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Determination of Anabolic Steroid as Doping Agent in Serum and Urine of Athletes by Using an Electrochemical Sensor Based on the Graphene-Gold Hybrid Nanostructure

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The current study focused on the creation of a gold and graphene hybrid nanostructure modified glassy carbon electrode (Au/GO/GCE) as an electrochemical sensor for the detection of nandrolone (ND) as a doping agent in blood and urine samples. The creation of a well-crystallized Au/GO nanohybrid structure was validated by the results of structural investigations. The electrochemical properties of Au/GO/GCE as ND sensors were studied using the DPV approach, which revealed a stable and selective response to ND, as well as a favorable and even superior performance of Au/GO/GCE than previous reported ND sensors. The linear range (1 to 100 μM), sensitivity (0.3547 μA/μM), and limit of detection (4 nM) of Au/GO/GCE were calculated. The practical application of a developed ND sensor was investigated in order to determine the ND in the serum and urine of four young athletes aged 20 to 30 years who were administered nandrolone decanoate subcutaneously. The results showed that the DPV and ELISA analyses agreed relatively well, and the resulting RSD values ranged from 3.10 percent to 4.33 percent, showing that the developed electrochemical ND sensor can be used as a viable sensing approach for determining ND in human biological fluids.

Keywords: Anabolic steroid; Nandrolone; Serum and urine samples; Au/GO nanohybrid

1. INTRODUCTION

Nandrolone (ND), commonly known as 19-nortestosterone, is an androgen and an anabolic steroid that has been alkylated [1, 2]. It is proposed for the mechanism of action of ND that it enters cells via receptor-mediated endocytosis and interacts with the androgen receptor[3-5]. After interacting with the androgen receptor, the androgen receptor undergoes a conformational shift, enters the nucleus, dimerizes, and can then bind to DNA sequences to regulate transcription [6, 7].

ND is used to treat anemia caused by renal insufficiency, as well as an adjuvant therapy in the treatment of senile and postmenopausal osteoporosis [8, 9]. It is also used to treat burns, allergic reactions, eye injuries, particularly to the cornea, and appetite loss caused by cancer, as well as prostate and breast malignancies [10, 11]. ND preferentially encourages the growth of skeletal muscle and lean body mass, which may result in a decrease in metabolic syndrome components [12, 13]. Furthermore, research on ND has suggested that it may play a role in the treatment of joint repair, particularly in rotator cuff injuries [14-16].

Headaches, fluid retention, gastrointestinal discomfort, diarrhea, stomach pain, jaundice, menstrual irregularities, and hypertension are all possible side effects of ND use [17]. Some professional athletes, as well as recreational athletes and even adolescents, abuse steroids like ND to gain muscle and improve performance [18]. However, research suggests that excessive or improper usage of ND or any other anabolic steroid can be harmful to human health [19, 20]. According to studies, ND taken at higher than clinical levels may be harmful to the liver, causing incipient fibrosis [21].

ND increases testosterone and epitestosterone levels, which improves muscle strength and endurance, and it was most likely used as a doping drug in sports in the 1960s. Since 1974, it has been prohibited at the Olympics [22, 23]. That is why determining the ND level in pharmaceutical and clinical samples is critical [24, 25]. As a result, many analytical methods for identifying and determining ND levels in biological fluids and clinical samples have been investigated, including liquid chromatography [26], mass spectrometry [27], gas chromatography [28], capillary electrophoresis [29], spectrophotometry [30], electrochemical methods [31-34], Raman spectroscopy [35], near-Infrared spectroscopy [36] and enzyme-linked immunosorbent assay kit (ELISA) [37, 38]. Electrochemical approaches outperformed the others in terms of selectivity and sensitivity for determining anabolic steroids in clinical samples. To the best of our knowledge, only a few studies on the electrochemical determination of ND have been conducted [31-34]. These studies are conducted to improve the detection limit for application electrochemical sensors in biological fluids, and the prepared sensors have been performed in a narrow and restricted linear range which limits further applications in pharmaceutical and food samples with a high level of ND. Thus, the present study have been conducted on the synthesis of Au/GO hybrid nanostructure as a wide linear range electrochemical sensor for the determination of ND as doping agents in serum and urine samples.

