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Screen printed electrode-based biosensor functionalized with magnetic cobalt/single-chain antibody fragments for cocaine biosensing in different matrices

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ABSTRACT

On-site detection of substance abuse is an important approach in the preventive and intervention protocols implementations. It is known that the traditional methods are heavy, time-consuming, and need a high level of logistical requirements. As such, biosensors represent great potential to simplify and improve substance abuse detection. In this study, we have designed a functionalized screen-printed electrode (SPE) electrochemical biosensor with cobalt oxide nanoparticles and single-chain antibody fragments (scFvs) for cocaine detection. Different electrochemical techniques such as differential pulse voltammetry, cyclic voltammetry, and electrochemical impedance spectrometry were used to examine the functionality of the designed biosensor. Furthermore, SEM observations were performed to observe the surface changes after functionalization. The results showed that the linearity ranged between 5.0 and 250 ng/mL and a detection limit of 3.6 ng/mL (n = 6). These results were compared to results obtained from Q-TOF/MS where four different matrices (serum, sweat, urine, and saliva) were spiked with 100 ng/mL cocaine and were analyzed by both methods (Biosensor and Q-TOF/MS). Results showed a higher performance of the biosensor compared to traditional methods. In addition, the selectivity of the biosensor was shown in the presence of different interferents where the designed platform showed a specific response to only cocaine. In conclusion, the designed biosensor proposes great potential for portable and on-site substance abuse detection in addition to boasting the capability of reuse of the SPE and thus, reducing the costs related to such applications.

1. Introduction

On-site detection of cocaine has been attracting a lot of interest in recent years due to the severity of cocaine abuse in society [1]. The current procedures for cocaine abuse detection and quantification in samples are mostly performed using heavy instruments such as gas or liquid chromatography followed by mass spectrometry (GC-MS and LC-MS/MS) [2]. These approaches are time-consuming, show a necessity for plenty of pretreatment procedures, costly, and needs highly trained staff members to perform. Although the cocaine limit of detection (LOD) is quite satisfying, a need for more advantageous tools having faster, easy to operate, and portability features are necessary for different fields of science and biomedicine [1].

Recently, biosensors became a focus for scientists for their flexibility to detect a myriad of target molecules, pathogens, and bacteria [3–5]. From the many possibilities, metallic nanoparticles represent a good candidate given their potential in enhancing the surface area, improving electron transfer, and the sensitivity of sensors [6–10]. Cobalt oxide nanoparticles are one of the metallic nanoparticles receiving the focus of many researchers due to many advantages seen. Cobalt oxide nanoparticles are known for their biocompatibility, stability, conductivity and electrocatalytic features. In addition, it is eco-friendly, easy to access, and of low cost [6,11–16].

In order to increase the biocompatibility and functional behavior of the nanoparticles, various materials are used for their functionalization (ie. polymers, antibodies, etc.) [17]. Thus, broadening the application

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of functionalized nanoparticles into different research fields such as drug delivery, biosensing, immunoassays, MRI, and many others [18–20].

Single-chain antibody fragments (scFvs) consist of different domains of the light and heavy chains that are linked by a flexible peptide connector. Given their small size and full antibody-like antigen-binding properties, scFv fragments have attracted great interest in therapeutic applications [21,22]. Furthermore, scFv fragments have served as sensing elements [23–25]. Compared to whole antibodies, antibody fragments have proved to be superior in terms of high customizability, high surface densities, and different immobilization options [26]. For instance, recombinant antibody fragments demonstrated higher sensitivity and specificity to prostate-specific antigen glycoprofiling than the full-length antibody [27]. The customizability of antibody fragments provides many advantages allowing controlled orientation on the biosensor's surface and thus improving its performance. For this purpose, various recombinant scFv fragments have been engineered, carrying hexahistidine tags [28,29], cysteine residues [30], polystyrene-binding peptides [31], biotins [32] or Cys3-tags [33].

Herein, we proposed a cobalt oxide/scFvs-based biosensor for the rapid detection of cocaine using surface printed electrode technology (SPE), which is a technology of recent and high interest in small molecules detection. The protocol was performed according to already established procedures in our laboratory, and the performance of the produced biosensor was found to be satisfactory for both sensitivity and selectivity.

