Int. J. Electrochem. Sci., 17 **(2022)** Article Number: 221178, doi: 10.20964/2022.11.72

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

A Sensitive Amperometric Sensor based on CuO and molecularly imprinted polymer composite for Determination of Danazol in human urine

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Received: 31 August 2022 / *Accepted:* 5 October 2022 / *Published:* 20 October 2022

The goal of this study was to create a new molecular imprinting-based sensor on a CuO nanostructured electrode for the selective detection of Danazol (DZ) as a doping agent in athlete biological fluid samples. CuO nanostructured film was electrodeposited on the screen-printed carbon electrode (CuO/SPCE) for the fabrication of modified electrodes, and molecularly imprinted polymer (MIP) was electropolymerized on CuO/SPCE. The analysis of FE-SEM images and XRD patterns on modified electrodes revealed that electropolymerization of amorphous MIP on CuO nanoparticles was successful. Electrochemical analyses using CV and amperometry revealed that the synergistic effect of CuO and MIPs significantly improved the electrocatalytic activity and selectivity of the MIP/CuO nanostructure. The results showed that a linear response from 0 to 6900 ng/mL was obtained. MIP/CuO/SPCE sensitivity was determined to be 0.02576 μA/ng.mL^{-1}, and the limit of detection was determined to be 0.10 ng/mL. When the proposed method was compared to some previously reported DZ sensors, MIP/CuO/SPCE demonstrated notable electrocatalytic performance with the lowest limit of detection value. The MIP/CuO/SPCE sensing system was studied for the analysis of DZ in real samples prepared from the urine of five bodybuilders, and the results showed that the RSD values (3.55% to 4.41%) are appropriate for valid and accurate practical analyses in urine and other biological fluids samples.

Keywords: CuO nanostructure; Molecularly *Imprinted* Polymers; Specificity; Danazol; bodybuilders; Amperometry

1. INTRODUCTION

Danazol (DZ), also known by the brand name Danocrine, is a synthetic steroid and testosterone derivative that belongs to the class of medications known as androgenic hormones [1, 2]. DZ acts as an

anterior pituitary suppressant by inhibiting pituitary gonadotropin output [3, 4]. It has biologic effects that are mediated by binding to steroid transport proteins in the circulation as well as specific receptors in target tissues [5, 6]. DZ inhibits gonadotropins centrally, suppressing gametogenesis and steroidogenesis. DZ treats endometriosis by shrinking the displaced uterine tissue. It works to treat fibrocystic breast disease by inhibiting the release of hormones that cause pain and lumps in the breast [7, 8].

DZ has been shown to displace testosterone from sex-hormone-binding globulin. Malevolent side effects such as acne, excessive hair growth, and voice deepening limit DZ use [9, 10]. The use of this medication may result in irregular monthly periods or the absence of a menstrual period. Weight gain, flushing, sweating, vaginal dryness or irritation, or decreased breast size are the side effects of DZ in women [11-13].

As a result, determining DZ levels in pharmaceutical and biological fluid samples is critical, and several studies have been conducted to study the determination of DZ concentration through liquid chromatography [14], high performance liquid chromatography [15], ultraviolet spectrophotometry [16], radioimmunoassay [17], thin-layer chromatographic densitometric method [18], micellar liquid chromatography method [19], gas chromatography/mass spectrometry [20], and electrochemical techniques [21-24]. Low selectivity is a significant issue that can arise as a result of interference from oxidizable species within the biological sample [25, 26]. The molecular imprinting technique has been shown in studies to be a method for forming a molecular lock to match a molecular key with tailor-made binding sites that induce separations based on the shape, size, and functional groups of the template molecules [27-30]. As a result, the goal of this study was to create a new molecular imprinting-based sensor on a CuO nanostructured electrode for the selective detection of DZ as a doping agent in athlete biological fluid samples.