2. EXPERIMENT

2.1. Synthesis hybrid nanostructure of gold and graphene

For the synthesis of the hybrid nanostructure of Au/GO [39], A mixture of 10 μL of hydrazine hydrate (65%, Sigma-Aldrich) and 200 L of ammonia solution was ultrasonically mixed with 100 mL of 0.5 g/L of GO (45 μm, Sigma-Aldrich) (28%, Sigma-Aldrich). The mixture was magnetically agitated for 35 minutes before being cooked in an oil bath at 85°C for 60 minutes. After centrifugation at 1000 rpm for 10 minutes, the precipitates were washed with deionized water and ovendried under

vacuum at 70°C. For the preparation of impregnated sulphur into a GO framework (S-GO), 34 mg of precipitates were added to a homogeneous mixture of 15 mg of elemental sulfur (95%, Sigma-Aldrich) and 4.25 mL of toluene (99.9%, Sigma-Aldrich) as solvent. The suspension was sonicated for 60 minutes. Next, the solvent of the suspension was evaporated in the air at room temperature. Then, 0.5 g of S-GO was ultrasonically dispersed in a 3 mL methanol (99%, Shandong S-Sailing Chemical Co., Ltd., China) solution containing 15 mg $HAuCl_4 \cdot 3H_2O$ (99.5 %, Sigma-Aldrich). The mixture was magnetically stirred for 15 minutes, and then, the solvent of the mixture was evaporated in air at room temperature. For reduction of the Au ions, the obtained mixture was transported into the heated quartz tube with a flow of argon at 300 ◦C for 120 minutes. Then, the obtained Au/GO hybrid nanostructure was dispersed in dimethylformamide (DMF, 99.9%, Dongying Qihao Chemical Co., Ltd., China) to achieve the 1.0 g/l suspension which dropped on the GCE surface and dried at room temperature.

2.2. Preparation of actual samples of urine and serums

Four young sportsmen aged 20 to 30 years old were given urine and serum samples after receiving subcutaneous injections of nandrolone decanoate. Because the half-life of ND is believed to be 6–12 days [40], urine and serum samples were collected 3 days after administration. The samples were centrifuged separately at 1500 rpm for 12 minutes before being used to prepare 0.1M PBS pH 7.2 as real samples for electrochemical investigations. The Nandrolone Racing ELISA kit (NEOGEN, detection range 3.12nmol/L-100nmol/L, NEOGEN Co., USA) was also used to determine the level of ND in urine and serum samples.

2.3. Crystallographic, morphological and electrochemical analyses

The crystallographic structure of produced nanostructures was studied using an X-ray difractometer (XRD; Philips PANalytical X'Pert, CuK, Holand). Morphological investigations were performed using field emission scanning electron microscopy (FE-SEM; S-4800, Hitachi, Japan). Electrochemical experiments were conducted out utilizing DPV techniques with an Autolab PGSTAT (M_20_4) potentiostat/galvanostat and Nova 1.1 software. The electrochemical cell used Ag/AgCl, platinum wire, and modified GCE as reference, counter, and working electrodes. The electrolyte used in the electrochemical tests was 0.1M phosphate buffer solution (PBS) with a pH of 7.4, which was made by combining stock solutions of 0.1 M NaCl (99%, Sigma-Aldrich) and 0.1M NaH_2PO_4–Na_2HPO_4 (99%, Sigma-Aldrich).

