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Aptamer-based electrochemical assay of 17β-estradiol using a glassy carbon electrode modified with copper sulfide nanosheets and gold nanoparticles, and applying enzyme-based signal amplification

Ke-Jing Huang · Yu-Jie Liu · Ji-Zong Zhang

Abstract We have developed an electrochemical method for the determination of 17β-estradiol. A glassy carbon electrode was modified with a composite made from copper sulfide nanosheets, gold nanoparticles, and glucose oxidase. The copper sulfide nanosheet was prepared by a single-step hydrothermal process, and its properties were characterized by X-ray powder diffraction, X-ray photoelectron spectroscopy, scanning electron microscopy and transmission electron microscopy. Finally, an estradiol-specific aptamer was assembled on the electrode. The copper sulfide nanosheet on the electrode surface acts as a relatively good electrical conductor. Glucose oxidase acts as an indicator, and the dual modification of glucose oxidase and gold nanoparticles for signal amplification. The determination of 17β-estradiol was performed by differential pulse voltammetry of glucose oxidase because the signal measured at typically −0.43 V depends on the concentration of 17β-estradiol because addition of 17β-estradiol at electrode hinders electron transfer. A linear relationship exists between the peak current and the logarithm of concentration of 17β-estradiol in the 0.5 pM to 5 nM range, with a 60 f. detection limit (at $3\sigma/S$). The method displays good selectivity over bisphenol A, 1-aminoanthraquinone and naphthalene even if present in 100-fold concentrations.

K.-J. Huang (✉) · Y.-J. Liu · J.-Z. Zhang
College of Chemistry and Chemical Engineering, Xinyang Normal University, Xinyang 464000, China
e-mail: kejinghuang11@163.com

K.-J. Huang
State Key Laboratory of Chemo/biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, China

Introduction

17β-estradiol has been listed as a typical environmental endogenous estrogen, which can disrupt the endocrine system, further causing adverse effects on the reproduction, growth and development of the body, and endanger the health of offspring [1]. 17β-estradiol is ubiquitous in the water, food and feed additive, and it can be accumulated in the environment and organism body owing to bad biodegradability and then interferes to the normal physiological processes and create many deleterious effects [2, 3]. So, analysis of 17β-estradiol at trace level is very important.

Usually, the methods for determination of 17β-estradiol are mainly based on HPLC assays [4, 5]. However, expensive instrumentation, time consuming extraction or preconcentration steps and skilled operators are required. Electrochemical methods overcome these difficulties and become the preferred choice due to their inherent advantages such as low cost of instrumentation and operation, simplicity for operators and on-site monitoring [6, 7]. But, an obstacle is that the bare electrode can not offer enough sensitivity to determine 17β-estradiol at ultra-trace level. The selectivity is another challenge for an electrochemical approach. Therefore, there are some works focused on biomolecules, such as antibodies, modified on the electrode to detect 17β-estradiol selectively [8]. Aptamers are artificial single-stranded DNA or RNA oligonucleotides which can recognize various targets including small species, sugars, proteins, and even whole cells with high specificity [9, 10].

Nanomaterials, such as nanoparticles, nanosheets and nanotubes, are widely applied for signal amplification in electrochemical assay. They usually provide the high specific

surface area for enhancement of mass transport, the high loading receptor molecules for synergistic amplification of the target response [11, 12]. Two-dimensional (2D) transition-metal chalcogenides, such as FeS, WS$_2$ and MoS$_2$, have received significant attention recently because they offers many advantages for electrochemical sensing [13–16]. This kind of material is composed of the metal layer and sulfur layer and stacked together by weak Van der Waals interactions. It has been expected to act as an excellent functional material because the 2D electron–electron correlations among metal atoms would be helpful in enhancing planar electric transportation [17–19].

Au nanoparticles (AuNPs) are mostly recommended owing to the fact that they can greatly increase the current response of the modified sensor with a good conductive ability and immobilization of biomolecular by Au-S bond, and have been widely used to construct aptamer sensors [20, 21].

In this work, a 2D CuS nanosheet was prepared by a simple hydrothermal method. Then a novel electrochemical sensing platform was fabricated for 17β-estradiol detection based on CuS nanosheets coupled with glucose oxidase (GOx) as indicator and aptamer as recognition group. GOx and gold nanoparticles (AuNPs) were dually modified on the electrode for providing signal amplification for electrochemical sensing. Under optimal experimental conditions, the developed electrochemical assay showed excellent sensitivity and good selectivity to 17β-estradiol, and has been applied for urine samples analysis.

