

Electrochemical Determination of Nandrolone as a Doping Agent in Sport by Molecularly Imprinted Polymers and Au Nanoparticles Hybrid Nanostructured Electrode in Biological Fluids

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The goal of this study was to develop a stable and broad-range electrochemical sensor for detecting nandrolone (19-nortestosterone (19-NT)) as a doping agent in clinical samples by combining molecularly imprinted polymers and Au nanoparticles hybrid film modified glassy carbon electrode (MIP/Au/GCE) using electrodeposition and electropolymerization techniques. According to crystallographic and morphological characterizations, electrodeposited Au nanoparticles were successfully covered by electropolymerized MIPs film. The MIP/Au/GCE demonstrated significant catalytic activity, stability, and selectivity for 19-NT sensing, according to electrochemical evaluation utilizing DPV and methods. The electrochemical electrocatalytic activity of MIP/Au/GCE was found to be favorable and even better than that of other reported 19-NT electrochemical sensors in the literature, with good sensitivity (0.5209 $\mu\text{A}/\mu\text{M}$), detection limit (3 nM), and linear range (5 to 95 μM) values. The accuracy and practical application of a newly developed amperometric sensing method for determining 19-NT in urine samples from young athletes aged 22 to 28 who were given Nandrodek subcutaneously were investigated, and the results showed that the findings of the amperometry and ELISA assays agreed fairly well. Furthermore, the RSD values obtained (3.09% to 4.38%) revealed that the proposed amperometric approach had excellent detection precision and validity for determining 19-NT in clinical samples and human bodily fluids.

Keywords: Graphene oxide; GO@Si nanocomposite; Lithium Ion Batteries; Capacity retention; Cycling stability

1. INTRODUCTION

Anabolic steroids are synthetic or human-made variants of the male sex hormone testosterone that can be taken as performance-enhancing medications to increase muscle growth and reduce fat

while also generating a variety of negative side effects [1, 2]. Anabolic steroids include nandrolone, oxandrolone, oxymetholone, stanozolol, and trenbolone acetate [3, 4]. The androgen receptor, which is the natural biological receptor for testosterone and its metabolite dihydrotestosterone, is the target of anabolic steroids [5-7].

Some athletes, weightlifters, and bodybuilders use them on a regular basis to improve their physical performance and physique [8-10]. Anabolic steroids, in addition to making muscles bigger, may minimize muscular damage that happens following a hard workout, allowing athletes to recover faster and go out harder and more frequently [11, 12]. Unfortunately, some sportsmen and bodybuilders abuse these medications to increase their performance or physical attractiveness, which is considered cheating [13, 14]. Drug use in sports affects principles such as fairness and teamwork. When athletes use drugs, they risk not only harming their own health, but also tarnishing the sport and setting a negative example for others [15-17].

Steroids diminish redness and swelling when taken at higher levels than the human body naturally produces (inflammation) [18, 19]. Inflammatory disorders like asthma and eczema can benefit from this. Steroids also lower the immune system's activity, which is the body's natural defense against illness and infection. Increased appetite, weight gain, mood swings, muscle weakness, blurred eyesight, increased body hair development, and easy bruising are all possible side effects of steroids [20-22].

The androgen and anabolic steroid 19-nortestosterone (19-NT), also known as nandrolone, promotes muscular growth, hunger stimulation, enhanced red blood cell formation, and bone density. Furthermore, clinical investigations have shown that 19-NT can help with anemia, osteoporosis, and breast cancer [23-25]. 19-NT is a doping substance used by athletes to accelerate muscular growth and strength, as well as allow for speedier recuperation between athletic performances, due to its anabolic qualities [26-28]. Thus, determination of the 19-NT level in pharmaceutical and biological fluid samples is critical, and many analytical methods such as liquid and gas chromatography [29, 30], mass spectrometry [31], capillary electrophoresis [32], electrochemical methods [33-37], and enzyme-linked immunosorbent assay kit (ELISA) [38-41] have been investigated for the determination of 19-NT levels in clinical samples. Many of these methods, however, demand the use of expensive equipment as well as time-consuming sample preparation. Electrochemical techniques demonstrate inexpensive, easy sample preparation, and high sensitivity systems for a wide range of both inorganic and organic substances.