3. RESULTS AND DISCUSSION

3.1. Crystallographic and morphological analyses

Figure 1 depicts the XRD patterns of GO and Au/GO hybrid nanostructures. According to the XRD pattern of GO, there are diffraction peaks at 25.19° and 43.16°, which correspond to the (002) and (100) planes of the hexagonal carbon structure of GO (JCPDS Card no. 75-1621) [41, 42]. XRD

patterns of Au/GO hybrid nanostructure reveal characteristic peaks at 37.87°, 43.68°, 64.09°, 77.13°, and 81.05°, which are indexed to (111), (200), (220), (311), and (222) planes, and are consistent with those of standard patterns of Au's usual fcc phase (JCPDS card no. 04-0783) [43-45]. Furthermore, the Au/GO XRD pattern shows an extra diffraction peak associated with the (002) reflection of GO, showing the presence of GO in the Au nanostructure [46, 47].

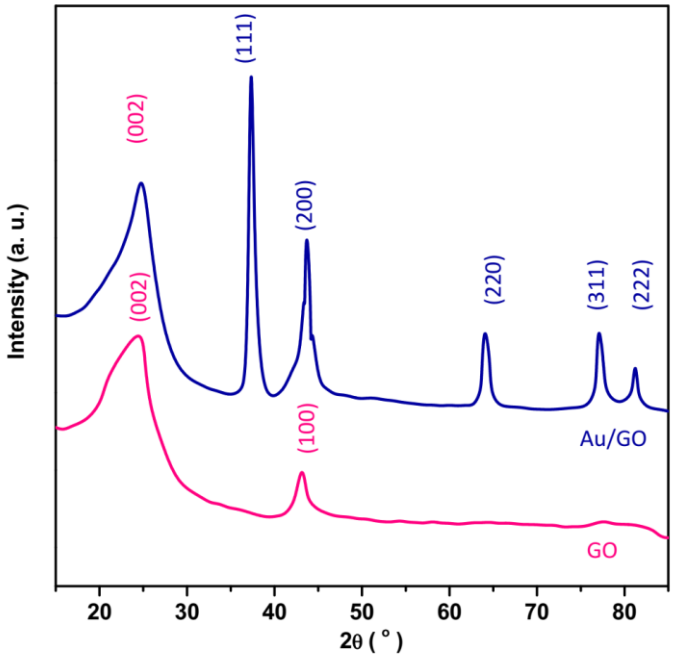

Figure 1. The XRD patterns of GO and Au/GO hybrid nanostructure.

The FE-SEM images of GO and Au/GO are shown in Figure 2. The wrinkled morphology and somewhat crumpled curved structure of the FE-SEM picture of the GO sample. The hybrid nanostructure of Au/GO is a combination of GO nanosheets and well-dispersed Au nanoparticles with spherical form, as revealed by FE-SEM pictures of Au/GO. The diameter of embedded Au nanoparticles on GO nanosheets is around 90nm. The Au/GO hybrid nanostructure provides numerous active sites, a rough and porous surface, and a large effective surface area, all of which improve electrocatalytic activity [48-51]. The results of the crystallographic and morphological characterizations confirm the creation of an Au/GO hybrid nanostructure.

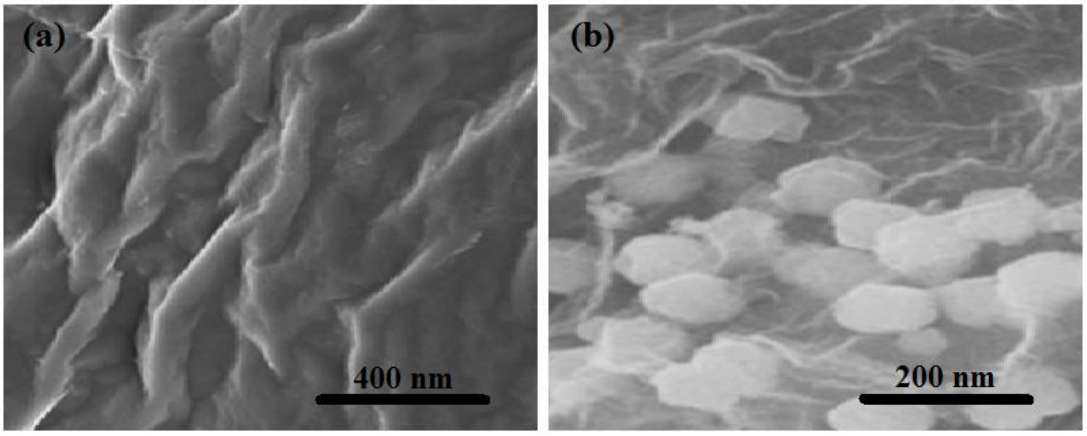

Figure 2. FE-SEM images of (a) GO nanosheets, (b) Au/GO hybrid nanostructure.