Experimental

Materials

Cu(NO$_3$)$_2$·3H$_2$O, H$_2$NCSNH$_2$, chitosan (deacetylation degree ≥95 %), HAuCl$_4$·4H$_2$O, 17β-estradiol and its aptamer were obtained from Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China, http://www.sangon.com/). The sequences of the aptamer was: 5'-SH-(CH$_2$)$_6$-TTT TTT TTT T GCT TCC AGC TTA TTG AAT TAC ACG CAG AGG GTA GCG GCT CTG CGC ATT CAA TTG CTG CGC GCT GAA GCG CGG AAG C-3'. It was dissolved in 100 mM Tris–HCl buffer (pH 8.0, containing 200 mM NaCl, 25 mM KCl, 10 mM MgCl$_2$ and 5 % ethanol) before use. 0.1 M phosphate buffer solution (pH 7.0) was prepared with 0.1 M Na$_2$HPO$_4$ and NaH$_2$PO$_4$ and adjusted by 0.1 M H$_3$PO$_4$ or 0.1 M NaOH solutions. All reagents were of analytical grade and used without further purification.

Apparatus

Electrochemical measurements were performed on a CHI 660E Electrochemical Workstation (Shanghai CH Instruments, China, http://www.instrument.com.cn/netshow/SH101344/). A conventional three-electrode system was used which composed of platinum wire as auxiliary, saturated calomel electrode (SCE) as reference and modified GCE as working electrode. A JEM 2100 transmission electron microscope (TEM, http://www.jeol.de/electronoptics-en/index.php) and a Hitachi S-4800 scanning electron microscope (SEM, http://www.hitachi.com/) were used to record the morphologies of the CuS samples. X-ray diffraction (XRD) spectroscopy was recorded on a Bruker Inc. (Germany, http://www.bruker.com/) AXS D8 ADVANCE diffractometer (Cu Ka radiation). Raman spectra were recorded on a Renishaw Raman system model 1000 spectrometer (www.renishaw.com) at an excitation wavelength of 514.5 nm. X-ray photoelectron spectroscopy (Thermo Electron Corp., USA, http://www.thermoscientific.com) was used to analysis of the composition of CuS.

Preparation of Au nanoparticles

AuNPs were prepared according to a previous protocol [22]. 100 mL 0.01 % HAuCl$_4$ solution was boiled with vigorous stirring, and 2.5 mL of 1 % trisodium citrate solution was then quickly added. The solution turned deep red, indicating the formation of AuNPs. Upon continued stirring and cooling own, the resulting Au colloidal solution was stored in brown glass bottles at 4 °C before use.

Synthesis of CuS nanosheets

CuS nanosheets were synthesized by a simple one-step hydrothermal process. Briefly, 0.243 g Cu(NO$_3$)$_2$·3H$_2$O was firstly dissolved in 40 mL glycol and stirred for 0.5 h. Then 0.125 g H$_2$NCSNH$_2$ was added and vigorously stirred for 30 min. The solution was subsequently transferred into a 100 mL Teflon-lined stainless steel autoclave and heated at 150 °C for 24 h. After cooling, the product was collected by filtration, washed with water and absolute ethanol, and dried in vacuum at 60 °C.

Fabrication of the aptamer based modified electrode

The GCE was polished carefully with 0.5 and 0.05 μm alumina slurry, and sonicated in ethanol and deionized water, respectively. A stock solution of 1 mg mL^{-1} CuS was prepared by dispersing 1.0 mg CuS nanosheets in 1 mL water using an ultrasonic bath until a homogeneous suspension resulted. Then 6 μL as-prepared suspension was applied on the pretreated GCE and dried to obtain the modified electrode CuS/GCE. Subsequently, AuNPs were formed on the CuS/GCE by electrochemical deposition at −0.2 V for 80 s in 1.0 mM HAuCl$_4$ containing

