

Novel self-powered sandwich type Aptamer sensor for estrogen detection assisted with peroxidase mimic Cu-MOF

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ARTICLE INFO

Keywords:

Electrochemical biosensor
Biofuel cell
Cu-MOF
17 β -estradiol
Sandwich

ABSTRACT

A novel self-powered and flexible enzymatic biofuel cell (EBFC)-based aptasensor was developed for the sensitive and selective detection of 17 β -estradiol (E2). A flexible polyvinyl alcohol (PVA)-tannic acid-carbon nanotube/reduced graphene oxide (PTCR) substrate was modified with gold nanoparticles (AuNPs) and thiolated aptamer 1 (Apt1) to yield Apt1@AuNPs/PTCR. A copper-based metal-organic framework (Cu-MOF) with peroxidase mimicking activity was employed to anchor glucose oxidase (GOD) and Apt2, forming the Cu-MOF@GOD/Apt2 tag. When E2 was recognized by Apt1, the anchored E2 quantitatively recognized Cu-MOF@GOD/Apt2 to create a Cu-MOF@GOD/Apt2-E2-Apt1 sandwich structure for glucose oxidation to generate electrical power. Increased E2 concentrations enhanced Cu-MOF@GOD/Apt2 capture and amplified the electrical signal. The electrical power increased linearly as the E2 concentration increased from 1.0 pM to 1.0 nM. The sensor was successfully applied to various food samples and blood serum detection. This work promoted the application of novel self-powered biosensors for food safety analysis.

1. Introduction

Steroid estrogen hormones are important for maintaining and developing female physiognomies (Biswas, Chen, Xie, Sun, & Wang, 2020). Among many estrogen hormones, *estra-1,3,5(10)-triene-3,17-diol* (E2), also known as 17 β -estradiol, plays a pivotal role in regulating the menstrual, reproductive and estrous cycles of females (Ye et al., 2024). E2 levels in women are also related to pathological variations in the reproductive system and imbalances in cellular immunity as well as humoral, cardiovascular, immune, and nervous systems (Arduini, 2024). E2 can also be detected in various commercial products, including food, medicine, cosmetics, and others. However, environmental pollution and the natural food supply of E2 are also well recognized. Chronic low exposure to E2 can have harmful biological effects on animals and humans (Wang et al., 2024). To avoid the abuse of E2 in food, it is necessary to develop simple, fast, sensitive and accurate methods for E2 detection.

At present, different approaches, such as fluorescence (Wang et al., 2024), gas phase/liquid chromatography-mass spectrometry (Dufour

et al., 2021), Raman spectroscopy (Chen et al., 2024), enzyme-linked immunoassay (Nuvoli et al., 2024) and electrochemical methods (Xie et al., 2022) are employed for the detection of E2. These methods are accurate and sensitive, but require expensive instruments, complex sample pre-treatment processes and well-trained operators. Simple and inexpensive measurement technologies will be of scientific and commercial importance for rapid and routine food analysis. Electrochemical methods have attracted considerable attention because of their low cost, fast response, portability for on-site detection and suitability for wearable applications (Chen et al., 2022; Hosseini et al., 2024). However, commonly used electrochemical sensors require external power sources which not only increase device volume but also compromise its portability (Xu, Liu, Li, Liu, & Huang, 2023; Yan, Ding, Liu, Liu, & Zhang, 2024; Yang et al., 2024). A promising solution is to integrate an energy-harvesting device into the detection system to realise self-powered sensing. In this regard, enzymatic biofuel cell (EBFC)-based self-powered sensors have demonstrated great potential for sensing applications.

The EBFC is a two-electrode device that utilises enzymes as catalysts

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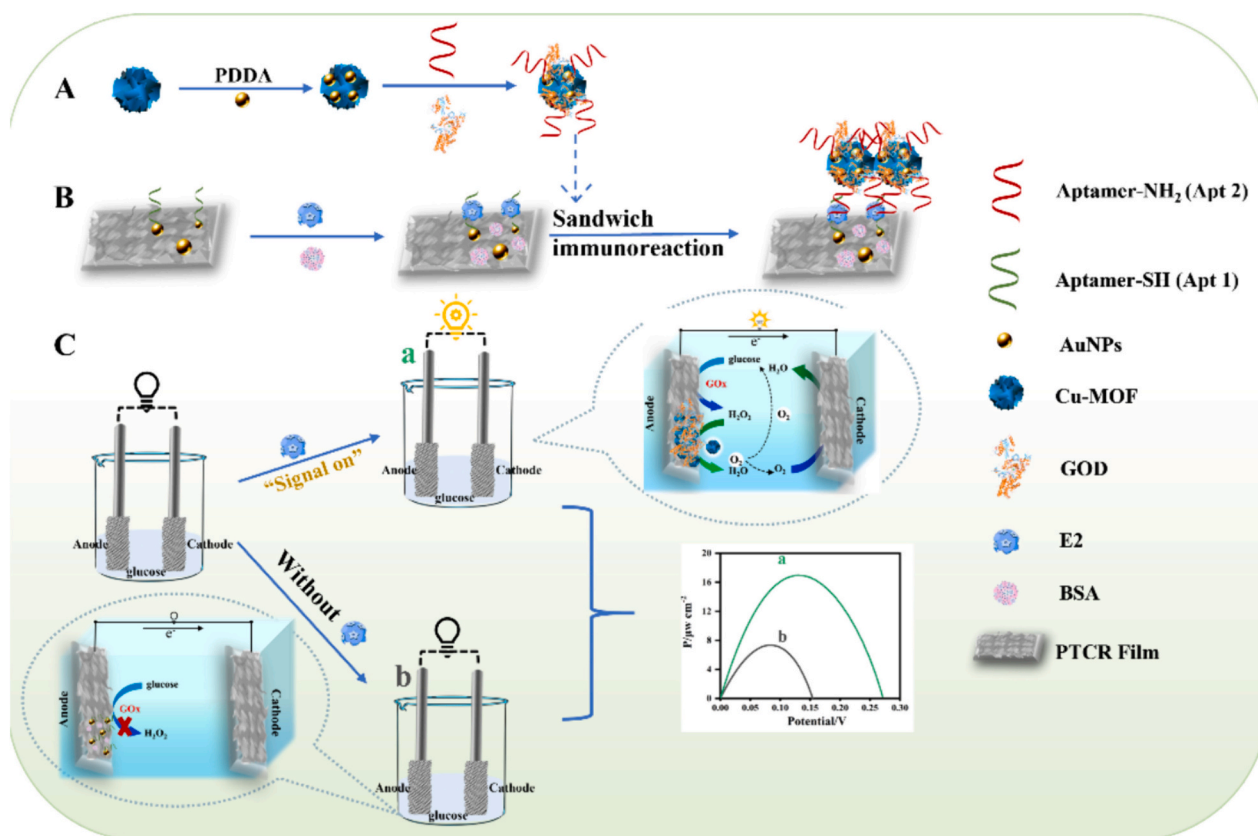
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to convert biofuels, such as glucose, into electricity. In an EBFC, current is generated by coupling the oxidation of glucose at the anode with the reduction of oxygen at the cathode, with the two electrodes connected through an external circuit (Hao, Sun, Zhang, Zhai, & Dong, 2020). No external power source is required, simplifying the apparatus and enabling miniature designs. Because the power output of the EBFCs is commonly proportional to the concentration of fuels or anchored enzymes, embedding EBFCs into the sensor platform can not only supplement the energy consumption of the sensing process but also obtain the concentration information of the target by monitoring the EBFCs' power and open circuit potential (OCP) output with low detection limits and feasibility in the field of portable online monitoring (Gu, Gai, & Li, 2022; Xu et al., 2024). Different strategies such as substrate or enzyme capacity, steric hindrance and gene regulatory effects have been applied to develop EBFC-based self-powered biosensors (EBCFs-SPBs) (Zhang et al., 2021). Based on the signal direction of the measured reaction, detection methods can be divided into 'signal-off' and 'signal-on' modes. The principle of 'signal-off' detection is based on the inhibition of electron transfer at the electrode, which is relatively straightforward; however, it suffers from drawbacks such as high false positive rates and imprecise experimental outcomes (Bai et al., 2023). In contrast, for the 'signal on' mode, the increase in the signal is directly proportional to the concentration of the analyte. 'Signal-on' architecture has a high potential to improve assay sensitivity because these assays are in theory not limited to the signal increase at the upper end of the signal range and selectivity. It is usually the preferred option (Kopra et al., 2019). Although our previous work (Qin, Wang, Yu, Xu, & Hu, 2022) has developed a 'signal-off' self-powered aptasensor to detect dibutyl phthalate based on the steric hindrance effect, we attempted to develop 'signal-on' EBCFs-SPBs with high sensitivity and selectivity. When the concentration of the target is connected to the quantity of enzymes loaded on either the anode or cathode, a 'signal-on' EBCFs-SPB can be