To our knowledge, only a few studies on the electrochemical determination of 19-NT have been undertaken [33-36, 42], and no publications on molecularly imprinted polymeric sensors based on gold nanostructures for the selective detection of 19-NT have been published. As a result, this study focused on the easy fabrication of MIP/Au hybrid film modified GCE as a stable and broad-range electrochemical sensor for assessing 19-NT in clinical samples utilizing electrodeposition and electropolymerization techniques.

2. EXPERIMENTAL

2.1. Preparation of MIP/Au hybrid film modified GCE

The GCE was modified by MIP/Au hybrid film as follows [43, 44]: first Au nanoparticles were electrodeposited on clean GCE surface (Au/GCE) from 1 g/L HAuCl₄ (99%, Sigma-Aldrich) aqueous solution at applying a potential of -0.2 V for 15 minutes using Autolab PGSTAT (M204) potentiostat/galvanostat and Nova 1.1 software equipped with electrochemical cell which contained reference (Ag/AgCl), counter (platinum wire), and working electrodes (clean GCE). Then, MIP layer was electropolymerized on Au/GCE using a cyclic voltammetry technique in a potentiostat/galvanostat in 30 mL of electrolyte prepared of 15 mL of 5 mM tetrabutylammonium perchlorate (>98.0%, Merck, Germany), 10 mL of 20 mM 2-aminothiophenol (99%, Sigma-Aldrich) and 5 mL of 10 Mm 19-NT (Sigma-Aldrich). Electropolymerization was conducted at potential range from -0.5 to 1.1 V at a scan rate of 40 mV/s during 40 cycles. After that, the MIP/Au/GCE was incubated in a mixture of methanol (99%, ChemFine International Co., Ltd., China) and acetic acid (99.9%, Sigma-Aldrich) in a volume ratio of (9:1) for 120 minutes to remove 19-NT.

2.2. Analysis of real sample

An amperometric technique was used to assess the level of 19-NT in human urine samples from five young athletes aged 20 to 30 who were taking Nandrodek (250 mg, Andro Medical Pharmaceuticals). Prior to analysis, all urine samples were centrifuged for 15 minutes at 1200 rpm, and the supernatant was used to make 0.1M PBS (pH 7.2) without purification. The 19-NT level in urine and serum samples was also determined using the Nandrolone Phenylpropionate ELISA Kit. The conventional addition method was used to calculate the RSD values.

2.3. Characterizations

The crystal structures and morphology of the samples were studied using an X-ray diffractometer (XRD; Rigaku D/max-2400, Japan) and scanning electron microscopy (Hitachi 3000, Tokyo, Japan). Differential pulse voltammetry (DPV) and amperometry measurements were performed on a potentiostat/galvanostat (Autolab® model PGSTAT 10N, Eco Chemie, Netherlands) equipped with an electrochemical cell containing bare or nanostructured modified GCE as the working electrode, a platinum plate, and an Ag/AgCl electrode. Electrochemical measurements were carried out in a 0.1 M phosphate buffer solution (PBS) with a pH of 7.2 that was made by combining 0.1 M NaCl (99%, Sigma-Aldrich) and 0.1 M KH₂PO₄-K₂HPO₄ (Sigma-Aldrich) solutions in an equal volume ratio.

3. RESULTS AND DISCUSSION

3.1. Crystallographic and morphological characterizations

Figure 1 shows the XRD patterns of powders of produced Au and MIP/Au hybrid films. There are diffraction peaks at 37.88° , 44.10° , 64.34° , 77.32° , and 81.28° in both the MIP/Au and electrodeposited Au nanoparticles XRD patterns, which correspond to the typical face-centered cubic (fcc) of Au with (111), (200), (220), (311), and (222) planes, respectively (JCPDS card no. 04-0783) [45-47]. The MIP/Au hybrid diffraction peaks, on the other hand, are less intense than those of the electrodeposited Au sample, showing that the electrodeposited Au nanoparticles were successfully covered by the electropolymerized MIPs film [48-50].