0.1 M KCl to obtain AuNPs/CuS/GCE. Then, 5 μL mixed solution containing 20 mg mL^{-1} GOx and 0.25 % chitosan was coated on the AuNPs/CuS/GCE and dried at 4 °C in refrigerator to obtain GOx/AuNPs/CuS/GCE. Afterwards, The GOx/AuNPs/CuS/GCE was incubated in AuNPs (3 mL, 2.5 nM) for 6 h at room temperature to prepare AuNPs/GOx/AuNPs/CuS/GCE. After that, 8 μL aptamer (0.1 μM) was applied on AuNPs/GOx/AuNPs/CuS/GCE and left to incubate for 12 h to obtain aptamer/AuNPs/GOx/AuNPs/CuS/GCE. To block the uncovered gold surface, 4 μL GOx (20 mg mL^{-1}) was applied on the electrode for 12 h, followed by washing with 0.1 M phosphate buffer solution to obtain GOx/aptamer/AuNPs/GOx/AuNPs/CuS/GCE. The electrode was then incubating in the 17β-estradiol solution at 37 °C for 3 h. The schematic diagram of the stepwise procedure of the modified electrode fabrication is shown in Scheme 1. The electrochemical measurement was performed in 0.1 M phosphate buffer solution (pH 7.0) containing 1.0 mmol L^{-1} [Fe(CN)$_6$]$^{3-/4-}$ and 0.1 mol L^{-1} KCl by cyclic voltammetry (CV) and differential pulse voltammetry (DPV).

Protocol for performing the assay

1 mL urine sample obtained from three volunteers was first diluted 100 times with phosphate buffer solution (pH 7.0), and then spiked with different concentrations of 17β-estradiol. Subsequently, 10.0 mL sample was added in an electrochemical cell and analyzed directly with the as-prepared the modified electrode 17β-estradiol/GOx/aptamer/AuNPs/GOx/AuNPs/CuS/GCE at the room temperature. The DPV signal was recorded at about −0.43 V for quantitative analysis.
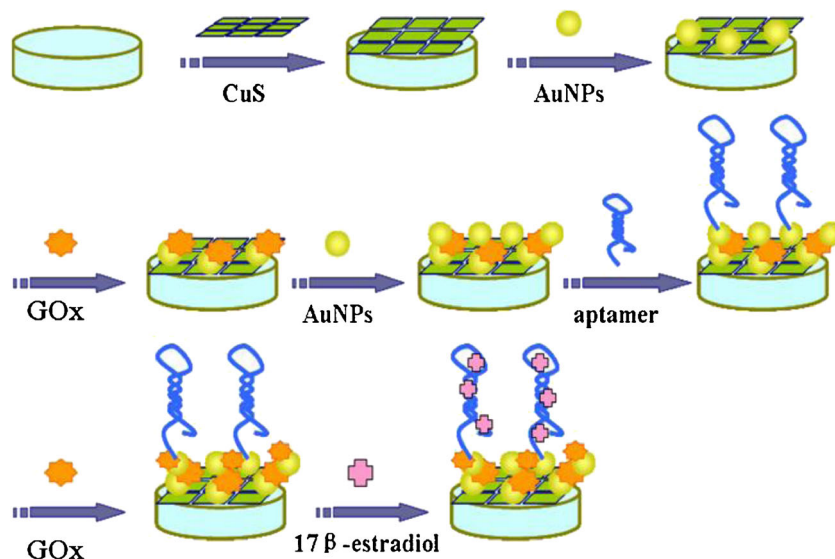
Results and discussion

Choice of materials

CuS, as an important transition-metal chalcogenide semiconductor, is inexpensive and abundant material with wide spread applications as optical materials [23], solar cell materials [24], catalysts [25]. In addition, CuS is also used as a cathode material for lithium ion batteries [26] and the materials in electrochemical sensing platform [27] due to its metal-like electronic conductivity (10^{-3} S cm^{-1}). Using 2D CuS nanosheets in the construction of electrochemical sensing platform will improve its response signal with due to their low background current, good conductivity and large electroactive surface area. Au nanoparticles (AuNPs) are mostly recommended owing to the fact that they can greatly increase the current response of the modified sensor with a good conductive ability and immobilization of biomolecular by Au-S bond, and have been widely used to construct aptamer based assays [28, 29]. As a redox enzyme, GOx is generally known to exhibit a reversible reaction between its oxidized quinone form (flavin adenine dinucleotide; FAD) and reduced hydroquinone form (FADH$_2$). In this work, upon immobilizing GOx to an AuNPs modified CuS/GCE, more AuNPs were again attached to the modified electrode and followed by more GOx to introduce signal amplification capability to the sensing platform, which would in turn lead to a low detection limit. A novel reagentless and mediatorless electrochemical assay was therefore developed based on the direct electron transfer (DET) of GOx for the detection of 17β-estradiol.