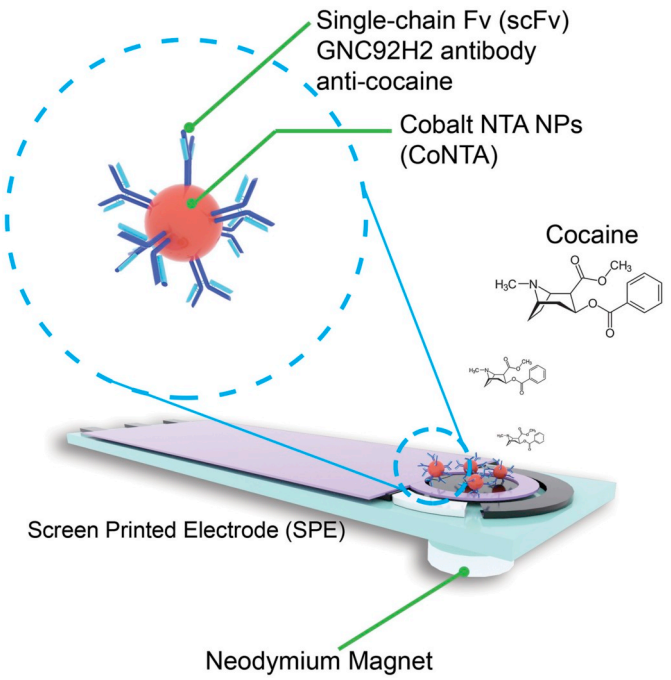

Fig. 1. Schematic description of the composition of the SPE-CoNTA-Ab biosensor.

2. Material and methods

2.1. Chemicals and reagents

Cobalt oxide nitrilotriacetate (CoNTA) nanoparticles were obtained from Jena Bioscience GmbH (Germany, reference number: AC-602-5). Cocaine (COC), JWH-073 (4-butanoic acid metabolite), methamphetamine (METH), and codeine (COD) were purchased from Cerilliant Corp (Round Rock, TX, USA). All the other reagents were from Sigma (St. Louis, MO, USA) unless described otherwise. All chemicals were of analytical grade. Milli-Q water was used in all solutions as a solvent.

2.2. Recombinant expression and purification of cocaine-binding antibody

Single-chain Fv (scFv) GNC92H2 antibody was expressed and purified as described before with some modifications [34]. The codon-optimized gene was synthesized and cloned into pET32b (+) vector (GenScript, Piscataway, NJ, USA). *Escherichia coli* BL21 GOLD (DE3) cells (Agilent Technologies, Santa Clara, CA, USA) with the transformed plasmid were incubated in 300 mL of Luria-Bertani (LB) broth medium at 37 °C until OD600 = 0.5–0.6 was reached. Expression of cocaine antibody with N-terminal thioredoxin tag and two His-tags at both N- and C-ends was induced by adding 0.3 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG). The cells were incubated for 24 h at 20 °C and were then harvested by centrifugation (4000 rpm, 30 min, 4 °C). To solubilize the antibody from inclusion bodies, the pellet was re-suspended in a buffer containing 50 mM NaH2PO4, 200 mM NaCl, 10 mM imidazole, and 1.0% sarkosyl (pH 7.9) [35]. The suspension was homogenized using ultrasonic homogenizer (Sonics & Materials Inc, USA) at 50% amplitude for 30 min at 4 °C. After centrifugation (15000 rpm, 30 min, 4 °C), the supernatant was loaded onto HisTrap™ FF crude column connected to ÄKTAprime plus system (GE Healthcare Life Sciences, Uppsala, Sweden). The column was washed with 50 mM NaH2PO4 buffer containing 200 mM NaCl, 10 mM imidazole and 0.5% sarkosyl (pH 7.9). Elution of the bound protein was achieved using 300 mM imidazole and the purity was checked by SDS-PAGE [36]. The purified antibody was desalted in 50 mM NaH2PO4 buffer (pH 7.4) using ultrafiltration centrifuge unit (Amicon Ultra-15 Centrifugal Filter Units, 10000 NMWL, Merck Millipore, Germany). Protein concentration was determined by Bradford's method using bovine serum albumin as the standard [37].