developed. Enzymes are the main components of the 'signal-on' EBCFs-SPB when used as probes of the target. However, enzymes are susceptible to denaturation or inactivation under certain conditions. Furthermore, enzymes can be expensive to produce and purify, which can increase the overall cost. To overcome these problems, it is necessary to introduce artificial or simulated enzymes into EBFC-based sensors.

Copper metal-organic frameworks have been investigated for their peroxidase-like activity in neutral solution (Qin et al., 2017; Wang et al., 2023) because Cu^{2+} in Cu-MOFs can function as a Lewis acid to form coordination bonds with substrate molecules (Huang et al., 2020). This coordination process facilitates the activation of substrate molecules, ultimately improving substrate utilisation efficiency as well as the rate and selectivity of the catalytic reactions. Cu-MOFs have various applications, including biosensors, biofuel cells, and environmental remediation (Pamei, Achumi, Kahmei, Sarkar, & Puzari, 2022; Wang et al., 2023; Wang et al., 2023; Yao et al., 2019). Furthermore, Cu-MOFs exhibit exceptional chemical stability and high porosity (Fang et al., 2024), enabling enhanced enzyme loading and efficient fuel adsorption. By combining Cu-MOFs and GOD to perform cascade reactions under neutral conditions, efficient conversion of fuel can be achieved. The peroxidase mimicking character of Cu-MOF also mitigates the adverse effects of H_2O_2 produced from glucose oxidation (Cui, Fang, Chen, & Tan, 2019) on EBFCs, ultimately enhancing the sensitivity and life of the developed sensor.

Herein, an EBFC-based self-powered biosensor was proposed for E2 detection with outstanding sensitivity via the assistance of peroxidase-mimicking Cu-MOF. A flexible PTCR paper electrode was fabricated through vacuum filtration for use as an EBFC substrate (Fig. S1). The flexible nature of the sensor extended its application to curved surfaces. Scheme 1 shows the construction process of the EBFC-based aptasensor and the E2 detection process and mechanism. Scheme 1A shows the formation process of the Cu-MOF@AuNPs/GOD/Apt2 tag. The Cu-MOF



Scheme 1. Schematic diagrams of (A) the construction process of Cu-MOF@AuNPs/GOD/Apt2 (B) the construction process of Cu-MOF@AuNPs/GOD/Apt2-E2-Apt1 sandwich structure and (C) the working principle of the self-powered sandwich type aptamer sensor for the detection of E2.

was functionalised with positively charged poly(diallyldimethylammonium chloride) (PDAA), which facilitated the assembly of gold nanoparticles (AuNPs) by electrostatic adsorption and led to the formation of Cu-MOF@AuNPs with good conductivity. Subsequently, Cu-MOF@AuNPs were used as the platform for assembling GOD and aptamer 2 (Apt2), resulting in the formation of Cu-MOF@AuNPs/GOD/Apt2 complexes. In this complex, Cu-MOF was selected not only as the immobilisation matrix for GOD and aptamer 2 due to its high surface area but also as a peroxidase mimicking character to eliminate the effect of the produced hydrogen peroxide (H_2O_2). In a typical EBFC process, glucose is converted into gluconolactone by GOD, producing H_2O_2 as a by-product. Because H_2O_2 inhibits GOD activity, the glucose conversion reaction is blocked. The Cu-MOF catalyzed the conversion of H_2O_2 to water and oxygen, improving the catalytic performance for glucose. Aptamers on the Cu-MOF@AuNPs/GOD/Apt2 complexes were able to recognise E2. A flexible PTCR was modified with AuNPs for immobilisation of aptamer 1 via the Au-S linkage to form a pole of the EBFC. E2 was first identified via aptamer 1, and then the trapped E2 served as the anchor site for the Cu-MOF@AuNPs/GOD/Apt2 complexes via interaction between Apt2 and E2 to form a Cu-MOF@AuNPs/GOD/Apt2-E2-Apt1 sandwich structure on the PTCR/AuNPs (Scheme 1B). The captured Cu-MOF@AuNPs/GOD/Apt2 was directly proportional to the concentration of E2. A two-electrode system was used for the construction of the EBFC using PTCR as the cathode and Cu-MOF@AuNPs/GOD/Apt2-E2-Apt1/AuNPs/PTCR as the anode (Scheme 1C). Glucose and oxygen were the fuels of the EBFC. GOD on the anode promotes glucose oxidation to generate protons and electrons. However, the H_2O_2 produced by the reaction needs to be addressed because H_2O_2 may affect the stability of GOD (Cui et al., 2019). Cu-MOF displays peroxidase activity. Therefore, it can accelerate the oxidation of glucose to produce more electrons. The electrons generated on the anode are then transferred to the cathode to reduce oxygen to water. The electrochemical reactions at both the anode and cathode result in the generation of current and power. The magnitude of the electrical current and its corresponding power are directly proportional to the amount of Cu-MOF@AuNPs/GOD/Apt2 tags captured by E2 accumulated on the Apt1/PTCR. This sensor is portable, simple to operate, and suitable for on-site food and environmental applications.