The morphologies and structures of CuS nanosheets

The morphology and particle size of the as-prepared CuS were investigated by SEM and TEM. As shown in Fig. 1A and B,

Scheme 1 Schematic diagram of the construction of modified electrode

Fig. 1 SEM (A, B) and TEM (C) images of the CuS nanosheets
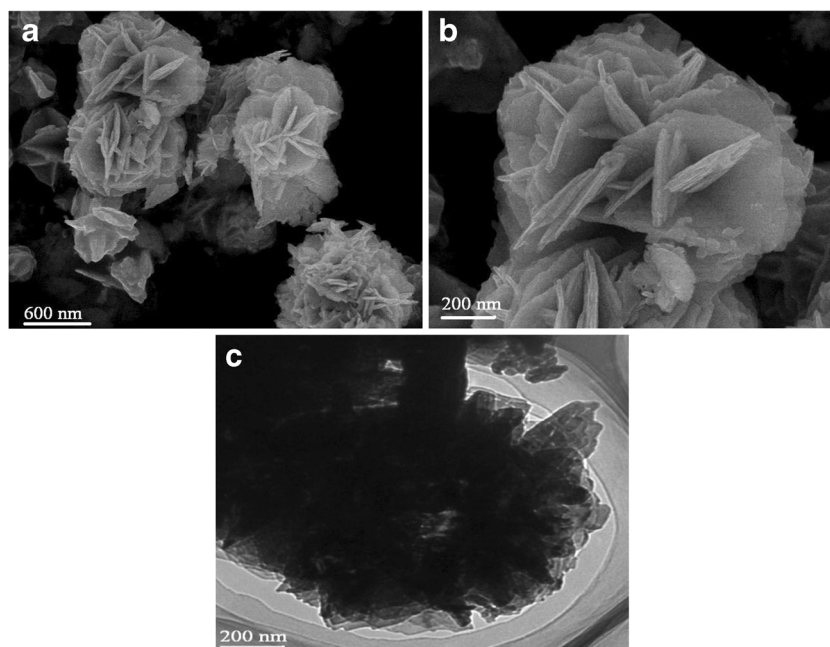
the CuS product demonstrates a structure of nanoflowers with diameters of 900–1000 nm, and the flower-like structures are composed of many self-assembled and uniform interlaced nanosheets. To further specify the microstructure of the CuS, the TEM image is shown in Fig. 1C. As shown in the figure, the nanoflowers lightly curve and gradually tenuate toward the edges. The nanoflowers are constituted by a great deal of stagger nanosheets and the mean thickness of the nanosheets is about 20 nm.

Figure 2A shows the XRD patterns of the as-prepared CuS samples. The XRD patterns shows main peaks with 2q values of 13.4, 28.1, 29.2, 31.8, 32.7, 48.0, 52.7, and 59.5° corresponding to (002), (101), (102), (103), (006), (110), (108), and (116) crystal planes of pure CuS, respectively. These can be perfectly identified as the pure hexagonal phase of CuS (JCPDS card no.06-0464). The absence of peaks corresponding to the precursors, copper oxide, or other phases of copper sulfide indicates the purity of the product. The strong and sharp diffraction peaks suggest that the as-obtained products are well crystalline.

Laser Raman spectroscopy is an effective method to study the material structure. It is applied to explore the surface layer structure of nanometer-sized crystals of CuS. As shown in Fig. 2B, a strong and sharp peak is measured at 474 cm^{-1}. The peak probably originates from the lattice vibration, which is very similar to the result reported previously [30].

Significant information on the surface electronic state and the chemical composition of CuS samples can be further provided by XPS measurements, as shown in Fig. 2C–E. The wide scan XPS spectra of CuS samples (Fig. 2C) clearly indicates that the sample is composed of Cu, S, C, and O, and no peaks of other elements were observed. The high resolution

XPS spectrum of Cu 2p region is shown in Fig. 2D. The measured binding energies of Cu 2p$_{3/2}$ and Cu 2p$_{1/2}$ are about 931.9 eV and 951.8 eV, respectively. These binding energies indicate that the element of copper presented in the products is Cu(II). In Fig. 3E, the close-up survey at the S 2p region shows the presence of a double of peaks at 162.3 and 168.7 eV, which indicates the presence of metal sulfides.