2.3. Synthesis, modification, and electrochemical surface characterization

100 µL of a well-mixed stock suspension of CoNTA was added to 800 µL of 50 mM NaH2PO4 pH8 buffer solution containing 300 mM NaCl. 200 µL of cocaine antibody solution (2.8 mg/mL) were added to the buffered CoNTA suspension. Components were mixed at room temperature for 2 h (20 rpm). The magnetic decantation method was used to wash the mixture with a buffer for removing non-bonded antibodies. The resulting cocaine antibody-attached CoNTA bioconjugates were named CoNTA-Ab and were re-dispersed in 500 µL phosphate buffer solution for further experiments.

In order to prepare the biosensor, surface printed electrodes (SPE) were put under extreme anodic and cathodic potentials using cyclic voltammetry (CV) to eliminate impurities from the electrode surface (-2.0 V to +2.0 V). Subsequently, SPE was rinsed by Milli-Q water. Afterward, 10 µl of CoNTA and CoNTA-Ab were fixed on the electrode surface by putting a neodymium magnet on the backside of the SPE directly under the working electrode (Fig. 1). Analytical performance and electrochemical characterization of CoNTA coated electrodes were analyzed by 100 µL of fresh prepared water-soluble redox probe (5.0 mM HCF, in 0.1 M KCl). DPV technique was used to demonstrate the signal responses with a potential range from -0.4 to +0.8 V. The biosensor responses were calculated by using the difference in DPV signals before and after the addition of analytes which gives the difference between current values (Δ µA). The calibration curve of cocaine was obtained from different standard concentrations (5, 25, 50, 100, 250, 500 ng/mL).

On another side, the different forms of nanoparticles (CoNTA, CoNTA-Ab, and CoNTA-Ab-COC) were analyzed under scanning electronic microscopy (SEM) to characterize the shape and structure of the resultant products using Quanta FEG 250 (FEI, Oregon, USA).

The biosensor platform made was tested for specificity using different interferents. Briefly, 100 ng/mL of benzoylecgonine (BE), JWH-073, codeine, and methamphetamine were tested in the same way described earlier for cocaine and the response signal of the sensor was monitored.

2.4. Confirmation of the biosensor with LC-QTOF/MS

For the confirmation analysis, synthetic urine, serum, sweat, and saliva samples were spiked with cocaine (100 ng/mL) and then injected into the LC-QTOF/MS system. A calibration curve (50–1000 ng/mL) of cocaine was prepared from a 1000 ng/mL stock solution. Calibration chromatogram (EIC) of each matrix sample was shown in the Supplementary Information Figs. S1–S3 according to m/z 304. Details on the experimental conditions are found in the supplementary information section.

2.5. Statistical analysis

Statistical analysis was performed using a one-way ANOVA test between the different groups (GraphPad Prizm Ver.8, San Diego). $p < 0.05$ was considered as statistically significant.

3. Results & discussion

Given the recent development in the biosensors technologies, many biocompatible, potent, and easy-to-use components have taken the front. This allowed the many obstacles seen in electrochemical biosensors development to be overcome and provide strong immobilization platforms. Amidst the many materials being focused on, metallic nanoparticles and proteins represent a great target for creating highly specific structures that are both stable and manageable in abuse drug detection and monitoring [38,39].

scFv cocaine-binding antibody was heterologously expressed in *E. coli* cells and successfully refolded from inclusion bodies using sarkosyl. *E. coli* has been proven as a cost-effective platform for the production of non-glycosylated antibody fragments [40]. However, recombinant antibody fragments are mostly expressed in insoluble form as inclusion bodies in *E. coli* cytoplasm. Different refolding technologies have been successfully used in order to obtain functional antibody fragments from inclusion bodies [41–44]. In this study, 1% sarkosyl was shown to be effective in refolding cocaine antibody. The solubilized His-tagged antibody was purified using nickel-chelate affinity chromatography (Fig. 2), and the yield was found to be 58.8 mg antibody per liter of *E. coli* culture.