2. EXPERIMENT

2.1. Fabrication of modified electrodes

The CuO nanostructured film was electrodeposited on the screen-printed carbon electrode (SPCE, DRP-110, Metrohm AG) surface using a CS2350 Bipotentiostat /Dual-channel electrochemical workstation (Xian Yima Optoelec Co., Ltd., China) in an electrochemical cell of SPCE (working electrode), a Pt plate (auxiliary electrode), and Ag/AgCl (reference electrode). The electrochemical deposition was carried out in a 0.1 M KCl (99.0%, Merck Millipore, Germany) solution containing 1 mM $CuCl_2$ (≥99%, Sigma-Aldrich) at a potential of 0.4 V for 2 minutes [31]. The obtained Cu nanostructured film was to be converted to CuO film through cyclic voltammetry (CV) in the potential range of 0.45 to 0.35 V at a scan rate of 50mV/s for 20 cycles in a 0.1 M NaOH (≥ 99.0 %) solution. A MIP-based sensor was created using an electropolymerization technique on the CuO/SPCE surface through the CV at a scan rate of 50 mV/s for 20 cycles [32]. The electrolyte was contained 0.6 g (Vinylbenzyl)trimethylammonium (99%, Sigma-Aldrich), 2 ml of danazol (≥98%, Sigma-Aldrich), 4 mL of divinylbenzene (55%, Sigma-Aldrich), 0.2 g of Azo-bis-isobutyronitrile (≥98%, Sigma-Aldrich) and 6 mL methanol (99.8%, Sigma-Aldrich) which added to a 25 mL of 0.1 M PBS. After

electropolymerization, the electrode was immersed in an ethanol solution (5% v/v) for the removal of target molecules (danazol). Finally, the electrode was rinsed with deionized water and dried at room temperature.

2.2. Instrument and characterization methods

CV and amperometry were the electrochemical techniques that were conducted on the CS2350 Bipotentiostat/Dual-channel Electrochemical Workstation in 0.1M phosphate buffer solution (PBS) electrolyte (pH 7.0) prepared from a mixture of 0.1M NaH_2PO_4 (99%) and 0.1M Na_2HPO_4 (99%). Field emission scanning electron microscopy (FE-SEM) and an X-ray diffractometer (XRD) were utilized to evaluate the morphological and crystallographic properties of the nanostructures.

2.3. Preparation of the actual sample from the urine of bodybuilders

To test the MIP/CuO/SPCE as a sensing system for DZ analysis in real samples, urine samples from five bodybuilders were provided. The bodybuilders were given Danocrine 200 mg capsules as gifts (200 mg Danazol, PT Aventis Pharma, Jakarta, Indonesia). Danazol's half-life has been reported to be 9.7 hours on average, so urine samples were collected 4 hours after taking Danocrine. The urine samples were centrifuged for 5 minutes at 1000 rpm, filtered, and then used to prepare 0.1 M PBS pH 7. Finally, urine-derived 0.1 M PBS was used as electrochemical electrolytes.

3. RESULTS AND DISCUSSION

3.1. Study of FE-SEM images and XRD patterns modified electrodes

Figure 1 shows FE-SEM images of SPCE, CuO/SPCE, and MIP/CuO/SPCE. The FE-SEM image of SPCE in Figure 1a exhibits a smooth and uniform surface. The FE-SEM micrograph of CuO/SPCE (Figure 1b) shows that CuO nanoparticles with an average diameter of 45 nm and uniform spherical shape are electrodeposited on the SPCE surface without aggregates.