3.2. Electrochemical behavior

Figure 3 depicts the electrochemical responses of bare and nanostructure modified GCE in a nitrogen-saturated 0.1 M PBS with pH 7.2 containing 60 M ND at a scan rate of 25 mV/s in the potential range of 0.1 V to 0.9 V. As can be seen, there is no visible redox peak in the DPV curves of bare GCE. However, GO/GCE and Au/GO/GCE exhibit anodic peaks at potentials of 0.53 V and 0.52 V, respectively, indicating that the hydroxyl group present at the 17^{th} position in ND was catalytically oxidized to create a cyclic keto group [31, 32, 52]. The anodic peak for Au/GO/GCE is detected at a lower potential, and its current is approximately 2-fold higher than that of GO/GCE. It demonstrates that the Au/GO/GCE exhibits significant catalytic attractiveness in the ND oxidative process due to the presence of Au nanoparticles, which can provide a high dispersion state of the metal particles on GO nanosheets and produce good chemical and mechanical stability [53-56]. GO nanosheets possess a high concentration of oxygen-containing polar groups and are the substantial active centers for electrocatalytic reactions [57-59]. And the presence of wrinkles and defectivd sites on the nanosheets surface and edges such as surface functionalities, vacancies, substrate-induced interactions, surface corrugations, atomic substitutions and grain boundaries enhances the density of electronic states and improves the heterogeneous electron transfer of GO modified electrodes [60-62]. Furthermore, the Au/GO hybrid nanostructure has a high electrical conductivity as well as a large accessible specific surface area for analyte adsorption in electrolytes, which facilitates charge transfer between analytes and the electrode surface [63-65]. Thus, in electrochemical processes, the combination of Au nanoparticles and GO nanosheets in an Au/GO nanohybrid structure can produce suitable electron-transfer routes [66, 67]. According to SEM data, anchoring Au nanoparticles into the GO structure prevents GO nanosheet self-aggregation and increases the number of strong electroactive and hot patches on the electrode surface [68-70].

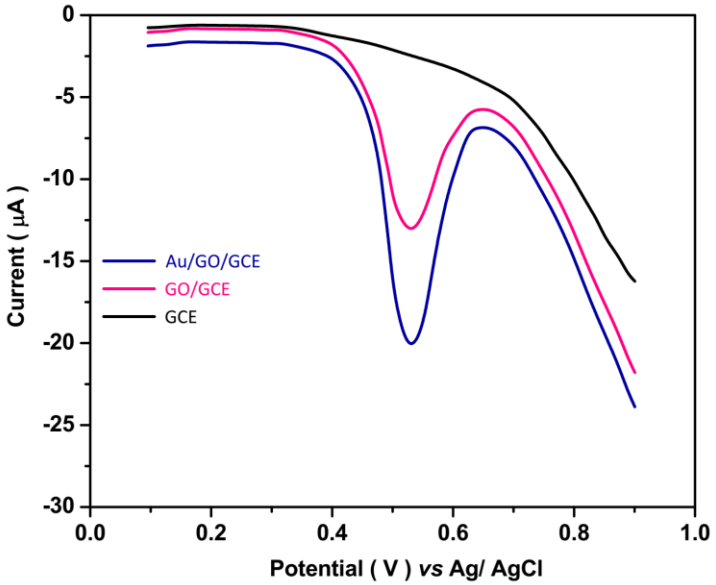

Figure 3. DPV curves of GCE, GO/GCE and Au/GO/GCE in a nitrogen-saturated 0.1 M PBS with pH 7.2 containing 60 µM ND at a scan rate of 25 mV/s.