The potential of selective cocaine immunosensors is of great interest to the affinity-based on-site detection devices. The adsorption of cobalt oxide nanoparticles (CoNTA) and their functionalization with scFv cocaine-binding antibody were characterized using microscopic and electrochemistry-based methods. Fig. 1 represents the approach taken to achieve the functionalized form (CoNTA-Ab) and the mobilization over the SPE.

The electrochemical assessment of the surface modification was demonstrated through CV and EIS measurements. Firstly, the electrochemical conduct of the bare SPE or the other modified forms (SPE-CoNTA, SPE-CoNTA-Ab, and SPE-CoNTA-Ab-COC) were estimated and compared with regard to their current responses showed in the CV diagrams (Fig. 3).

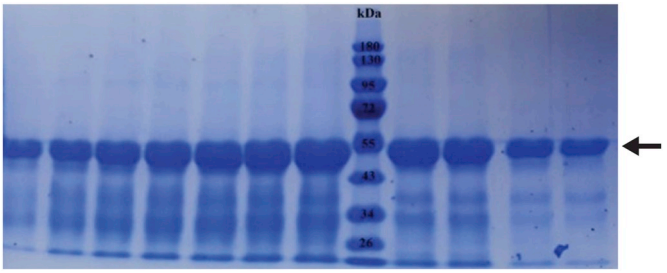

Fig. 2. SDS-PAGE analysis of the eluted fractions of His-tagged scFv cocaine antibody eluted with 300 mM imidazole.

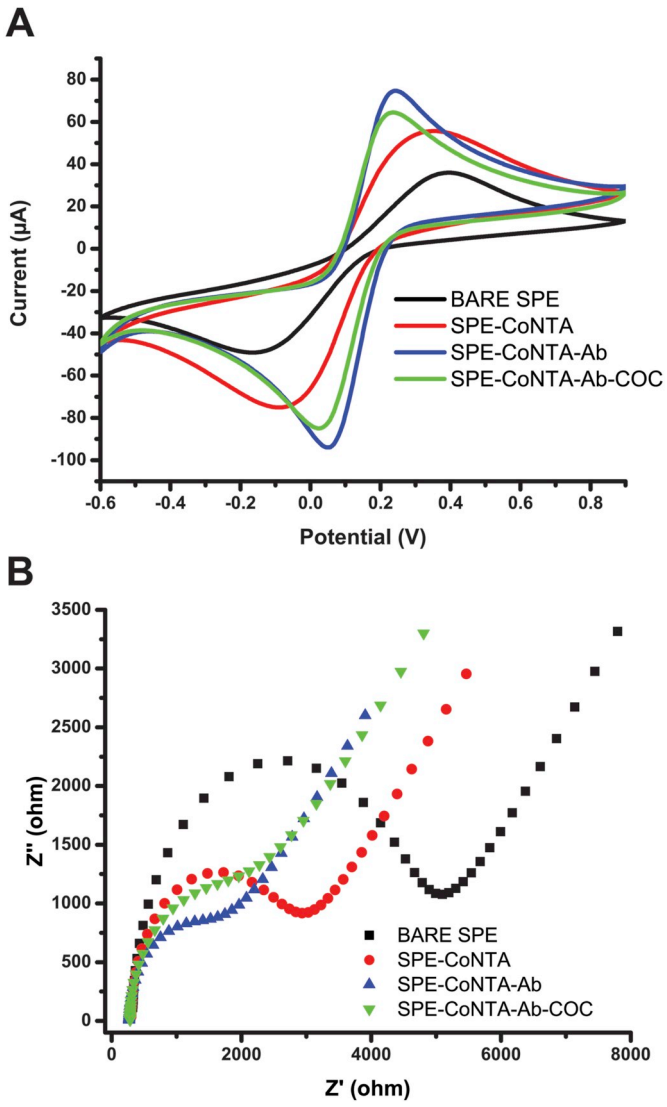

Fig. 3. Cyclic voltammetry (CV) (A) and electrochemical impedance spectrometry (EIS) (B) of Bare SPE, SPE-CoNTA, SPE-CoNTA-Ab, and SPE-CoNTA-Ab-COC surfaces.