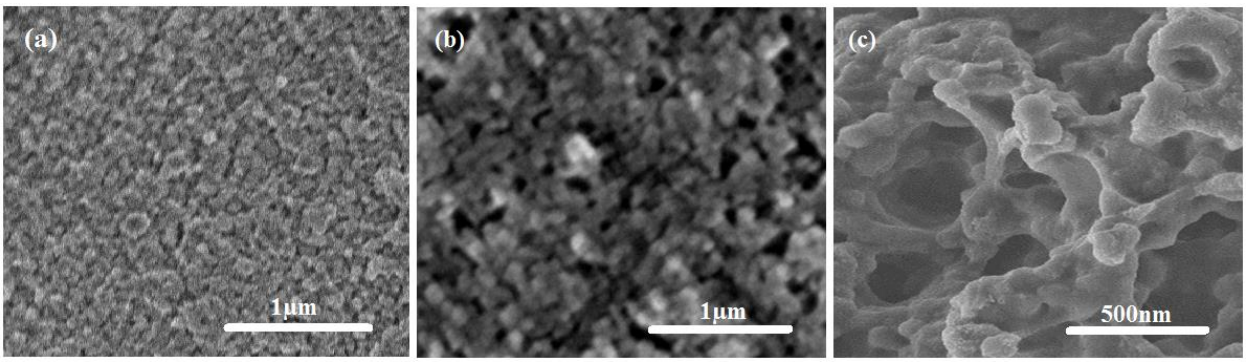

Figure 1. FE-SEM images of (a) SPCE, (b) CuO/SPCE and (c) MIP/CuO/SPCE.

The FE-SEM micrograph of MIP/CuO/SPCE (Figure 1c) shows that after electropolymerization, the porous thin polymeric film is coated onto the electrode surface and MIP nanoparticles can be evenly dispersed on the CuO/SPCE surface. The cavities with specific space are expected to mimic the exact grooves of the template and act as a recognizer center for target molecules with the same or similar properties as the template molecules [33-35]. In addition, the large surface area of MIP/CuO/SPCE provides more recognition sites on the surface and facilitates the electron transfer kinetics [36-38].

Figure 2 depicts XRD patterns of MIP, CuO, and MIP/CuO deposited powders. The XRD pattern of MIP shows broad diffraction peaks at approximately 23.7° and 23.8°, which are assigned to MIP's amorphous structure [39, 40]. CuO and MIP/CuO XRD patterns show strong diffraction peaks at 32.79°, 35.69°, 38.97°, 48.81°, 53.71°, 58.15°, and 67.31°, which correspond to the (1 1 0), (1 1 1), (2 0 2), (0 2 0), (2 0 2), and (1 1 3) planes of CuO with monoclinic phase (JCPDS card no. 05-06 The MIP/CuO XRD pattern also shows a large shoulder, indicating that electropolymerization of amorphous MIP on CuO nanoparticles was successful electropolymerization of amorphous MIP on CuO nanoparticles.

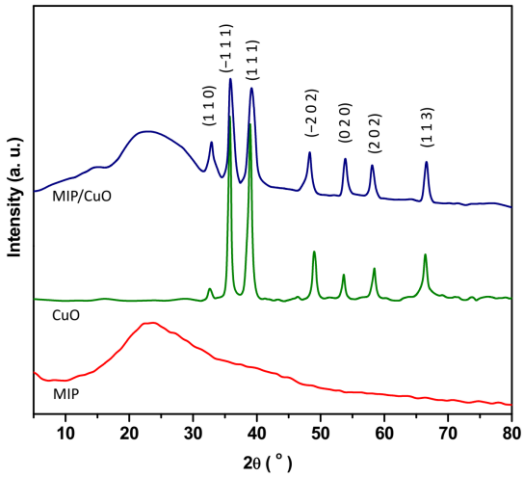

Figure 2. XRD pattern of the deposited powder of MIP, CuO and MIP/CuO.

3.2. Electrochemical analyses

The CV curves of SPCE and CuO/SPCE and MIP/CuO/SPCE in 0.1 M PBS pH 7 are shown in Figure 3 by scanning the potential between -1.0 and 0.0 V vs. Ag/AgCl at a scan rate of 50 mV/s. In an electrochemical cell, CV responses were recorded in both the absence and presence of DZ solutions. There is no obvious peak in the CV curves of CuO/SPCE and MIP/CuO/SPCE for DZ-free solutions. After adding the DZ solution to the electrochemical cell, the CV curves of SPCE, CuO/SPCE, and MIP/CuO/SPCE exhibit well-defined peaks at -0.64, -0.55, and -0.49 V, respectively, which may be related to the cathodic reduction of the isoxazole ring present in a steroidal nucleus of heterocyclic compound DZ [41-43]. Furthermore, the peak current of MIP/CuO/SPCE appears at a lower potential