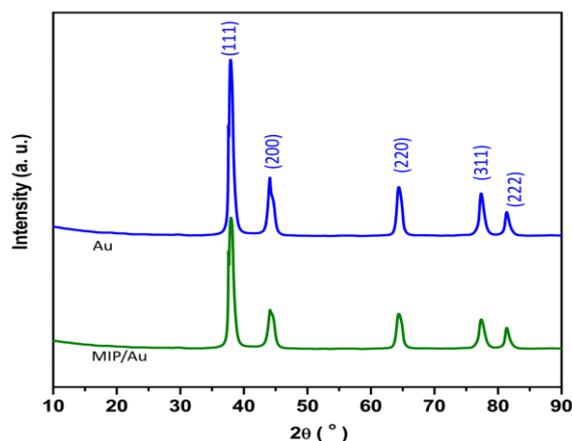


Figure 1. XRD patterns of powders of synthesized Au and MIP/Au hybrid films.

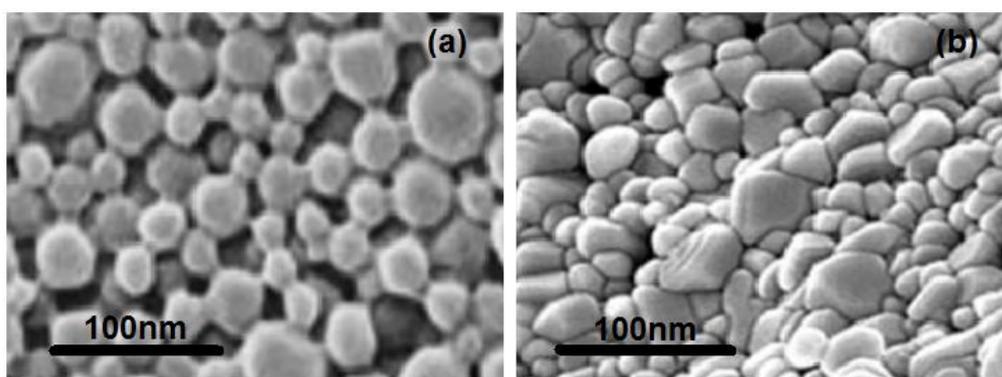


Figure 2. FE-SEM images of (a) Au and (b) MIP/Au hybrid films modified GCE.

Figure 2 shows SEM images of produced Au and MIP/Au hybrid films modified by GCE. The electrodeposition approach was used to successfully construct well-ordered Au nanoparticle arrays, as shown. There are hexagonal close-packed Au arrays with homogenous spherical nanoparticles (Figure 2a). The size dispersion of electrodeposited Au nanoparticles is lower, with an average diameter of 45

nm. Figure 2b indicates that the spherical form of Au nanoparticles and MIP/Au hybrid does not differ significantly. However, due to the electropolymerization process, the surface of MIP/Au hybrid nanoparticles has a small aggregate, and the average size of hybrid nanoparticles has increased to 50 nm. It shows that the Au nanoparticles were coated successfully with MIP film.

3.2. Electrochemical characterization

Figure 3 exhibits the electrochemical DPV responses of bare and modified GCE in a nitrogen-saturated solution of 0.1 M PBS (pH 7.2) containing 20 μ M 19-NT within the potential range of 0.0 V to 1.0 V at a scanning rate of 30 mV/s. It is found that the DPV curve of bare GCE does not show any obvious peak. However, both Au/GCE and MIP/Au/GCE show anodic peaks at potentials of 0.49 V and 0.47 V, which are linked to the synthesis of cyclic keto groups via catalytic oxidation of the beta-hydroxy group at position 17 of 19-NT as 3-oxo Delta(4)-steroid [33, 34, 51]. MIP/Au/GCE has an anodic peak that is somewhat lower in potential than Au/GCE. Because mono-layered Au/GCE has a smaller barrier to electron transmission than multi-layered MIP/Au/GCE, it exhibits faster electron transfer kinetics.