Characterization

The electrochemical properties of different modified electrodes were investigated by CV in solution containing 1 mM [Fe(CN)$_6$]$^{3-/4-}$ as the indicator substance. As shown in Fig. 3A, there is a pair of redox peaks at the bare GCE (curve a). After modification of CuS nanosheets, the redox peaks obviously increase and the peak potential difference (ΔE_p) between anodic and cathodic peak decrease (curve b). These indicate CuS nanosheet increases the effective surface area of electrode and improves the electrocatalytic performance. After attachment of AuNPs on the CuS/GCE, the redox peaks greatly increase and ΔE_p further decreases (curve c). The phenomena may be attributed to the excellent electrocatalytic activity, high conductivity, large specific surface area and synergistic effect of the AuNPs and the CuS nanosheets.

For studying the effect of signal amplification of the developed assay, the CVs of different modified electrodes in 0.1 M phosphate buffer solution (pH 7.0) containing 0.9 % NaCl are shown in Fig. 3B. Obviously, no redox peak is observed at AuNPs/CuS/GCE (curve a). After the modification of GOx on the AuNPs/CuS/GCE, a pair of well defined redox peaks is obtained, attributing to the redox reaction between FAD and FADH$_2$ (curve b). When AuNPs are remodified on the GOx/

Fig. 2 XRD patterns (A) and Raman spectra (B) of the CuS nanosheets; XPS survey spectra of the CuS nanosheets (C); high-resolution spectra for Cu 2p (D); high-resolution spectra for S 2p (E)
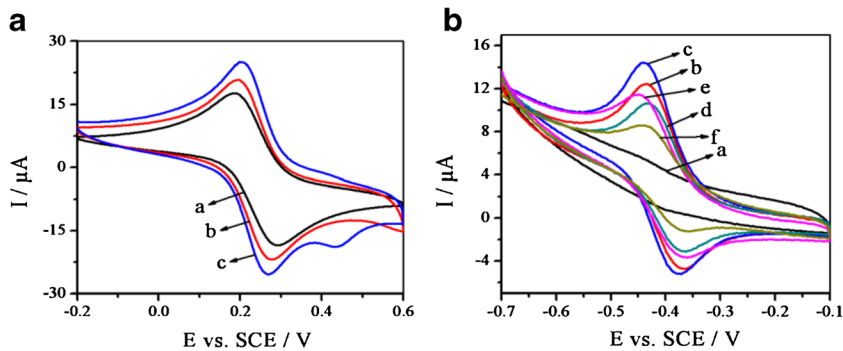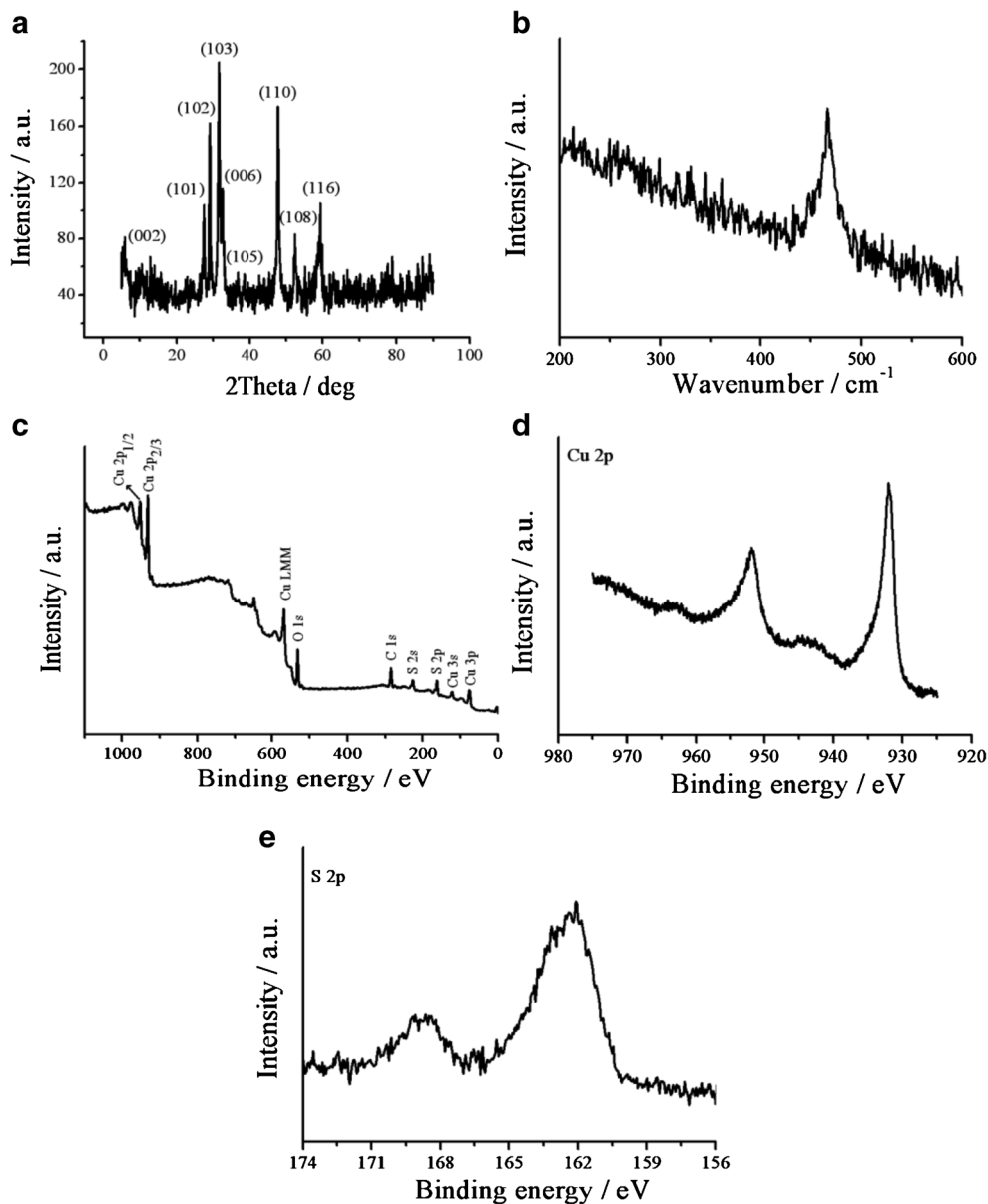