2. Experimental section

2.1. Reagents and materials

Gold chloride trihydrate (III) ($HAuCl_4 \cdot 3H_2O$) and polyvinyl alcohol with an alcoholysis degree of 87.0–89.0% were purchased from Sigma-Aldrich. Sodium hydroxide (NaOH), 4,4-bipyridine and Tannic acid were purchased from Shanghai Macklin Co. Ltd. (Shanghai, China). Cellulose membrane (pore size 0.22 μm) was purchased from Xingya Material Co. Ltd. (Shanghai, China). Potassium ferricyanide ($K_3Fe(CN)_6$), potassium ferrocyanide ($K_4Fe(CN)_6$), potassium chloride (KCl), magnesium chloride ($MgCl_2$), sodium chloride (NaCl), hydrogen peroxide, copper chloride ($CuCl_2 \cdot 2H_2O$), ethanol (C_2H_5OH), carbon nanotubes (CNTs), reduced graphene oxide (rGO), monosodium dihydrogen orthophosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), 17 β -estradiol, magnesium sulfate ($Mg(SO_4)_2$), estriol (E3), progesterone (P4), bisphenol A (BPA), dibutyl phthalate (DBP), uric acid, atrazine, ascorbic acid, naphthalene and genistein were all purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Glucose oxidase, bovine serum albumin (BSA), glutaraldehyde, 3,3',5,5'-tetramethylbenzidine (TMB), and all aptamers employed in the study were obtained from Sangon Biotech Co. Ltd. (Shanghai, China). The aptamers were synthesized according to references (Nameghi et al., 2019) and the sequences are shown in Table S1. Phosphate buffer solution (PBS, 0.1 M, pH 7.0) containing Na_2HPO_4 and NaH_2PO_4 was used as the supporting electrolyte. A Tris-HCl (0.1 M, pH 8.0) solution was prepared by the mixture containing 95% water and 5% ethanol, 10 mM

$MgCl_2$, 25 mM KCl, and 20 mM NaCl. The original 1.0×10^{-3} M E2 standard solution was prepared by dissolving a suitable amount of E2 in 1 mL of acetonitrile and then diluted with Tris-HCl. A series of E2 standard solutions were prepared by serially diluting the original E2 standard solution with Tris-HCl. Glucose solution (80 mM) was prepared with PBS 7.0. $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ solution (2.5 mM) was prepared with PBS containing 0.1 M KCl. The aptamer standard solution was diluted with PBS 7.0 to 1.0 μM , and then stored at 4 °C for the following experiments.

2.2. Instruments

All the electrochemical experiments were performed on a CHI 660B (Shanghai, Chenhua) electrochemical workstation. Cyclic voltammetry (CV) measurements were tested in a 0.1 M PBS (pH 7.0) solution containing 2.5 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ and 0.1 M KCl. A three-electrode system was used, with the fabricated anode serving as the working electrode, an Ag/AgCl electrode as the reference electrode, and a platinum electrode as the auxiliary electrode. The current and potential output of the biofuel cell system were measured by linear cyclic voltammetry (LSV). The Cu-MOF@AuNPs/GOD/Apt2-E2-Apt1/PTCR anode and the PTCR cathode were connected and studied in PBS with glucose to evaluate the performance of the EBFC. All tests were conducted at room temperature. The scanning electron microscopy (SEM) image was determined with the Zeiss Supra 55. The accelerating voltage was 20 keV. The morphology of different materials was observed by a transmission electron microscope (TEM: JEM 2100, Japan). The pH adjustment of the buffer solution was conducted with a pH S-25 model pH meter (Leici Instruments, Shanghai). Fourier transform infrared spectra (FTIR) were obtained using a Shimadzu IRPrestige-21 spectrometer (Japan).

2.3. Fabrication of flexible PVA-TA/CNTs-rGO paper film

Fig. S1 described the preparation process of the flexible PTCR paper films. The details are as follows: PVA (54 mg) and TA (6 mg) were added to 10 mL of deionized water and stirred at 90 °C for 2.5 h (Chen, Jiao, Zhao, Zhang, & Wang, 2018). Subsequently, CNTs-rGO (210 mg) was added to the above PVA-TA aqueous solution, and it was kept stirring for 24 h. The mixture (400 μL) was deposited on the cellulose membrane by vacuum filtration and dried at 60 °C for 1 h to obtain PTCR paper film electrodes (Lu et al., 2021). The obtained PTCR paper films were cut to 5 mm $\times$ 10 mm and rinsed three times in deionized water and ethanol before they were dried at 25 °C for further use.

2.4. Synthesis of Cu-MOF and AuNPs

Cu-MOF was synthesized by a simple in-situ formation process (Wang, Gao, Cao, & Tan, 2018). The 4,4-bipyridine alcoholic solution (1 mL, 0.1 M) was dropwisely added into 9 mL of $CuCl_2$ aqueous solution (5.6 mM) and the mixture was reacted for 120 min at 25 °C with vigorous stirring. The products were centrifuged (10,000 rpm, 5 min) and washed three times with ethanol and water before being vacuum dried at 60 °C for 12 h.

Au nanoparticles were prepared by a sodium citrate reduction method as described in a previous study (Shu et al., 2020). $HAuCl_4 \cdot 3H_2O$ (1.5 mL, 1%) was dispersed into 150 mL of deionized water, trisodium citrate solution (7.5 mL, 38.8 mM) was added, and the reaction was carried out at 110 °C for 10 min. The reaction solution was cooled to room temperature, centrifuged (10,000 rpm, 10 min), washed and dispersed to 1000 μL deionized water and stored at 4 °C for further use.

2.5. Preparation of Cu-MOF@AuNPs/GOD bioconjugate modified with Apt2

PDAA (10 mL, 2.5 mg mL⁻¹) dispersed with 5 mg Cu-MOF were

sonicated at room temperature for 30 min, followed by centrifugation at 10000 rpm for 10 min and then discarding the supernatant. The composite was washed with distilled water three times to remove the loosely bound PDDA. Subsequently, AuNPs (100 μL) was added to the above obtained composite with continuous stirring for 2 h. The Cu-MOF@AuNPs was separated by centrifugation and washed three times to remove the loosely bound AuNPs. Glutaraldehyde solution (1 mL, 5%) was added to the dispersion of Cu-MOF@AuNPs and stirred for 1 h. This step aims to activate the carboxyl groups on the surface of AuNPs. Subsequently, GOD (1 mL, 5 mg mL⁻¹) and Apt2 (50 μL , 1.0 μM) were used to disperse the obtained Cu-MOF@AuNPs and the mixture was shaken at 37 °C for 12 h. To prevent non-specific binding, bovine serum albumin solution (40 μL , 20 mg mL⁻¹) was added and allowed to incubate for 0.5 h. Excess aptamer, enzyme, and BSA were removed by centrifugation with PBS. The reaction mixture was then centrifuged at 10000 rpm for 15 min and washed three times with water to remove the unbound GOD and Apt 2. The resulting precipitate was dispersed in 100 μL of 0.1 M PBS (pH 7.0) and stored at 4 °C for further use.