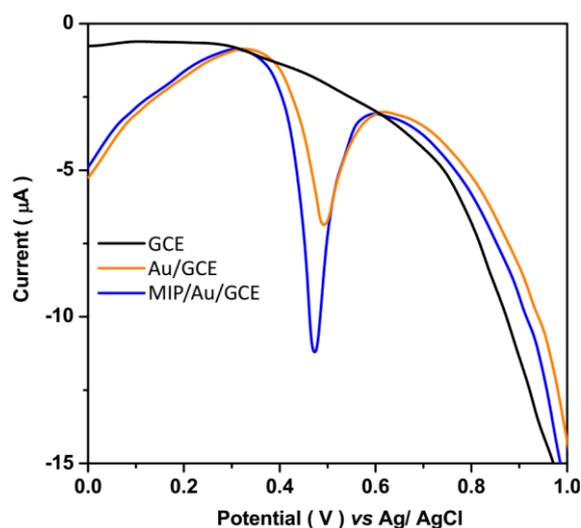


Figure 3. DPV responses of bare and modified GCE in a nitrogen-saturated solution of 0.1 M PBS (pH 7.2) containing 20 μ M 19-NT within potential range 0.0 V to 1.0 V at a scanning rate of 30 mV/s.

Due to the synergetic impact of the presence of MIP and Au nanoparticles, which may give high electrical conductivity and outstanding chemical and mechanical durability, the MIP/Au/GCE exhibits extraordinary electrochemical sensitivity toward the 19-N oxidative process. The electrocatalytic oxidation ability of the electrode to the template molecules [52-54], the presence of imprinted sites in the imprinted polymer [55, 56], and the great electron transfer ability from the recognition sites to the electrode surface are all factors that improve the selectivity and sensitivity of

molecularly imprinted sensors [39, 57]. Because of their large effective surface area, high catalytic capability, and electrical properties, Au nanoparticles can increase conductivity and the number of electron transfer sites and imprinted cavities, facilitating electron transfer through imprinted cavities and binding sites to the electrode surface [58-60]. It has been proposed that the presence of cavities in the imprinted electrode, as well as the imprinted polymer's unique binding capacity to the 19-NT molecules, can promote ion diffusion at the electrode/electrolyte interface, hence increasing electrochemical current [59, 61]. According to SEM data, coating Au nanoparticles by MIP molecules, increases the porous and number of strong electroactive and hot patches on the MIP/Au/GCE surface.

The stability of the DPV response of Au and MIP/Au modified GCE was studied with 100 sweeps in the potential range 0.0 V to 1.0 V at a scanning rate of 30 mV/s in a nitrogen-saturated solution of 0.1 M PBS (pH 7.2) containing 20 μ M 19-NT. Figure 4 shows the first and 50th recorded DPV curves for both electrodes, revealing a 3% and 4% decrease in the current peak of Au/GCE and MIP/Au/GCE, respectively. This research shows that both modified electrodes are quite stable. Because of the chemical and mechanical stability of Au, the Au/GCE demonstrates slightly superior stability [62-64]. Moreover, the good stability of the electrochemical response of MIP/Au/GCE is associated with an electropolymerization technique that allows MIP particles to be covalently immobilized on Au nanoparticles [65, 66]. As a result, the above electrochemical experiments obviously indicate that the MIP/Au/GCE shows considerably more catalytic activity toward 19-NT sensing than the bare GCE and Au/GCE, and it was selected as a preferred catalyst for further electrochemical measurements of 19-NT sensing.