The stability of the DPV response of GO/GCE and Au/GO/GCE was investigated in nitrogen saturated 0.1 M PBS with pH 7.2 containing 60 M ND at a scan rate of 25 mV/s with 100 sweeps in the potential range of 0.1 V to 0.9 V. Figure 4 shows the first and 80th recorded DPV curves of changed electrodes, which show a 7% and 4% drop in the current peak of GO/GCE and Au/GO/GCE, respectively. This study demonstrates the increased stability of the electrochemical response of Au/GO/GCE due to the strong chemical stability of Au nanoparticles and their covalent attachment to the GO structure [71, 72]. The connection between Au and GO is minimal, but incorporating sulphur into a GO framework results in a substantially greater interaction between Au and S-GO [73, 74]. The homogeneity of the Au nanoparticles anchored into the GO framework suggests that the sulphur is distributed uniformly across the GO nanosheets [75-77]. As a result, the above electrochemical experiments clearly show that the Au/C hybrid nanostructure has greater catalytic activity toward ND sensing than the naked GCE and GO modified GCE, and it was chosen as a preferred catalyst for further electrochemical measurements of ND sensing.

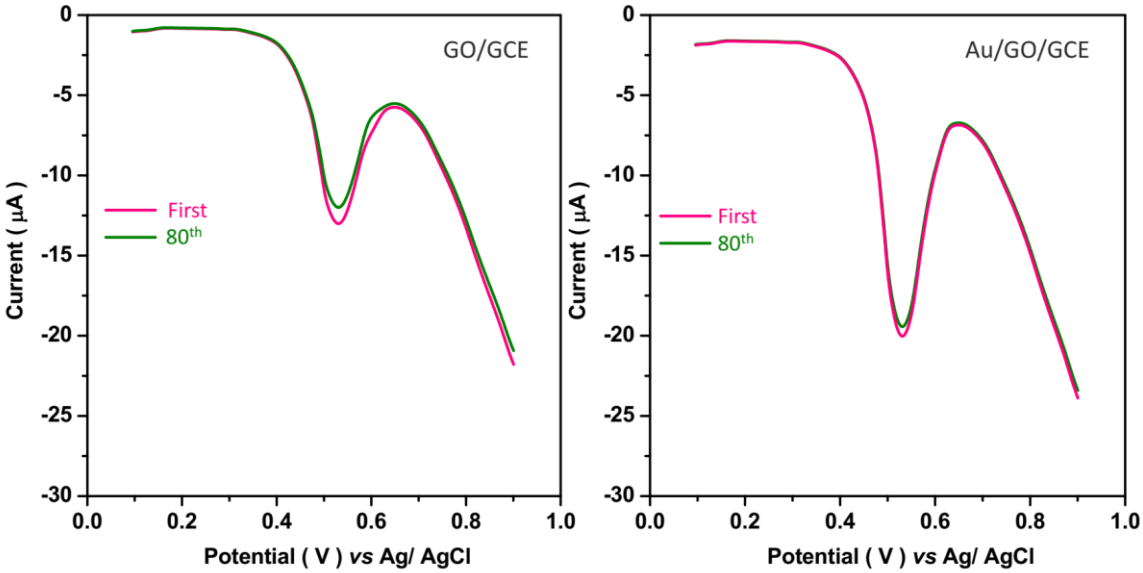

Figure 4. The first and 80th recorded DPV response of GO/GCE and Au/GO/GCE in a nitrogen saturated 0.1 M PBS with pH 7.2 containing 60 µM ND at a scan rate of 25 mV/s with successive 100 sweeps in the potential range from 0.1 V to 0.9 V.