The changes in the anodic and cathodic current peaks were followed for each form. The cathodic peak current value for bare SPE was registered as -44.6 μA. As for SPE-CoNTA, it reached -71.2 μA. SPE-CoNTA-Ab reached -101.1 while SPE-CoNTA-Ab-COC showed a value of -89.8. On the other hand, the anodic peak current values observed were 35.8 μA for bare SPE, 58.1 μA for SPE-CoNTA, 87.1 μA for SPE-CoNTA-Ab, 76.3 μA for SPE-CoNTA-Ab-COC. The obtained peak to peak separations measured 0.519 V for bare SPE, 0.356 V for SPE-CoNTA, 0.185 V for SPE-CoNTA-Ab, and 0.210 V for SPE-CoNTA-Ab-COC.

As observed in Fig. 3A, the bare SPE surface showed the initial oxidation and reduction current peak which was quite broader than the peaks obtained after surface modification. The cobalt oxide nanoparticles (CoNTA) layer increased and narrowed the current peak. Furthermore, scFv cocaine-binding antibody coating further increased and narrowed the observed current peak. These observations are similar to our previous findings where polyamidoamine (PAMAM) dendrimers were able to increase the peak potential due to the positively charged amine groups [45,46]. On the other hand, the addition of cocaine decreased the peak due to a hindering of electron transfer.

EIS is another approach to further confirm the successful electrocatalytic interface modification. Raw EIS data were fitted according to the parameters described in our previous work [47]. The parameters