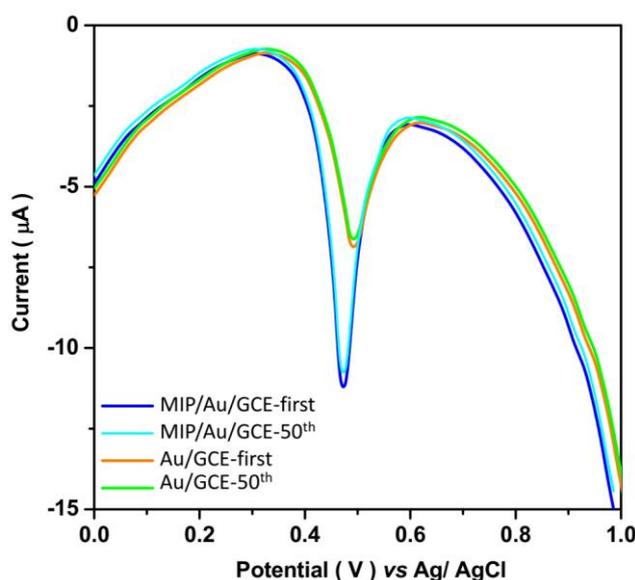


Figure 4. The first and 50th recorded DPV response of Au and MIP/Au modified GCE with successive 100 sweeps within potential range 0.0 V to 1.0 V at a scanning rate of 30 mV/s in a nitrogen-saturated solution of 0.1 M PBS (pH 7.2) containing 20 μ M 19-NT.

Figure 5 shows the MIP/Au/GCE amperometric response and calibration graph in a nitrogen-saturated solution of 0.1 M PBS after adding 60 μM 19-NT at a potential of 0.47 V. (pH 7.2). As can be seen, the MIP/Au/GCE responds quickly to any addition of 19-NT solution, signaling rapid electron transfer on the MIP/Au/GCE surface, as well as quick desorption of analytes in the MIP coating and a porous morphological structure of the MIP coating, which can speed up analyte diffusion [67, 68].

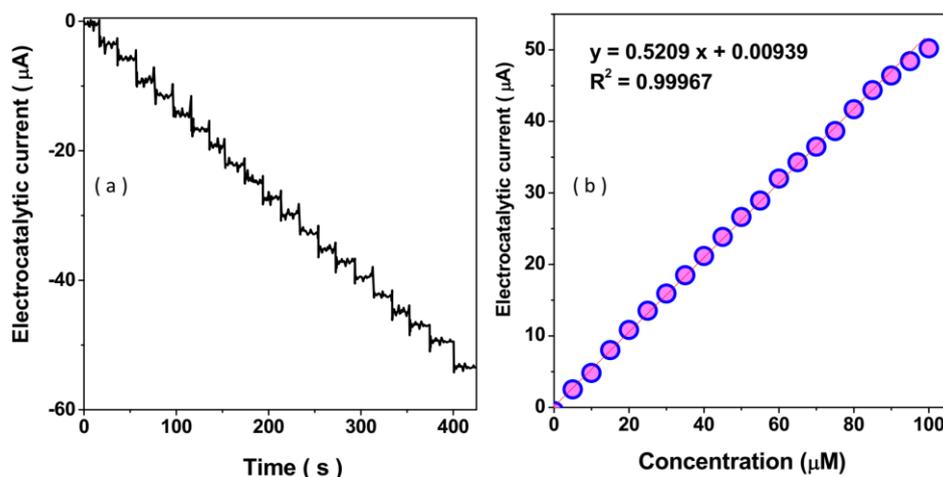


Figure 5. MIP/Au/GCE amperometric response and calibration graph to consecutive additions of 60 μM 19-NT at potential of 0.47 V in a nitrogen-saturated solution of 0.1 M PBS (pH 7.2).

Table 1. Comparison between electrochemical performance of MIP/Au/GCE and with other reported 19-NT electrochemical sensors in the literature.

Electrode	Technique	LOD (nM)	Linear range (μM)	Ref.
MIP/Au/GCE	AMP	3	5 to 95	This work
C ₆₀ /GCE	DPV	0.42	10^{-4} to 50	[33]
C ₆₀ /EPPGE	OSWV	0.015	10^{-5} to 0.05	[34]
Au/ITO	DPV	136	0.05 to 1.5	[35]
Hanging mercury drop electrode	LSAV	0.5	8×10^{-4} to 0.5	[36]
Screen printed electrode	ELISA	0.004	2.6×10^{-5} to 0.017	[38]
-----	icELISA	0.004	1.3×10^{-5} to 0.016	[40]
-----	MAic-ELISA	0.24	1.7×10^{-6} to 0.037	[41]