Fig. 3 (**A**) CVs of (a) GCE, (b) CuS/GCE and (c) Au/CuS/GCE in 1 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.1 M KCl; (**B**) CVs of different electrodes in 0.1 M phosphate buffer solution (pH 7.0) containing 0.9 % NaCl: (a) AuNPs/CuS/GCE, (b) GOx/AuNPs/CuS/GCE, (c) AuNPs/GOx/AuNPs/ CuS/GCE, (d) aptamer/AuNPs/GOx/AuNPs/CuS/GCE, (e) GOx/ aptamer/AuNPs/GOx/Au/NPs/CuS/GCE, (f) 17β-estradiol/GOx/ aptamer/AuNPs/GOx/AuNPs/CuS/GCE

AuNPs/CuS/GCE, the peak currents greatly improve (curve c), suggesting that AuNPs facilitate electron transfer. However, the redox peak currents significantly decrease after aptamer is attached to the electrode (curve d), which is likely to have arisen from blockage by the large oligonucleotides for electron transfer on the electrode. Subsequently, when the modified electrode is blocked by GOx, the peak currents obviously increase (curve e). Following the reaction between 17β-estradiol and the aptamer, a dramatic decrease in peak currents is observed (curve f), attributing to the product on the surface of electrode retardation of the electron transfer. The results prove that the modified electrode works indeed as described in the principle scheme.

Detection scheme

As shown in Scheme 1, a novel reagentless and mediatorless electrochemical aptasensor was developed based on the DET of GOx for the detection of 17β-estradiol. AuNPs was firstly immobilized on WS_2 modified GCE surface by electrochemical deposition, and then GOx was immobilized based on Au-thiol interaction. Then, Au nanoparticle as an electron relay were deposited on it for immobilization of 17β-estradiol aptamer. GOx was subsequently served as blocking reagent to block remaining active sites. GOx as one of the redox enzymes could exhibit redox activity from the reversible redox reaction of $FAD/FADH_2$ redox couple, which was the oxidized and reduced form of the GOx active redox center, respectively. After the target 17β-estradiol reacted with aptamer to form the estradiol/aptamer complex on the electrode surface, the DET signal decreased due to the increasing spatial blocking around GOx molecules, giving the quantitative foundation for 17β-estradiol detection.

Optimization of variable parameters

To obtain good analytical performance, several experimental conditions were optimized. The effect of aptamer concentration was evaluated by varying its concentration on the AuNPs/GOx/AuNPs/CuS/GCE. The relationship between the DPV response and the aptamer concentration is shown in Fig. 4A.