Figure 5 shows the Au/GO/GCE DPV response and calibration graph after adding 10 µM ND solution into 0.1 M PBS with pH 7.2 in the potential range of 0.1 V to 0.9 V at a scan rate of 25 mV/s. The electrocatalytic response of Au/GO/GCE is shown to grow linearly with each addition of 10 µM ND solution in the range of 1 to 100 µM, with a correlation coefficient of 0.99990. The linear relationship is realized as follows [78-80]:

$$I\ (\mu A) = 0.3547\ [ND]\ (\mu A/\mu M) + 0.01728 \quad (1)$$

Where [ND] denotes the concentration of ND. The calculated sensitivity and limit of detection (LOD) values are 0.3547 µA/µM and 4 nM, respectively. Table 1 compares the sensing performance of the developed ND sensor in this work to that of other reported ND sensors in the literature, implying a wider linear range response to ND and favorably and even better performance of Au/GO/GCE than that of other reported ND sensors, which can be associated with higher conductivity and excellent electrocatalytic activity of Au nanoparticles in the Au/GO nanohybrid structure [81-83]. This wide linear range response to ND can be used not only to determine ND levels in clinical samples, but also to determine ND levels in pharmaceutical and food samples with high levels of ND.

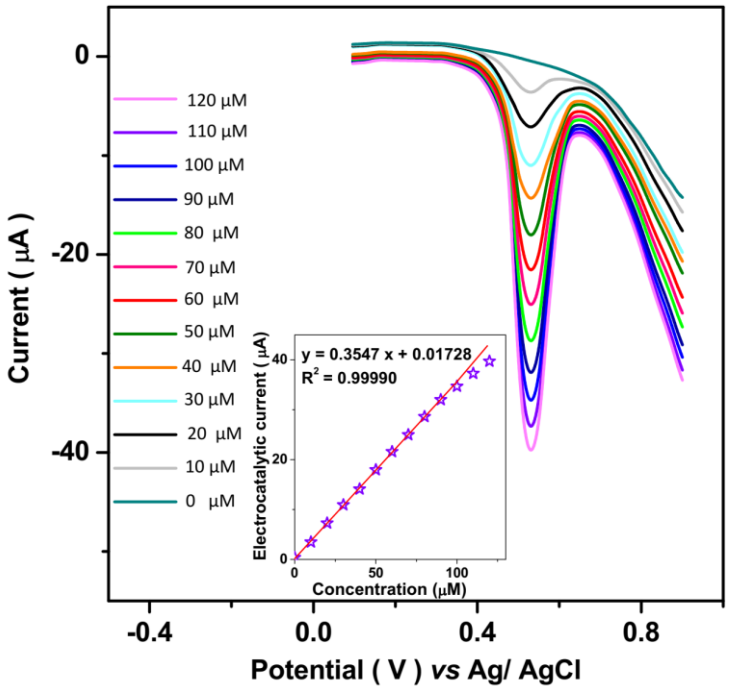

Figure 5. DPV response and calibration graph of Au/GO/GCE for addition of 10 µM ND solution into 0.1 M PBS with pH 7.2 in the potential range from 0.1 V to 0.9 V at a scan rate of 25 mV/s.

Table 1. Comparison between sensing performance of developed ND sensor in this work and the other reported ND sensors in the literature.

Electrode	**Technique**	**LOD (nM)**	**Linear range (µM)**	**Ref.**
Au/GO/GCE	DPV	4	1 to 100	This work
C_{60}/GCE	DPV	0.42	10^{-4} to 50	[31]
C_{60}/EPPGE	OSWV	0.015	10^{-5} to 0.05	[32]
Au/ITO	DPV	136	0.05 to 1.5	[33]
Hanging mercury drop electrode	LSAV	0.5	8×10^{-4} to 0.5	[34]
Screen printed electrode	ELISA	0.038	2.18×10^{-4} to 0.145	[37]

LSAV: Linear sweep adsorption voltammetry; OSWV: Osteryoung square wave voltammetry

The potentially interfering influence of several pharmacological and biological fluid substances on ND detection was also investigated. Figure 6 shows the results of DPV electrocatalytic currents of Au/GO/GCE at 0.52 V in 0.1 M PBS with pH 7.2 under the addition of ND and 4-fold excesses interfering compounds at a scan rate of 25 mV/s, demonstrating that the sensor response to 1 µM ND solution is significantly greater than that of interfering species, and indicating no significant changes in electrocatalytic signal of ND at 0.52 V, implying that the addition 4-fold excess These findings show that the proposed sensor has a high selectivity for ND measurement in pharmaceutical and biological fluid samples [84, 85].