OSWV: Osteryoung square wave voltammetry; LSAV: Linear sweep adsorption voltammetry; icELISA: indirect competitive ELISA; MAic-ELISA: monoclonal antibody based indirect competitive ELISA

The corresponded calibration graph demonstrates the linear increase of electrocatalytic current with successive additions of 19-NT solution from 5 to 95 μM . Besides, the sensitivity and detection

limit (LOD, calculated with a signal-to-noise ratio of 3) are found to be 0.5209 $\mu\text{A}/\mu\text{M}$ and 3 nM, respectively. Table 1 compares the results to other 19-NT electrochemical sensors previously described in the literature. The comparison shows that MIP/Au/GCE has a wider linear range response to 19-NT and favorably and even better electrochemical electrocatalytic activity, which can be attributed to the strategy of electrodeposition of Au nanoparticles onto the surface of GCE and electropolymerization of MIP onto the surface of Au/GCE, which not only enhances the electrochemical signal but also plants the cavities and binding sites as functional monomers of the MIP sensor [58].

Using amperometric measurements of MIP/Au/GCE under consecutive additions of 19-NT and 10-fold of interfering substances at a potential of 0.47 V in a nitrogen-saturated solution of 0.1 M PBS, the effect of various substances from pharmaceuticals and/or in biological fluids as compounds potentially interfering with the detection of 19-NT was evaluated (pH 7.2). Table 2 shows the results of amperometric electrocatalytic current at 0.47 V, which shows a significant amperometric response to the addition of 19-NT in the electrochemical cell and a poor electrochemical signal to subsequent additions of interfering chemicals. As a result, there is no discernible interference effect when determining 19-NT in the presence of the various pharmaceutical substances given, and the 19-NT sensor created in this study exhibits good specificity. In the presence of the template molecules, the electropolymerization approach for molecular imprinting produces a functional monomer, which is then extracted from the polymer network [69, 70]. This results in the formation of nanocavities that are complementarity in binding sites and shape and act as artificial antibodies toward the imprinted molecules, which, as a consequence, enhance the selectively recognizing the target molecules in any matrix of interest [58, 71].

Table 2. Results of evaluation of interfering effect of various substances with the detection of 19-NT using amperometric measurements of MIP/Au/GCE under consecutive additions of 19-NT and 10-fold of interfering substances at potential of 0.47 V in nitrogen-saturated solution of 0.1 M PBS (pH 7.2).

Substance	Added (μM)	Amperometric current (μA) at 0.47 V	RSD (%)
19-NT	1	0.5213	± 0.0187
Caffeine	10	0.0364	± 0.0033
4-Nitrobenzoic Acid	10	0.0119	± 0.0025
Cystine	10	0.0153	± 0.0019
Hypoxanthine	10	0.0233	± 0.0015
Glucose	10	0.0279	± 0.0021
Xanthine	10	0.0310	± 0.0015
Dopamine	10	0.0121	± 0.0009
Oxalic Acid	10	0.0222	± 0.0020
Ascorbic Acid	10	0.0231	± 0.0022
Uric Acid	10	0.0125	± 0.0013
KCl	10	0.0198	± 0.0017
NaNO_3	10	0.0088	± 0.0018
MgSO_4	10	0.0272	± 0.0019
CaCl_2	10	0.0188	± 0.0015

For the detection of 19-NT in five different urine samples from young athletes aged 22 to 28 years who were supplied Nandrodek subcutaneously, the accuracy and practical use of the established amperometric sensing method was investigated. Figure 6 shows the amperometric reaction of MIP/Au/GCE to adding 19-NT solution in prepared 0.1 M PBS (pH 7.2) from human urine samples at 0.47 V, as well as the calibration plot. It shows that the concentration of 19-NT in the urine of the first athlete (A1) is 0.079 μM . It's worth mentioning that the viability value obtained by amperometric measurements is extremely close to that obtained by the ELISA kit assay (Table 3). For the other four samples, both amperometric and ELISA kit assays were performed, and the results of an average of four times of assays to determine the 19-NT level are provided in Table 3. As may be seen, amperometry and ELISA assays agree fairly well. Additionally, the range of obtained RSD values in Table 3 are 3.09% to 4.38% which indicated to great detection precision of the proposed amperometric method, and excellent validity for determination 19-NT in clinical samples and human biological fluids.