2.6. Fabrication of EBFC bioanode

The flexible PTCR film was used as the substrate electrode. AuNPs, featuring high electrical conductivity, good biocompatibility, ease of functionalization, and a high surface-area-to-volume ratio, have been widely applied to transfer electrons. In this work, AuNPs (20 μL) was dropped onto the PTCR film electrode with an effective surface area of 25 mm² and dried at 37 °C for 2 h. Then, Apt1 (20 μL , 1.0 μM) was dropped onto the surface of the AuNPs/PTCR electrode. After incubation at 4 °C overnight, Apt1 was immobilized on the electrode surface by the Au—S bond. Subsequently, E2 solutions (20 μL) with different concentrations were drop-cast onto the Apt1/AuNPs/PTCR electrode. After incubating at 37 °C for 20 min, unbound target molecules were washed off with PBS (0.1 M, pH 7.0). Then 20 μL of BSA solution (10 mg mL⁻¹) was added and left at room temperature for 40 min to block inactive sites. The electrode was then washed with PBS three times to remove excess BSA. After the accumulation of E2, Cu-MOF@AuNPs/GOD/Apt2 biocomplex (20 μL) was drop-cast onto the electrode to incubate 40 min. The obtained Cu-MOF@AuNPs/GOD/Apt2/E2/Apt1/AuNPs/PTCR sandwich electrode was applied as the anode for further use.

A membrane-less EBFC-based self-powered aptasensor was constructed by using Cu-MOF@AuNPs/GOD/Apt2/E2/Apt1/AuNPs/PTCR as the bioanode and PTCR film as the cathode. The output power and OCP of the EBFC-based self-powered aptasensor were measured by connecting the cathode and bioanode in the electrolytic cell. The supporting electrolyte was 20 mL of 0.1 M PBS (pH 7.0) containing 80 mM glucose. The polarization curves of the EBFC-based self-powered biosensor were measured by the LSV method. According to the polarization curve, the relationship between power output and current was calculated by the formula $P = UI$.

2.7. Determination of E2 in actual samples

To evaluate the practicability of the proposed sensor, it was used to detect E2 in real sample matrices. For milk analysis, milk (3 mL) and Mg (SO₄)₂ (0.1 g) were placed into a 15 mL centrifuge tube. The mixture was vigorously shaken for 10 min to precipitate protein, fat and other organic substances in the sample matrix and then centrifuged at 12000 rpm for 10 min to remove the precipitate again. The same method was used for the determination of milk powder samples. For the treatment of royal jelly, water containing 10 g of royal jelly was fixed to 1000 mL, and then 0.44 μm filter paper was used for filtration. The final solution was used for E2 detection. The spiked samples were prepared by adding E2 of different concentration into the samples. The human serum experiments were approved by the Ethics Committee of Northern Jiangsu People's Hospital and were analyzed in accordance with the guidelines of the Declaration of Helsinki. The serum samples were centrifuged at 13,000

rpm for 10 min to obtain the supernatant. Then, the samples were diluted 100-fold with PBS (pH 7.0) for E2 detection.

3. Results and discussion

3.1. Characterisation of Cu-MOF and Cu-MOF-based composites

Fig. 1A and B show the SEM and TEM images of Cu-MOF, respectively. Both reveal that Cu-MOF displays two-dimensional sheet-like nanostructures with an approximate size of 200 nm, which agrees well with the previous work (Wang et al., 2022). Fig. 1C shows that AuNPs are distributed evenly on the Cu-MOF surface. No AuNP agglomeration is observed. These AuNPs exhibit good conductivity and carboxyl groups, providing effective surfaces for the immobilisation of GOD and aptamers. Fig. 1D shows the Cu-MOF@AuNPs/GOD/Apt2 composites. Cu-MOFs exhibiting two-dimensional nanostructures remained discernible; however, some blurriness is noticeable due to the presence of GOD and Apt2. The FTIR spectrum of the Cu-MOF is shown in Fig. 1E, revealing the C—N (1491 cm⁻¹), C—C (1604 cm⁻¹) and pyridine ring (1400–1600 cm⁻¹) stretching vibrations of the Cu-MOF. Moreover, Cu-MOF@AuNPs exhibit a powder XRD pattern substantially identical to that of Cu-MOF (Fig. 1F), indicating that the main crystal structure of Cu-MOF remains unchanged after the integration of AuNPs. Fig. 1G shows the high peroxidase-mimicking activity of Cu-MOF towards TMB under neutral conditions (pH 7.0). The oxidative reaction of TMB was conducted in PBS buffer (0.1 M, pH 7.0) in the presence of H₂O₂. As shown in the inset of Fig. 1G, a typical colorimetric reaction occurs in the mixed solution containing TMB, H₂O₂, and Cu-MOF, resulting in the colour change of the TMB solution from colourless to blue. An absorption peak at approximately 650 nm was also observed. In contrast, no colorimetric reaction was observed in the control experiment, confirming the peroxidase-like activity of the Cu-MOF. Because nearly neutral pH conditions are generally required in many biological systems, Cu-MOF with excellent catalytic activity at nearly neutral pH values may potentially serve as a peroxidase mimic for applications.

The affinity between the Cu-MOF and H₂O₂ was studied using Michaelis-Menten curves in a particular concentration range of H₂O₂ (Fig. S2). The enzymatic kinetic parameters K_m (0.854) and V_{max} (6.77×10^{-8} M s⁻¹) were calculated and compared with those of other peroxidase mimics and horseradish peroxidase (Table S2). The Cu-MOF exhibited a relatively fast catalytic rate and high affinity towards the substrate compared to previously reported nanozymes, indicating that the Cu-MOF had a stronger affinity for H₂O₂. It can catalyse the decomposition of H₂O₂ into water and oxygen, thereby enhancing the catalytic efficiency for glucose and performance of the EBFC.

3.2. Morphology and electrochemical characterisation of the bioanode formation process

The design of the anode plays a vital role in the construction of EBFC-based self-powered biosensing platforms for E2 analysis. The morphological features of the electrodes obtained by the stepwise fabrication processes were characterised by SEM. The PTCR film exhibits an interconnected microporous structure with micron-sized pores (Fig. 2A). The PTCR film had diverse macrostructures, which were conducive to fixing biomolecules on its surface and the exposure of active sites. Fig. 2B shows the SEM image of AuNPs/PTCR film. Some AuNPs were dispersed on the film, which was beneficial for the immobilisation of Apt1 and promoted electron transfer between the enzyme and the electrode. The SEM images of Apt1/AuNPs/PTCR are shown in Fig. 2C. The surface exhibits minimal alterations in its characteristics compared to that shown in Fig. 2B. This is because the aptamer is a single-stranded DNA molecule. Its immobilisation has little effect on the surface of AuNPs/PTCR. Consequently, the surface morphology towards the target molecule E2 exhibits negligible variations, suggesting a lack of significant surface perturbations during specific recognition events (Fig. 2D).