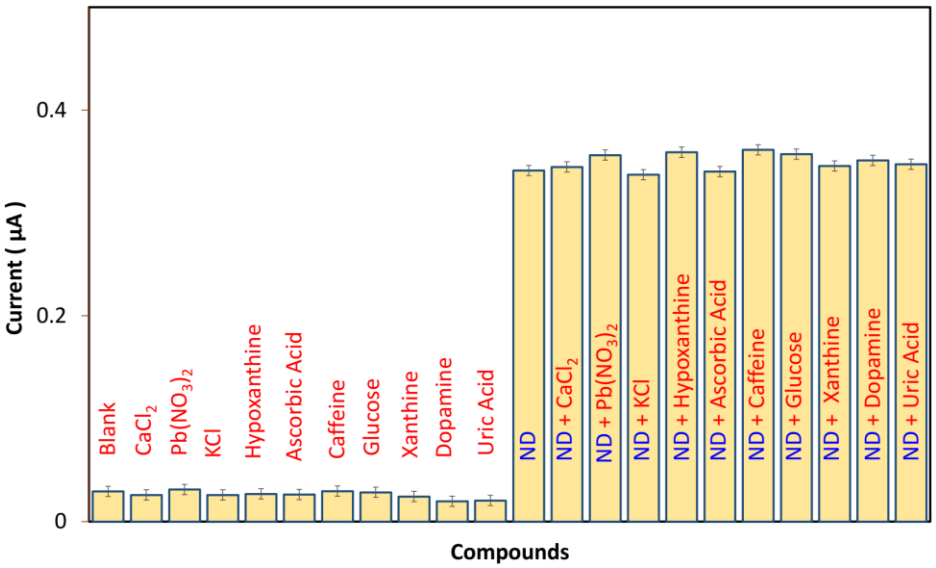

Figure 6. The results of DPV electrocatalytic currents of Au/GO/GCE at 0.52 V under successive additions of 0.1 M PBS with pH 7.2 under addition 1 µM ND and 4-fold excesses interfering compounds at scan rate of 25 mV/s; (Blank sample is referred to the 0.1 M PBS without any analyte)