with a higher current intensity than SPCE. It demonstrates that the synergistic effect of CuO and MIPs significantly improves the electrocatalytic activity of MIP/CuO nanostructures. MIP-based electrodes have recognition sites that are complementary in shape to the target molecule, resulting in high electrocatalytic activity as well as improved selectivity, sensitivity, and durability [44, 45]. When subjecting these MIPs to the target analyte, molecules get trapped in the cavities and thus get detected [46-48]. CuO nanoparticles are a simple way to improve electronic conductivity, provide an efficient pathway, accelerate electron diffusion, and facilitate faster electron transfer during electrochemical reactions [49, 50]. In comparison between the CV curves of SPCE and CuO/SPCE, it is observed that CuO nanoparticles increase the peak current and decrease the peak potential position toward the lower potential, which indicates the higher electron transfer efficiency of CuO nanoparticles. Here, MIP was selected due to its selectivity, low price, environmental friendliness, and synthesis with proper yield. MIP shows a lack of stability on the SPCE surface. Studies have indicated a strong interaction between MIP and CuO nanoparticles which improves the stability of MIP-based sensors [51, 52]. Moreover, the electrodeposited CuO nanoparticles on the SPCE surface increase the surface area of MIP [52]. Furthermore, CuO nanostructures could increase the number of active sites to achieve impressive sensitivity. As a result, CuO nanostructures can act as a suitable support for anchoring MIP molecules in sensors and improve the electron transfer behavior of MIP/CuO nanostructured electrodes [53-56]. Therefore, the further electrochemical experiments were conducted on MIP/CuO/SPCE.

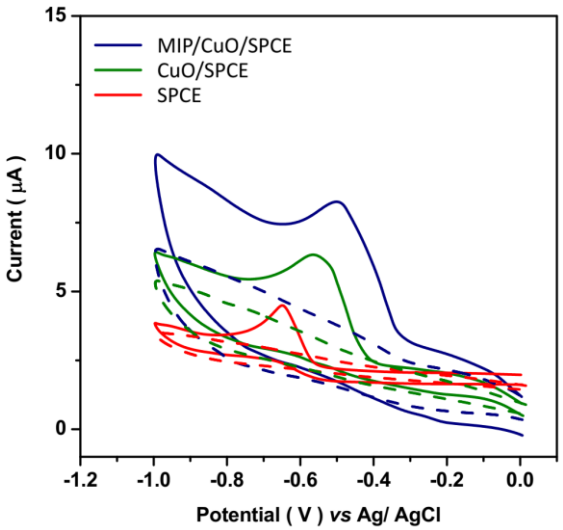

Figure 3. CV curves of SPCE and CuO/SPCE and MIP/CuO/SPCE in 0.1 M PBS pH 7 by scanning the potential between −1.0 and 0.0 V vs. Ag/AgCl at a scan rate of 50 mV/s in both of absence (dashed line) and presence (solid line) of DZ solutions conditions in electrocvhemical cell.

Figure 4 depicts the results of MIP/CuO/SPCE amperometric experiments with consecutive injections of a solution containing 300 ng/mL DZ solution in 0.1 M PBS pH 7 electrolyte solution at an applied potential of -0.49 V vs. Ag/AgCl. The results show that each addition of DZ solution results in rapid and stable reactions. In an electrochemical cell, increasing the DZ concentration increases the amperometric current linearly. The calibration plot in Figure 4's inset shows a linear response from 0 to 6900 ng/mL. The sensitivity of MIP/CuO/SPCE is determined to be 0.02576 µA/ng.mL^{-1}, and the limit

of detection is determined to be 0.10 ng/L using a signal-to-noise ratio (S/N = 3). The proposed method is compared to some previously reported DZ sensors in Table 1. As seen, MIP/CuO/SPCE exhibits notable electrocatalytic performance with the lowest limit of detection value, which can be attributed to the MIP electropolymerized on the SPCE covered with CuO nanoparticles having an enlarged active surface and being more homogeneous, which minimized steric hindrance and thus improved MIP/CuO/SPCE sensing performance [57-59].