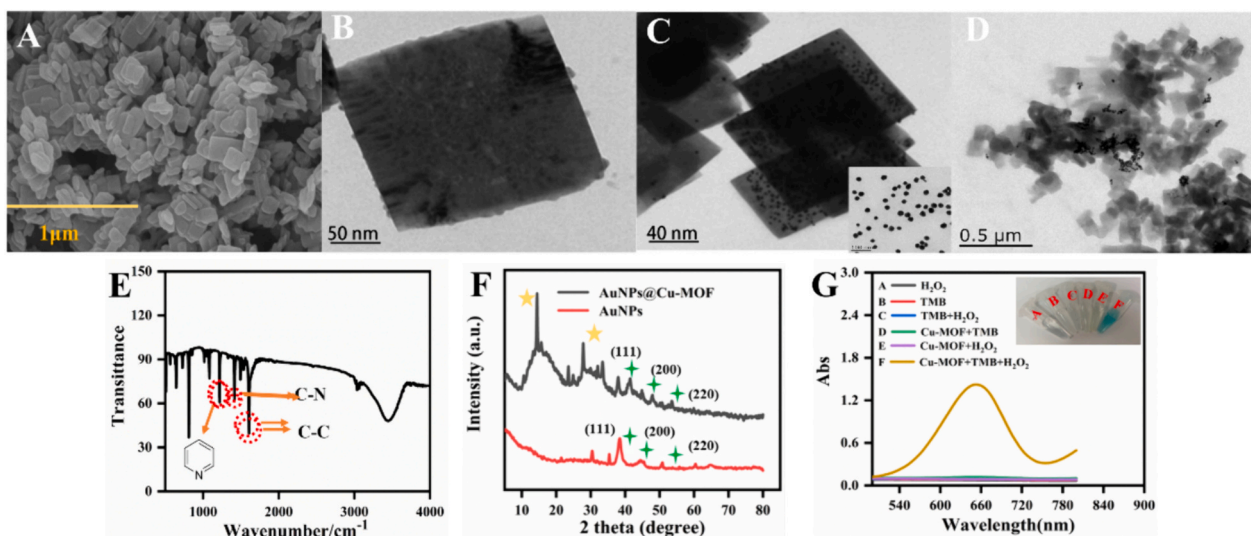


Fig. 1. (A) SEM of Cu-MOF. (B) TEM of Cu-MOF. (C) TEM of Cu-MOF@AuNPs (Inset was the TEM image of the AuNPs). (D) TEM of Cu-MOF@AuNPs/GOD/Apt2 bioconjugate. (E) FTIR profiles of Cu-MOF. (F) XRD patterns of Cu-MOF@AuNPs (black line) and AuNPs (red line). (G) Absorption spectra and digital photos (inset) of (A) H_2O_2 , (B) TMB, (C) TMB + H_2O_2 , (D) Cu-MOF + TMB, (E) Cu-MOF + H_2O_2 and (F) Cu-MOF + TMB + H_2O_2 . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

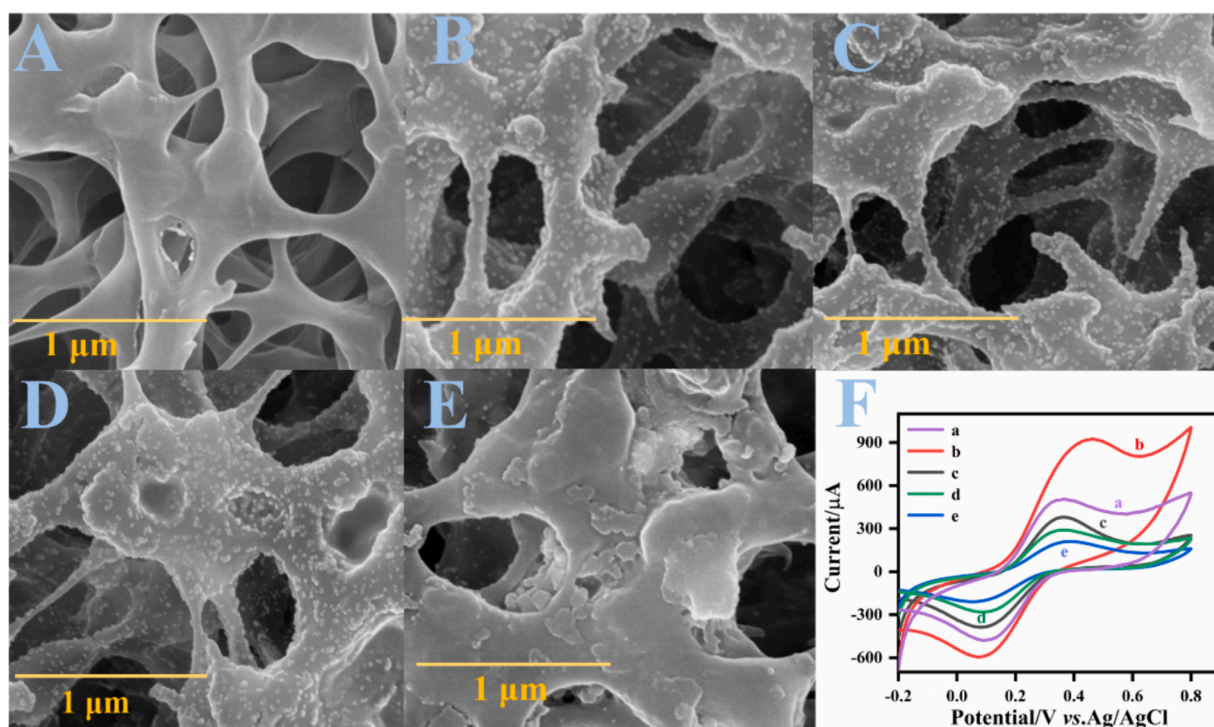


Fig. 2. SEM of (A) PTCR film; (B) AuNPs/PTCR film; (C) Apt1/AuNPs/PTCR film; (D) E2/Apt1/AuNPs/PTCR film; (E) Cu-MOF@AuNPs/GOD/Apt2/E2/Apt1/AuNPs/PTCR film. (F) CV curves of bare PTCR film (a), AuNPs/PTCR (b), Apt1/AuNPs/PTCR (c), E2/Apt1/AuNPs/PTCR (d) Cu-MOF@AuNPs/GOD/Apt2/E2/Apt1/AuNPs/PTCR (e) in $[\text{Fe}(\text{CN})_6]^{3-/4-}$.