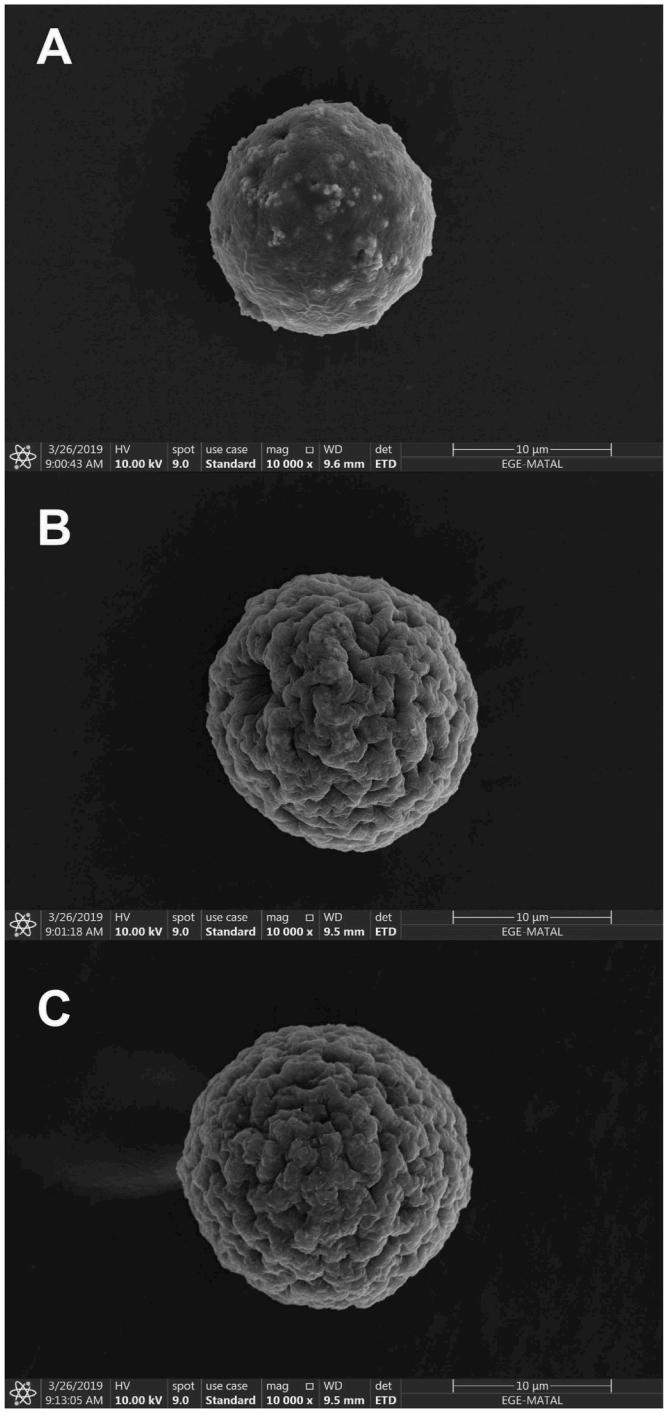

Fig. 4. SEM micrographs of (A) CoNTA, (B) CoNTA-Ab and (C) SPE-CoNTA-Ab-COC.

included in the Nyquist plot include solution resistance (Rs), Warburg impedance (Zw) from [Fe(CN)6](3-/4-) redox probe diffusion, the double-layer capacitance (Cdl) and the charge transfer resistance (Rct). The Rct values observed in Fig. 3B showed an overall significant decrease. Bare SPE reached an Rct value of 4299 Ω, 2303 Ω for SPE-CoNTA, 1244 Ω for SPE-CoNTA-Ab, and 1591 Ω for SPE-CoNTA-Ab-COC. Further characterization of the modified structure of the cobalt oxide nanoparticle with the antibody fragments before and after interacting with the analyte was performed using SEM technique monitoring surface morphology (Fig. 4). The shape of the CoNTA beads can be confirmed as spherical shape (Fig. 4A) to which the biofunctionalization with the scFv chains through the interaction with their poly histidine-tags can be confirmed via the increase in the size and the texture on the surface of the particles (Fig. 4B). Similarly, the addition of cocaine could be confirmed through the observation of crystal-shaped structures on the surface as well as a further increase of the particle size (Fig. 4C). It has been suggested that the shape of the used nanoparticles could influence the biosensor. Spherical NPs demonstrate ample adsorbing sites and high surface which can reserve biocompatibility and consequently, by improving the amount loaded, there is a great enhancement of the biosensing activity [48].

In order to evaluate the analytical performance of the SPE-CoNTA-Ab electrode in sensing cocaine, the DPV method, which is considered more consistent than CV, was performed on the electrode using different concentrations of cocaine. The procedure is based on the current signals calculated from the difference between the peak signals of SPE-CoNTA-Ab and in the presence of different concentrations of the analyte (cocaine 5.0–500 ng/mL). After addition of the different concentration of cocaine to the SPE-CoNTA-Ab, DPV signals decreased as demonstrated in (Fig. 5A). Furthermore, a standard curve in the range of 5–250 ng/mL of cocaine was obtained with a linear equation of $y = 0.216x + 6.905$ ($R2 = 0.998$) (Fig. 5B). Given the range and sensitivity of the current biosensor, we can stipulate its potential as a good candidate for cocaine detection.

The repeatability and limit of detection (LOD) of the SPE-CoNTA-Ab were also explored. The repeatability of the platform was investigated after 6 successive measurements where a ± 1.15% S. D and a 3.87% coefficient of variations (c.v) were observed. As for cocaine LOD, it was calculated using the 3Sb/m formula and gave a value of 3.6 ng/mL ($n = 6$) based on the standard deviation of the intercept and the slope of the calibration curve. The use of magnetic nanoparticles over SPE confers a reusable criterion to the platform. The elimination of the magnet from the back of the electrode surface followed by a simple wash makes the SPE reusable. Though a fully-reusable biosensor has not been described, the current platform can show a cost-effective performance and make a stable base for the development of reusable biosensors.

The concentrations of 100 ng/mL spiked matrices samples was measured with LC-QTOF/MS analysis and our biosensor as described in the experimental section. The concentrations obtained from both methods are summarized in Table 1. The results showed a good sensitivity of our biosensor compared to the traditional method. All results of the current biosensor are close to the initial 100 ng/mL cocaine deposited before ($p > 0.05$). While the only instance where the biosensor showed lower performance than the traditional method is on synthetic urine. These results further confirm the successful performance of the designed biosensor. The raw data of the EIC chromatogram and mass spectra obtained from LC-QTOF/MS were included in the Supporting Information (Figs. S4–S7).