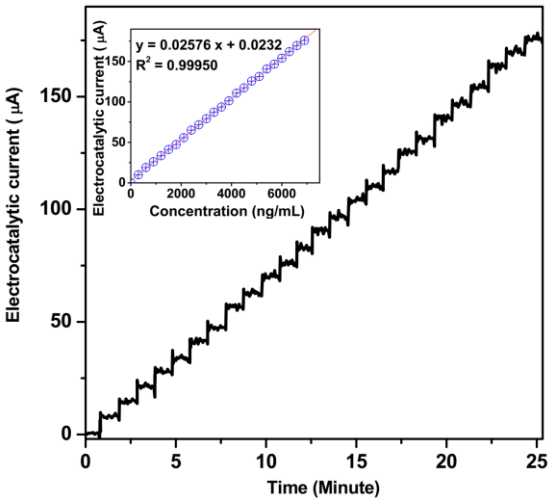

Figure 4. The results of amperometric experiments of MIP/CuO/SPCE under consecutive injections of a solution containing 300 ng/mL DZ solution in 0.1 M PBS pH 7 electrolyte solution at an applied potential of −0.49 V vs. Ag/AgCl.

Table 1. The performance of electrochemical sensor for determination of DZ in present work and released outcomes of DZ sensors in literetures.

Electrode	**Technique**	**Linear range (ng/ml)**	limit of detection **(ng/ml)**	**Ref.**
MIP/CuO/SPCE	Amperometry	0–6900	0.10	This work
Hanging mercury drop electrode	SW-AdSV	25.3–126.5	1.92	[21]
Hanging mercury drop electrode	DPP	1687.5−33750	337.5	[22]
Polyaniline film electrode	CV	0.675−3.375	---	[23]
Reversed-phase C8 and C8 columns	LC	1.0−150	---	[14]
ODS column	HPLC	---	2	[15]
C18 column	HPLC-UV spectroscopy	620−25000	55	[60]
Agilent Zorbax Eclipse XDB-C18 column	HPLC	31.25−2500	31.25	[61]

SW-AdSV: square-wave adsorptive stripping voltammetry; DPP: differential pulse polarography; LC: liquid chromatography; HPLC: high performance liquid chromatography

Before examining the fabricated sensor for determining DZ in human urine samples, the selectivity of the MIP/CuO/SPCE sensor in the presence of interfering substances in biological fluids was investigated. Table 2 summarizes the results of amperometric measurements of MIP/CuO/SPCE after consecutive injections of 100 ng/mL DZ and 400 ng/mL interfering substance solutions in 0.1 M PBS pH 7 electrolyte solution at an applied potential of -0.49 V vs. Ag/AgCl. The results in Table 2 show that a significant electrocatalytic signal is generated after adding DZ solution to an electrochemical cell, but no obvious amperometric responses are observed when interfering substances are added. The results show that the MIP/CuO/SPCE system is an excellent, specific sensor for determining DZ in biological fluid samples. This specificity is associated with MIP because it is prepared using an electropolymerization method that produces covalent or noncovalent interactions between monomers and template molecules [62, 63]. The removal of the template causes the formation of binding sites and molecular cavities with molecular memory, mirroring size and shape, allowing the polymer to bind target analytes with high affinity and specificity [64, 65].

Table 2. the obtained data from amperometric measurements of MIP/CuO/SPCE under consecutive injections of a solution containing 100 ng/mL DZ and 400 ng/mL interfering substances solutions in 0.1 M PBS pH 7 electrolyte solution at an applied potential of −0.49 V vs. Ag/AgCl.

Substance	**Added (ng/mL)**	**Amperometric signal (µA) at −0.49 V**	**RSD**
DZ	100	2.5777	±0.0035
Testosterone	400	0.1039	±0.0028
Glutamic acid,	400	0.0727	±0.0019
Guanosine	400	0.0746	±0.0018
Boldenone	400	0.0462	±0.0014
Stanozolol	400	0.0533	±0.0017
Ascorbic acid	400	0.0450	±0.0012
Epinephrine	400	0.0342	±0.0015
Progesterone	400	0.0325	±0.0019
Serotonin	400	0.0349	±0.0012
Dopamine	400	0.0292	±0.0012
Urea	400	0.0248	±0.0014
Urea	400	0.0222	±0.0013
Acetaminophen	400	0.0275	±0.0011
Uric acid	400	0.0159	±0.0010
Lactose	400	0.0178	±0.0012
Cellulose	400	0.0158	±0.0013
Citric acid	400	0.0181	±0.0012