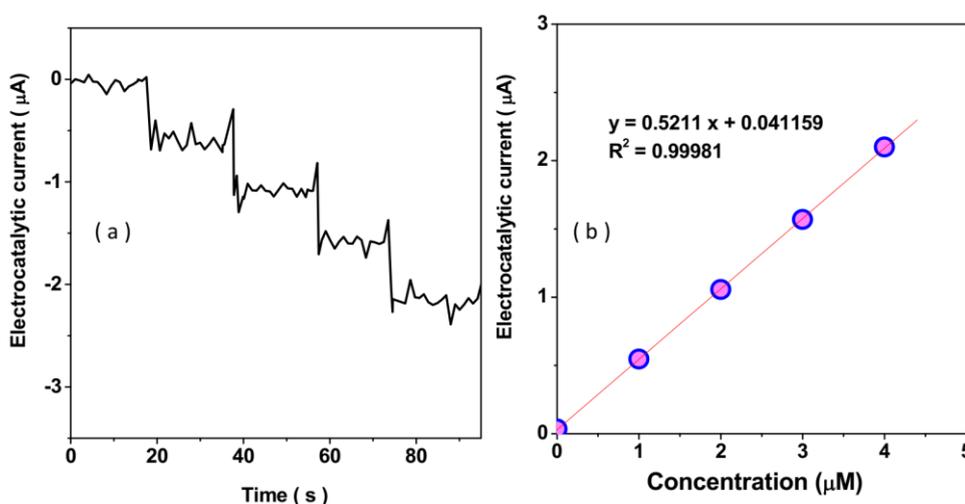


Figure 6. Amperometric response and corresponded calibration plot of MIP/Au/GCE to successive adding 19-NT solution in prepared 0.1 M PBS (pH 7.2) from human urine samples at 0.47 V.

Table 3. The results of determination of 19-NT in five different in urine samples from young athletes aged 22 to 28 years who administered Nandrodek subcutaneously using the ELISA and amperometry assays.

Sample	Concentration of 19-NT in prepared urine samples (μM)			
	Amperometry		ELISA kit	
	MIP/Au/GCE	RSD (%)	ELISA	RSD (%)
A1	0.079	± 3.27	0.081	± 3.22
A2	0.050	± 3.42	0.048	± 4.02
A3	0.049	± 4.38	0.046	± 4.31
A4	0.061	± 3.12	0.063	± 3.15
A5	0.039	± 3.09	0.037	± 3.21

4. CONCLUSION

The simple production of MIP/Au hybrid film adapted to GCE as a stable and broad-range electrochemical sensor for assessing 19-NT as a doping agent in clinical samples was the subject of this study. The MIP/Au hybrid film was created using electrodeposition and electropolymerization techniques. According to crystallographic and morphological characterizations, electrodeposited Au nanoparticles were successfully covered by an electropolymerized MIPs film. The MIP/Au/GCE demonstrated significant catalytic activity, stability, and selectivity for 19-NT sensing, according to electrochemical evaluation utilizing DPV and methods. The favorably and even better electrochemical electrocatalytic activity of MIP/Au/GCE toward other reported 19-NT electrochemical sensors in the literature with good values of sensitivity ($0.5209 \mu\text{A}/\mu\text{M}$), detection limit (3 nM) and linear range (5 to $95 \mu\text{M}$) toward other reported 19-NT was obtained. The accuracy and practical application of a newly developed amperometric sensing method for determining 19-NT in urine samples from young athletes aged 22 to 28 who were given Nandrodek subcutaneously were investigated, and the results showed that the findings of the amperometry and ELISA assays agreed fairly well. Furthermore, the RSD values obtained (3.09% to 4.38%) revealed that the proposed amperometric approach had excellent detection precision and validity for determining 19-NT in clinical samples and human bodily fluids.

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