The peak current increases when the aptamer concentration is in the range of 0.01-0.1 μM and then decreases when it exceeds 0.1 μM. Thus 0.1 μM aptamer was used.

The pH values of the substrate solution play the important role in the electrochemical reaction since the activity of the immobilized GOx is pH dependent. As shown in Fig. 4B, the peak current increases with the increasing of pH value from 4.0 to 7.0 and then decreases when it exceeds 7.0. So 0.1 M phosphate buffer solution (pH 7.0) was used in subsequent studies.

Determination of 17β-estradiol

Under the optimized conditions, DPV was applied for the determination of 17β-estradiol with the developed electrochemical assay. Figure 5A shows the DPVs obtained at GOx/aptamer/AuNPs/GOx/AuNPs/CuS/GCE for different 17β-estradiol concentrations. The peak current decreases linearly with the logarithm of the concentration of 17β-estradiol in the range of 5.0×10^{-13}-5.0×10^{-9} M (inset of Fig. 5A). The corresponding regression equation is i (μA)$=-4.74 - 0.72 \log c$ (M) with a correlation coefficient of 0.992. The detection limit of this method, estimated as three times the standard deviation of the blank sample measurements, was 6.0×10^{-14} M. The analytical performance of this fabricated electrode was compared with previously reported different modified electrodes for the determination of 17β-estradiol [31–38]. The comparison results are listed in Table 1. The developed electrochemical assay based on CuS nanosheets, GOx and AuNPs shows significant improvement of the detection limit and linear range. The results indicate that the double modification of GOx and AuNPs greatly enhances the electrochemical signal, which improves the sensitivity of the assay.

The specificity and repeatability

The selectivity of the as-developed electrochemical assay was evaluated and three small organic chemicals (bisphenol A, napthalene and 1-aminoanthraquinone) which bear structural similarity to 17β-estradiol were chosen as control samples. As shown in Fig. 5B, only 17β-estradiol can cause an obvious

Fig. 4 Effects of the 17β-estradiol aptamer concentration (A) and (B) pH value of the substrate solution on the amperometric responses
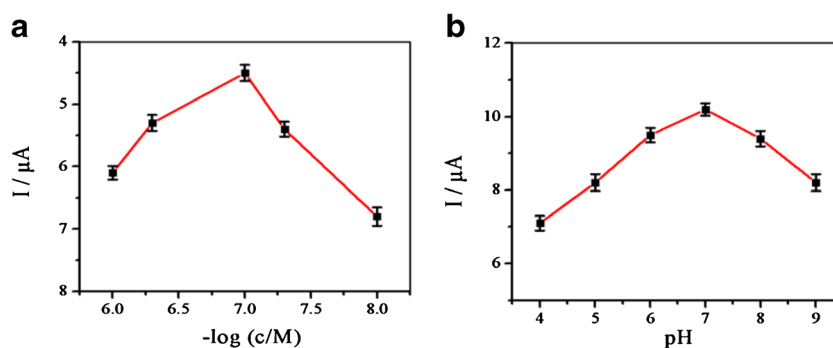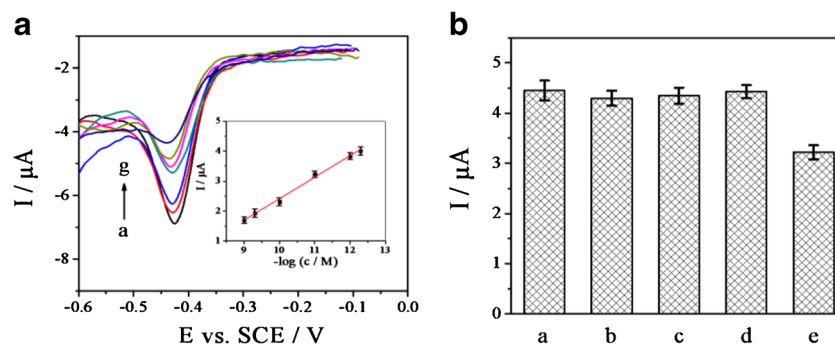
Fig. 5 (**A**) DPVs of the modified electrode after reaction with different concentrations of 17β-estradiol (from a to g): 0, 5.0×10^{-13}, 1.0×10^{-12}, 1.0×10^{-11}, 1.0×10^{-10}, 5.0×10^{-9}, 1.0×10^{-9} M. Inset: the relationship of the peak current with the concentration of 17β-estradiol; (**B**) DPVs of GOx/aptamer/AuNPs/GOx/Au/NPs/CuS/GCE (a) and after reaction with 1.0×10^{-9} M BPA (b), 1.0×10^{-9} M 1-aminoanthraquinone (c), 1.0×10^{-9} M naphthalene (d), and 1.0×10^{-11} M 17β-estradiol (e)

decrease in peak current even when the three control samples are presented at 100-fold concentrations, indicating the developed assay has a good selectivity for the 17β-estradiol detection.