The MIP/CuO/SPCE sensing system was investigated for the analysis of DZ in real samples prepared from the urine of five bodybuilders. Figure 5a shows the results of amperometric measurements of MIP/CuO/SPCE at an applied potential of 0.62 V vs. Ag/AgCl after consecutive injections of a solution containing 10 ng/mL DZ solution in 0.1 M PBS pH 7 electrolyte solution

prepared from the first bodybuilder's urine sample (BB1). According to the calibration plot in Figure 5b, the DZ level in the processed sample is 3.53ng/mL. This method was used to analyze samples BB2 to BB5, and the results of the analytical studies are tabulated in Table 3 to show that the RSD values (3.55% to 4.41%) are appropriate for valid and accurate practical analyses in urine and other biological fluid samples.

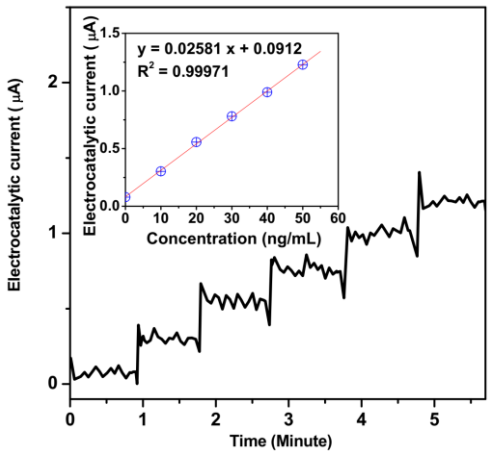

Figure 5. The obtained data from amperometric measurements of MIP/CuO/SPCE at an applied potential of −0.49 V vs. Ag/AgCl under consecutive injections of a solution containing 10 ng/mL DZ solution in 0.1 M PBS pH 7 electrolyte solution prepared from urine sample of first bodybuilder (BB1).

Table 3. The obtained data from amperometric measurements to determination of DZ level in prepared real samples from bodybuilders's urine samples undergoing taking Danazol.

Sample No.	Detected content of DZ in prepared urine samples (ng/mL)	RSD (%)
BB1	3.53	±4.41
BB2	3.41	±4.35
BB3	2.87	±4.15
BB4	3.05	±3.55
BB5	2.96	±3.77

4. CONCULUSION

The current manuscript describes the development of a new molecular imprinting-based sensor on a CuO nanostructured electrode for the selective determination of DZ as a doping agent in athlete biological fluid samples. To make MIP/CuO/SPCE, a CuO nanostructured film was electrodeposited on the SPCE surface, and the MIP layer was electropolymerized on the CuO/SPCE. Structural studies revealed that electropolymerization of amorphous MIP on CuO nanoparticles was successful. Electrochemical analyses revealed that the synergistic effect of CuO and MIPs significantly improved the electrocatalytic activity and selectivity of the MIP/CuO nanostructure. The results showed that a

linear response from 0 to 6900 ng/mL was obtained. MIP/CuO/SPCE sensitivity was determined to be 0.02576 μA/ng.mL^{-1}, and the limit of detection was determined to be 0.10 ng/mL. The comparison of the proposed method with some of the previously reported DZ sensors revealed that MIP/CuO/SPCE exhibited notable electrocatalytic performance with the lowest limit of detection value, which can be attributed to the MIP electropolymerized on the SPCE covered with CuO nanoparticles having an enlarged active surface and being more homogeneous, which minimized steric hindrance, leading to improvement of MIP/CuO/SPCE sensing performance. The MIP/CuO/SPCE sensing system was studied for the analysis of DZ in real samples prepared from the urine of five bodybuilders, and the results demonstrated that the RSD values are appropriate for valid and accurate practical analyses in urine and other biological fluids samples.

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