Fig. 2E shows the SEM image after the formation of the sandwich structure of Cu-MOF@AuNPs/GOD/Apt2/E2/Apt1/AuNPs/PTCR. It does not exhibit any observable AuNPs, indicating successful encapsulation of the AuNPs within the constructed sandwich structure. For comparison, the interaction between Cu-MOF@AuNPs/GOD/Apt2 and Apt1/AuNPs/PTCR in the absence of E2 was studied. Fig. S3 shows that only minor differences are observed between AuNPs/PTCR and Cu-MOF@AuNPs/GOD/Apt2/Apt1/AuNPs/PTCR. Therefore, E2 plays an important role in the formation of the sandwich structure. In the absence

of E2, no Cu-MOF pellets could bind to the PTCE. Cu-MOF@AuNPs/GOD/Apt2 were effectively and quantitatively fixed on the Apt1/AuNPs/PTCE electrode to form the bioanode.

The anode assembly process was also investigated by CV in a 2.5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution containing 0.1 M KCl. The CV curve of the PTCE flexible electrode (curve a) exhibits two distinct peak currents, as shown in Fig. 2F, indicating good electron transfer of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ probe on the PTCE electrode. This was due to the good conductivity of the carbon nanotubes/reduced graphene oxide. When the PTCE electrode

was modified with AuNPs, a higher current response was observed (curve b). This enhancement can be attributed to the substantial enlargement of the effective surface area provided by the AuNPs, which promotes improved contact between the electrode and the electrolyte solution. Additionally, the conductive network formed on the electrode surface by the AuNPs further enhanced the charge transmission and overall conductivity. After assembling Apt1 onto the electrode surface, the peak current of the Apt1/AuNPs/PTCR flexible electrode was significantly reduced (curve c) due to electrostatic repulsion between the negatively charged aptamer and $[\text{Fe}(\text{CN})_6]^{3-/4-}$ anions in the electrolyte. In the presence of the negative aptamer, the negatively charged $[\text{Fe}(\text{CN})_6]^{3-/4-}$ difficultly approached the proximity of the electrode surface, leading to a diminished ion transport rate and subsequently reducing current. After E2 was identified on the Apt1/AuNPs/PTCR flexible electrode, a further reduction in peak current was observed (curve d). This phenomenon could be attributed to the steric hindrance caused by the identification of E2, which impeded the charge transfer between the electrode and electrolyte interface. When Cu-MOF@AuNPs/GOD/Apt2 were captured by Apt 1 on the anode surface (curve e), the peak current was further reduced due to the non-conductive shell and steric hindrance of the enzyme molecules, which impeded electron transfer.

CV measurements were conducted to assess the electrocatalytic activities of the Cu-MOF@AuNPs/GOD/Apt2/E2/Apt1/AuNPs/PTCR anode and PTCR cathode. The voltage ranges for the anode and cathode were from -0.8 to 0 V and 0 to -1.0 V, respectively, with a scan rate of 10 mV/s. CV scanning of the bioanode was conducted in blank PBS solution in the presence of glucose, as depicted in Fig. 3A. A significant increase in the peak current was observed on the bioanode in the presence of glucose, indicating the catalytic oxidation activity towards the fuel (glucose) by the Cu-MOF@AuNPs/GOD/Apt2/E2/Apt1/AuNPs/PTCR anode. The excellent oxidation activity of the bioanode towards glucose is attributed to the large surface area and peroxidase-like activities of the two-dimensional sheet-like Cu-MOF. Cu-MOF facilitates superior catalytic activity of the bioanode towards glucose, highlighting the potential of Cu-MOF-based structures in bio electrochemical applications. The CV curves of the cathodes in PBS solution under N_2 and air are shown in Fig. 3B. In the presence of air, the reduction peak current at the cathode surface increased, indicating the effective reduction of O_2 at the cathode. The electrochemical reactions at both the anode and the cathode resulted in the generation of current and power. The magnitude of the electrical current and its corresponding power is directly proportional to the amount of captured Cu-MOF@AuNPs/GOD/Apt2 tag. The influence of oxygen on the performance of the EBFC is an important factor to consider when constructing an EBFC-based sensor. To monitor the effects of oxygen on the performance of the EBFC, the fuel cell was fed with nitrogen, air and oxygen. The corresponding power output curves are shown in Fig. 3C. As shown in Fig. 3C, the presence of

nitrogen reduces the power output of the EBFC. The decrease in power output is associated with the reduced amount of O_2 that is reduced at the cathode. However, the power output of the EBFC changed only slightly upon purging O_2 (curve a) and air (curve b). Therefore, the concentration of the dissolved O_2 is sufficient for the cathodic reduction reaction. Moreover, the immobilized Cu-MOF reproduced O_2 from H_2O_2 accompanying the glucose oxidation catalyzed by glucose oxidase. Constant O_2 supply from the produced H_2O_2 could also prevent the interference by O_2 (Gu, Gai, Kong, Hou, & Li, 2020). Therefore, O_2 did not affect the detection results in this work.

3.3. Feasibility of EBFCs for E2 sensing

The feasibility of the EBFCs for E2 detection was evaluated by combining the Cu-MOF@AuNPs/GOD/Apt2/E2/Apt1/AuNPs/PTCR anode and the PTCR cathode into a membrane-less cell. Glucose (0.08 M) was added to the cell as fuel. The OCP curves of the PTCR cathode and different anodes are shown in Fig. 4A. The OCP of the PTCR cathode reached approximately 0.15 V. In the absence of E2, even when the Apt1@AuNPs@PTCR anode was incubated with Cu-MOF@AuNPs/GOD/Apt2 for 40 min, the OCP was approximately 0.13 V. The potential difference between the cathode and anode was negative, indicating that the EBFC was not effective in the absence of target E2. However, when the Apt1@AuNPs@PTCR anode was incubated with E2 and Cu-MOF@AuNPs/GOD/Apt2 step-by-step, the OCP of the formed cathode was decreased to -0.12 V. When the cathode potential exceeded the anode potential, oxidation occurred at the anode. Electrons are generated on the anode after the formation of the sandwich structure. The potential difference between the cathode and the Cu-MOF@AuNPs/GOD/Apt2/E2/Apt1/AuNPs/PTCR anode verified the thermodynamic feasibility of the EBFCs. Fig. 4B shows that the OCP of the constructed EBFC is approximately 0.27 V, which is close to the potential difference between the cathode and anode shown in Fig. 4A. The results indicated that the constructed biofuel cell was thermodynamically favourable.

The signal output performance of the EBFCs was further evaluated by the polarization curve and power-density (P – V) measurement, and the results are shown in Fig. 4C and D. In the absence of E2, the OCP and P_{max} value of the resulting cell were only 0.02 V and $0.5 \mu\text{W cm}^{-2}$, respectively. In contrast, those of the resulting cells increased to 0.154 V and $7.5 \mu\text{W cm}^{-2}$ after the Apt1/AuNPs/PTCR was incubated with E2 and Cu-MOF@AuNPs/GOD/Apt2. The signal improvement was due to the recognition of Apt1 on the AuNPs/PTCR anode to E2 and the formation of the Cu-MOF@AuNPs/GOD/Apt2/E2/Apt1 sandwich structure. These results indicated that a self-powered EBFC-based aptasensor could be realised for E2 detection by measuring the OCP and P_{max} values of the assembled biofuel cells.