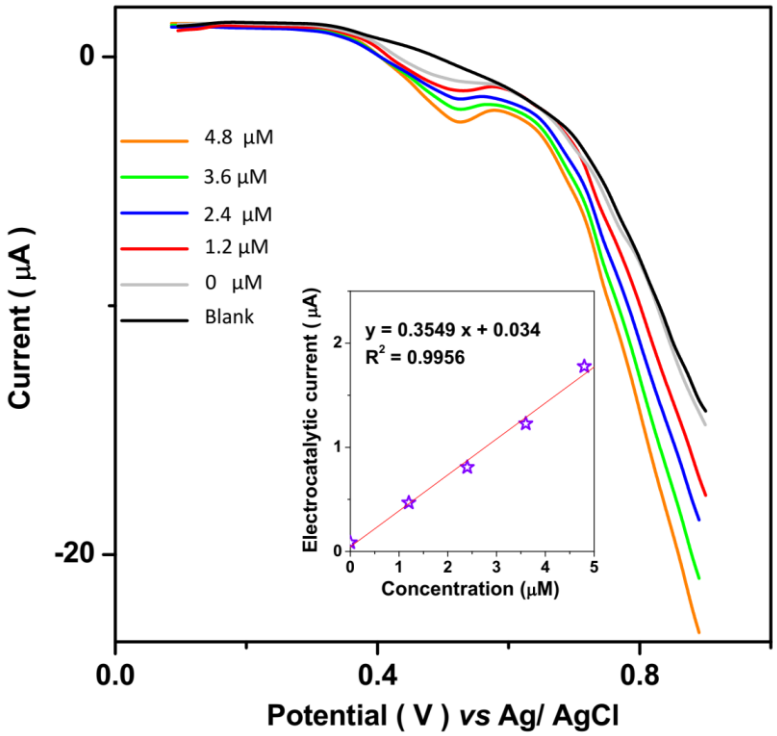

Figure 7. DPV measurements and corresponding calibration graph of Au/GO/GCE to successive additions of ND in the potential range from 0.1 V to 0.9 V at a scan rate of 25 mV/s in 0.1 M PBS with pH 7.2 prepared from serum sample of first volunteer; (Blank sample is referred to the 0.1 M PBS without any analyte)

Table 2. The findings of DPV and ELISA analyses to determination of ND in prepared real samples from serum and urine samples originating from young athletes aged 20 to 30 years who administrated nandrolone decanoate by subcutaneous injection.

Content of ND in prepared serum samples (µM)				
Volunteer No.	**Amperometry**		**ND ELISA kit**	
	Au/GO/GCE	**RSD (%)**	**ELISA**	**RSD (%)**
V1	0.095	±3.47	0.097	±3.62
V2	0.097	±3.59	0.099	±3.79
V3	0.096	±4.33	0.094	±4.11
V4	0.086	±4.29	0.090	±3.95
Content of ND in prepared urine samples (µM)				
V1	0.082	±3.62	0.080	±3.55
V2	0.078	±3.11	0.075	±4.13
V3	0.077	±3.10	0.075	±4.10
V4	0.080	±4.10	0.081	±3.84

The practical application of a developed ND sensor was investigated in order to determine the ND in the serum and urine of four young athletes aged 20 to 30 years who were administered nandrolone decanoate subcutaneously [86]. Figure 7 depicts the DPV measurements and calibration graph of a produced genuine sample of serum from one of the athletes (V1) using Au/GO/GCE in 0.1 M PBS with pH 7.2 and ND in the potential range of 0.1 V to 0.9 V at a scan rate of 25mV/s. As can be shown, the ND concentration in a manufactured genuine sample of serum is 0.095 M, which is extremely close to the ND content acquired by the ELISA assay (Table 2). The DPV and ELISA analyses were also performed on all prepared genuine samples from serum and urine samples of other athletes, and the results are reported in Table 2, suggesting that the two analyses agree reasonably well [87]. Furthermore, the RSD values reported in Table 2 range from 3.10 to 4.33%, demonstrating that the developed electrochemical ND sensor can be used as a reliable sensing approach for determining ND in human biological fluids [88, 89].

4. CONCLUSION

In conclusion, this study focused on the fabrication of Au/GO hybrid nanostructure modified GCE as an electrochemical sensor for determining ND as doping agents in the serum and urine samples. The findings of crystallographic and morphological investigations revealed the production of a well-crystallized Au/GO nanohybrid structure with numerous active sites, a rough and porous surface, and a large effective surface area. The electrochemical properties of Au/GO/GCE as ND sensors revealed a stable, selective, and sensitive response to ND, as well as favorably and even better performance than other reported ND sensors, which can be attributed to the higher conductivity and excellent electrocatalytic activity of Au nanoparticles in the Au/GO nanohybrid structure. The linear

range (1 to 100 µM), sensitivity (0.3547 µA/µM), and limit of detection (4 nM) of Au/GO/GCE were determined. The practical application of a developed ND sensor was investigated in order to determine the ND in the serum and urine of four young athletes aged 20 to 30 years who were administered nandrolone decanoate subcutaneously. The results showed that the DPV and ELISA analyses agreed pretty well, and the acceptable RSD values indicated that the developed electrochemical ND sensor can be used as a viable sensing approach for determining ND in human biological fluids.

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