the specific recognition of testosterone by imprinted ANI/MSAN copolymers. This work also demonstrated the importance of monomer ratio in creating films with specific and selective recognition, and detailed the distinctive surface morphologies of both imprinted and non-imprinted films for effective and accurate in electrochemical detection of testosterone in urine.

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