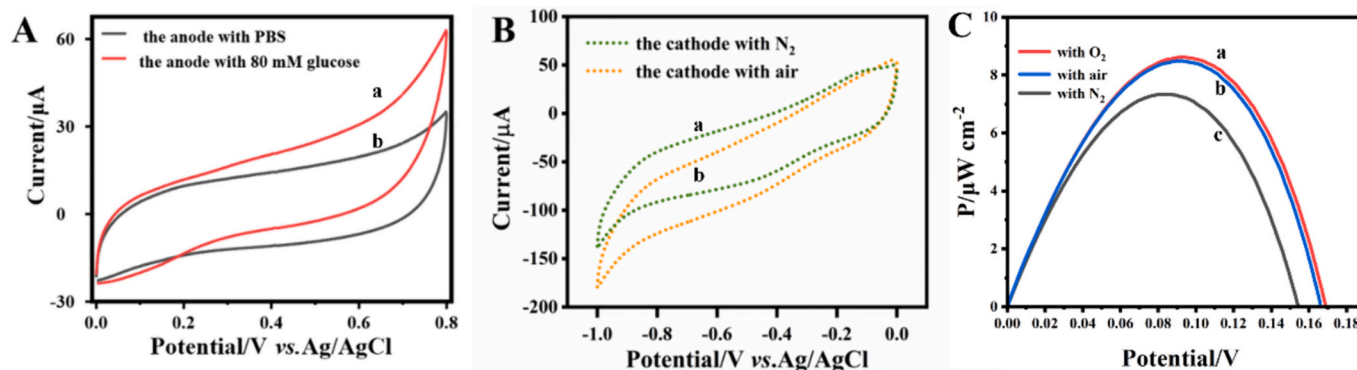


Fig. 3. (A) CV curves of the Cu-MOF@AuNPs/GOD/Apt2/E2/Apt1/AuNPs/PTCR anode in PBS with (curve a) and without 80 mM glucose (curve b) (B) CV curve of PTCR cathode in PBS with N_2 (curve a) and air (curve b) saturated. (C) Power-density curves of EBFCs in glucose with O_2 (curve a), air (curve b) and N_2 (curve c).

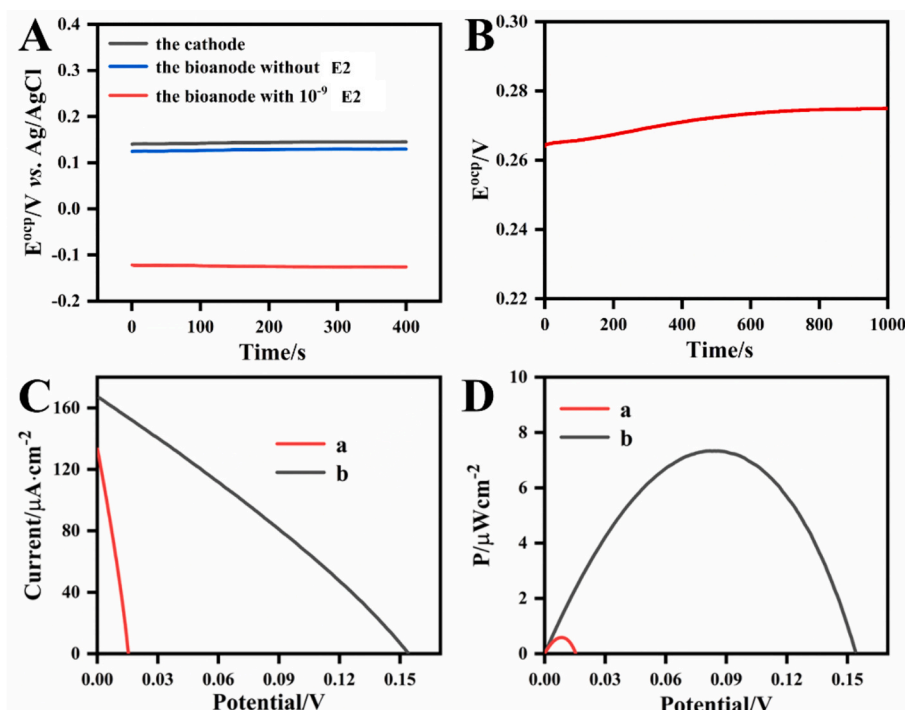


Fig. 4. (A) OCPs of the PTCR cathode (blank line) and the anode before and after the incubation of E2. (B) OCP of the EBFC by using PTCR as the cathode and Cu-MOF@AuNPs/GOD/Apt2/E2/Apt1/AuNPs/PTCR as the anode. Polarization curves (C) and power-density curves (D) of EBFCs before and after the incubation of E2.

3.4. Performance of the EBFC-based self-powered aptasensor for E2 detection

To fabricate an aptasensor with the best performance, the parameters including the preparation of PTCR flexible electrodes (mass of CNT-rGO,

filtration volume of viscous slurry) and the detection conditions (incubation time between Apt1 and E2, identification time between E2 and Cu-MOF@AuNPs/GOD/Apt2, glucose concentration, and pH of the detection solution), which influence the performances of the aptasensor, were optimised (Figs. S4, S5). Detailed discussion about the