In order to evaluate the selectivity of the current cocaine biosensor, the performance of the sensor was analyzed in the presence of some molecules that can interfere in the detection process due to structural similarities and other factors. We have tested the sensor platform with Benzoylecgonine (BE), JWH-073, Codeine, and methamphetamine. 100 ng/mL of each molecule was added to the biosensor in the same experimental conditions performed for cocaine. The results showed no significant positive change for these molecules compared with cocaine (Fig. 6). This data gives a strong statement on the specificity and selectivity of the designed SPE-CoNTA-Ab sensor for cocaine detection. The observed specificity is consistent with the binding properties of the scFv antibody, which has been originally designed for use in cocaine abuse immunotherapy [34]. The scFv antibody shows excellent specificity for cocaine compared to chemically similar metabolites. In this study, we aimed to show that the scFv antibody could be useful for not only immunotherapy but also biosensor construction. As a future improvement, the cocaine-binding scFv antibody could be subjected to protein engineering and the ligand-binding pocket could be redesigned

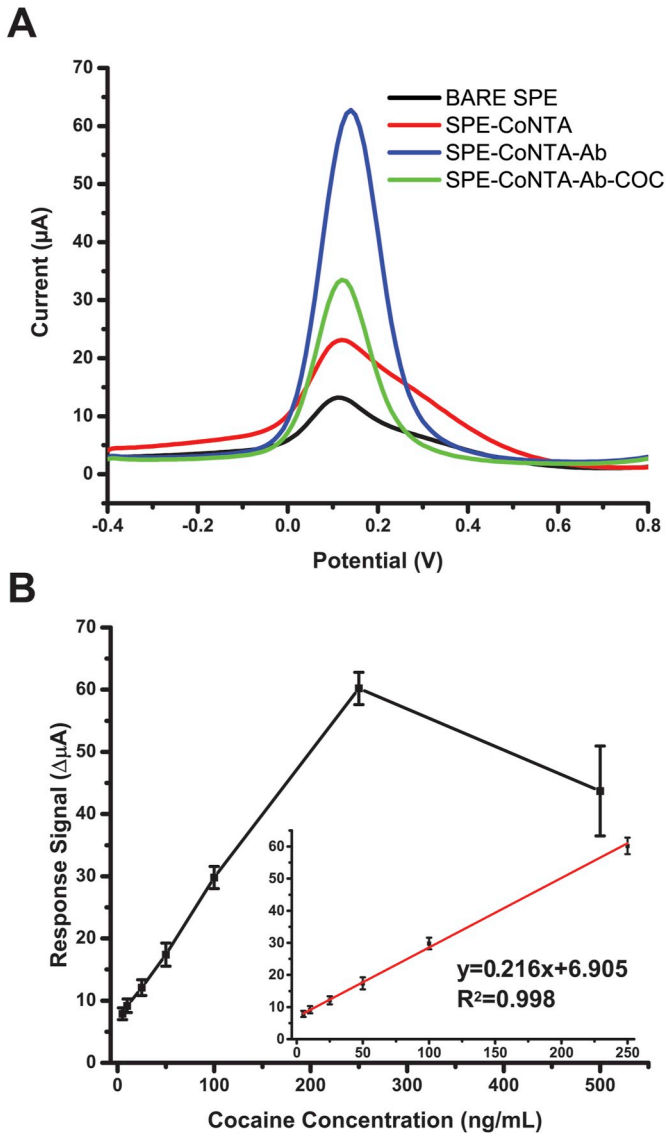

Fig. 5. Differential pulse voltammetry (DPV) curves of Bare SPE, SPE-CoNTA, SPE-CoNTA-Ab and SPE-CoNTA-Ab-COC surfaces (A). Calibration curve of SPE-CoNTA-Ab-COC obtained by DPV technique (B).

Table 1
Comparison of cocaine sensing using our biosensor and LC-QTOF/MS analyses.