To evaluate the repeatability of the electrochemical assay, the peak currents of ten successive measurements of 5.0×10^{-12} M 17β-estradiol by DPV was determined. The relative standard deviation (RSD) of 2.1 % was obtained. Six parallel-made GOx/aptamer/AuNPs/GOx/AuNPs/CuS/GCEs were also applied to detect a 5.0×10^{-12} M 17β-estradiol and the RSD of 4.3 % was achieved, suggesting good reproducibility of the developed sensing platform.

The stability of the developed electrochemical assay was estimated by detecting 5.0×10^{-12} M 17β-estradiol. When the electrode was stored in the refrigerator at 4 °C for 1 week, it retained 95.2 % of its initial current response, demonstrating good stability.

Practical application

In order to evaluate the performance of developed assay in practical analytical applications, the determination of 17β-

estradiol in urine samples was carried out via a recovery study according to the above-described analytical procedure. The obtained urine samples was diluted 100 times with phosphate buffer solution and then directly detected. The results are shown in Table S1 (Electronic Supporting Material). The recoveries are in the range of 96.3–104.3 % with the RSD in the range of 1.4–3.5 %, indicating good potential of the developed assay for clinical applications.

Conclusion

In this work, CuS nanosheets were prepared by a simple one-step hydrothermal process. A novel electrochemical assay for 17β-estradiol detection was then developed by assembling an aptamer on CuS film modified GCE using GOx as indicator and dual modification of GOx and AuNPs for signal amplification. The combining of CuS nanosheets and AuNPs in the construction of modified electrode efficiently accelerated the electron transfer and enhanced the detection signal, which led to a high sensitivity with a detection limit of 60 fM. The

Table 1 Comparison between the developed assay and other reported method for 17β-estradiol detection

Electrodes	Analytical technique	Linear range (M)	LOD (M)	Ref.
Aptamer/Au electrode	EIS	$1 \times 10^{-8} - 1 \times 10^{-11}$	2×10^{-12}	[31]
PEDOT/AuNPs/Au electrode	SWV	$1 \times 10^{-10} - 1 \times 10^{-7}$	2×10^{-11}	[32]
Bare GCE	DPV	$4 \times 10^{-5} - 1 \times 10^{-3}$	1×10^{-5}	[33]
Poly-serine/GCE	DPV	$1 \times 10^{-7} - 3 \times 10^{-5}$	2×10^{-8}	[34]
nanoPt-MWNT/GCE	SWV	$5 \times 10^{-7} - 1.5 \times 10^{-5}$	1.8×10^{-7}	[35]
Aptamer/AuNPs/VS$_2$/GCE	DPV	$1.0 \times 10^{-11} - 1.0 \times 10^{-8}$	1.0×10^{-12}	[36]
Ag/PAMAM-Au/GR-PANI/GCE	DPV	$1.4 \times 10^{-10} - 2.5 \times 10^{-8}$	7.2×10^{-11}	[37]
GR-dihexadecylphosphate/GCE	LSV	$4.0 \times 10^{-7} - 2.0 \times 10^{-5}$	7.7×10^{-8}	[38]
GOx/Aptamer/AuNPs/GOx/AuNPs/CuS/GCE	DPV	$5.0 \times 10^{-13} - 5.0 \times 10^{-9}$	6.0×10^{-14}	This work

PEDOT, poly(3,4-ethylenedioxylthiopene); *MWNT*, Multi-walled carbon nanotubes; *SWV*, Square wave voltammograms; *PAMAM*, poly(amino-amine) dendrimers; *GR*, graphene; *PANI*, polyaniline; *LSV*, linear sweep voltammetry

electrochemical assay also displayed excellent precision and accuracy, wide linear range, high selectivity and good repeatability, and was applied successfully for 17β-estradiol determination in real samples.

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