	Biosensor (ng/mL)	LC-QTOF/MS (ng/mL)	Statistical significance
Urine	85 ± 1	101	***
Sweat	92 ± 4	85	*
Saliva	107 ± 1	88	***
Serum	96 ± 2	70	***

Statistical significance was calculated for Biosensor vs. Standard method. $^{*}p < 0.05$; $^{***}p < 0.001$.

to detect cocaine metabolites.

Cocaine testing is a common practice in companies, therapy programs, and legal and forensic investigations [49]. There exist many advantages for on-site substance abuse testing including the simplicity of the devices, and no training is needed to perform the analysis. The time needed for collecting samples to obtaining results is relatively short which can circumvent the inefficiencies seen with off-site protocols. On-site tests have an important potential in public safety and drug crimes which can be used legally as evidence during legal proceedings and making arrests [50]. It should be noted that cocaine has a short life

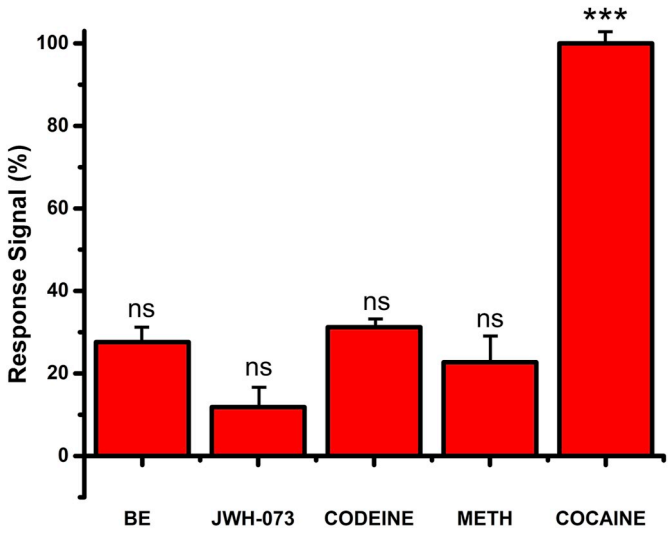

Fig. 6. Biosensor selectivity in the presence of different interferents. ns = non-significant; $^{***}p < 0.001$ vs. SPE-CoNTA-Ab.

in the bloodstream (around 1 h) which compels the authorities and biomedical people to explores metabolites that can subsist for a longer period in body fluids such as urine [49]. However, in some cases, forensic scientists and medico-legal investigators are often advised to consider further these results due to being presumptive. Surface contamination especially seen in the hair are sometimes misinterpreted as substance use and may result in wrong accusations. For all the mentioned above, a need for devices able to detect parent drugs or their metabolites is necessary depending on the type of the application intended for the device which further highlights the importance of the currently proposed cocaine biosensor.

4. Conclusion

A novel Single-chain antibody fragments Functionalized Cobalt nanoparticle biosensor for cocaine detection has been reported in the current work. We have described the bio-functionalization of cobalt oxide nanoparticles and immobilization over an SPE electrode platform. This novel biosensor showed interesting selectivity and sensitivity to cocaine compared to other abuse drugs molecules. Our proposed biosensor presents great potential for mobile detection devices due to its good affinity, easy preparation, adaptability, and portability. Furthermore, given the small size of the biosensor, it gives a great advantage thanks to the necessity of low sample volumes. Given the positive performance of the current biosensor, we believe such devices will contribute greatly to the on-site detection of abuse drugs use.

Author contributions

The manuscript was written through the contributions of all authors who read and approved the final version of the manuscript.

CRediT authorship contribution statement

Serdar Sanli: Conceptualization, Investigation, Methodology, Validation. **Hichem Moulahoum:** Conceptualization, Formal analysis, Writing - review & editing, Visualization. **Ozge Ugurlu:** Conceptualization, Investigation, Methodology, Validation. **Faezeh Ghorbanizamani:** Conceptualization, Writing - original draft. **Zinar Pinar Gumus:** Formal analysis, Methodology. **Serap Evran:** Writing - review & editing, Supervision. **Hakan Coskunol:** Writing - review & editing, Supervision. **Suna Timur:** Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SEM and Chromatographic analyses were performed at EGE MATAL (Ege University/Izmir).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2020.121111.

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