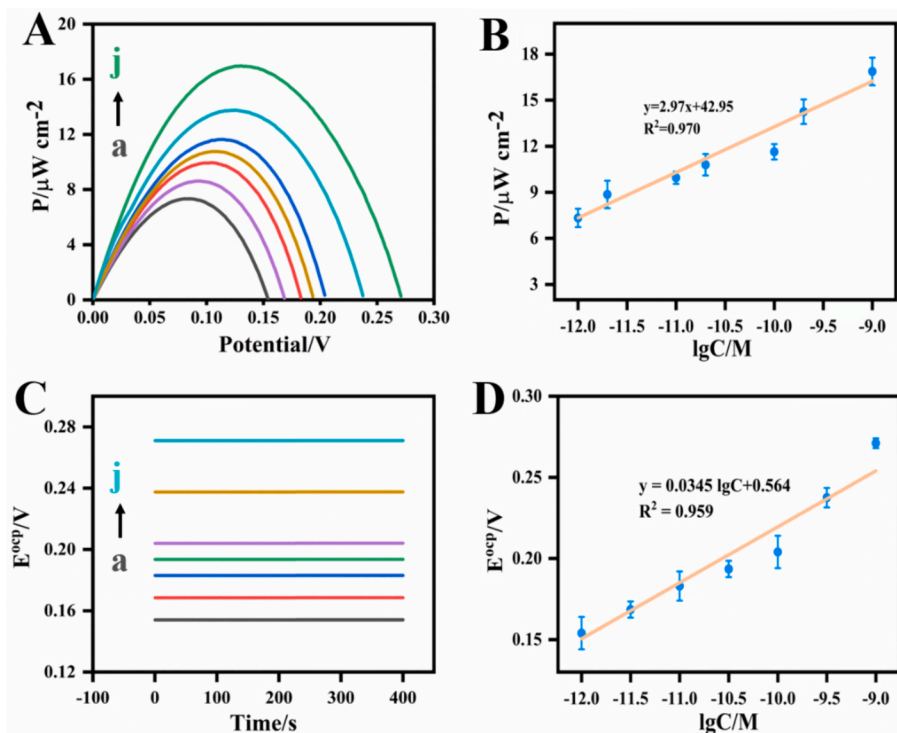


Fig. 5. (A) P_{max} responses to different concentrations of E2: from a to j (1.0×10^{-12} M to 1.0×10^{-9} M) (B) Calibration curve of P_{max} and E2. (C) OCP responses to different concentrations of E2: from a to j (1×10^{-12} M to 1×10^{-9} M) (D) Calibration curve of OCP and E2. ($n = 5$, the error bars are the width of confidence interval at the 95% confidence level.)

optimisation of the PTCR electrode fabrication and the experimental conditions for E2 detection is provided in the supporting information. The optimised conditions for the preparation of the flexible PTCR film were as follows: 0.21 g of CNTs-rGO and 400 μ L filtration volume of the CNTs-rGO solution were selected to prepare the flexible PTCR films. For the detection of E2, the incubation time between Apt1 and E2 was 20 min, the identification time between Apt1-E2 and Cu-MOF@AuNPs/GOD/Apt2 was 40 min and 80 mM glucose at pH 7.0 was selected as the fuel.

Under optimal experimental conditions, the performance of the proposed self-powered aptasensor for E2 analysis was investigated. P_{max} and OCP were employed as the response signals in the proposed biosensing platform, thereby simplifying the instrumentation process, which involves only two electrodes. The proposed simple instrumentation facilitated the construction of portable devices. As shown in Fig. 5A, P_{max} gradually enhances with increasing concentrations of E2 ranging from 1.0 pM to 1.0 nM, and the linear relationship between P_{max} and the E2 concentration (c) is fitted to a linear equation of $P_{max} = 42.95 + 2.97 \log C$ ($R^2 = 0.970$) (Fig. 5B). The E^{OCP} value also increases with an increase in the amount of target E2 (Fig. 5C) with a relationship of $E^{OCP} = 0.564 + 0.0345 \log C$ ($R^2 = 0.959$) in the concentration range of 1.0 pM to 1.0 nM (Fig. 5D). The LOD was estimated to be 0.12 pM ($S/N=3$). Compared to some established methods (Table S3), this sensor showed superior sensitivity, selectivity and detection limit. In this sensor, two different recognition units are used to form a sandwich structure that ensures a high degree of specificity and selectivity. In addition, appropriate labels such as enzymes and nanoparticles are usually employed to enhance the detectable signal. These amplification methods enhance the signal generated by the sandwich complex, improving the sensitivity of the assay while maintaining selectivity (Pei et al., 2013). In this study, the sandwich structure of Apt1-E2-Apt2 enables the assembly of Cu-MOF@AuNPs/GOD binding onto the anode surface, serving as a signal amplification probe to enhance the catalytic activity of the GOD, thereby improving the sensitivity of the sensor. Consequently, this method exhibits good sensitivity, selectivity, and detection limits than the established methods.

To confirm that the observed responses were due to specific interactions between the aptamers and E2, the selectivity of the aptasensor was studied. We determined the power output of the aptasensor after the accumulation of 10 pM E2 and the mixed liquor of 10 pM E2 with 100 pM E3, P₄, BPA, DBP, uric acid, atrazine, ascorbic acid, naphthalene and genistein. Fig. S6A shows that the effects of these interferences are lower than 4%, indicating satisfactory selectivity. The good selectivity of the sensor may be attributed to the sandwich structure formed between aptamers 1 and 2. The reproducibility of the self-powered aptasensor was examined by detecting 0.1 nM E2 with five aptasensors (Fig. S6B). The relative standard deviation was 7.6%, indicating good reproducibility of the aptasensors. In addition, the long-term storage stability of the proposed aptasensor is an important index that should be considered. Moreover, the stability of the aptasensor was evaluated by storing them in a refrigerator at 4 °C and measuring their response every 3 days. The results are shown in Fig. S6C. The aptasensor retained 64.8% of the original response even after 21 days of storage. This result implied that the proposed aptasensor had acceptable stability. However, because the activities of GOD and aptamers were affected by temperature, the power output (21.1% of the original) of the sensors decreased significantly over the same time period when the sensor was kept in air. It is assumed that the activities of GOD and aptamer decrease in ambient air. Therefore, the sensor should be stored in a refrigerator at 4 °C.

The fabricated self-powered aptasensors were also applied to detect E2 in milk, royal jelly and milk powder samples. No E2 was detected in these samples. Recovery experiments were also performed to evaluate the practical utility of the proposed aptasensor. The results are shown in Table S4. Meanwhile, two serum samples were utilised for testing. To assess the precision of the test outcomes, standard determination was performed using the chemiluminescence method. The resulting values

were compared to those measured by the constructed sensor, exhibiting negligible discrepancies (Table S5). Furthermore, Student's t -test was performed using the serum detection data. The experimental results in Table S5 indicate that the estimated values of the t -test are consistently lower than the theoretical values. Therefore, there is no significant difference between the developed aptasensor and the electrochemical luminescence method. However, note that the electrochemical luminescence method is associated with a longer analysis time. In summary, the developed aptasensor effectively fulfills the requirements for rapid and precise analysis of authentic specimens. Recovery tests are employed to assess whether the assay influences the sample matrix. The recovery rate is calculated as the quotient of the measured value divided by the added value, accounting for both positive and negative errors in the measurement process. Consequently, the recovery rate exhibits a positive (>100%) or negative (<100%) outcome, indicating a normal distribution pattern (Garrido-Frenich et al., 2006; Li, Qiu, Pang, & Ashbolt, 2019). The recovery values obtained by the aptasensor are in the range of 94.0–105.8%, suggesting that this sensor is applicable to E2 determination in real samples.

3.5. Bending and twisting stability test of the flexible PTCR film electrode

The CVs of the PTCR film electrodes under different levels of bending and twisting were recorded in 2.5 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] containing 0.1 M KCl solution at a scan rate of 10 mV/s. As shown in Fig. S7A, even when the film is bent to 100°, the peak current changes only by approximately 5%, and the relative resistance R/R_0 changes by approximately 18% (Fig. S7C). With regard to the twisting properties, even at a twisting angle of 100°, the flexible sensor retained good electron transport capabilities. In addition, the relative resistance of the PTCR film has not changed significantly upon twisting at different angles (Figs. S7B and S7D). These results indicated that the flexible electrochemical sensor has outstanding mechanical bendability and durability, broadening the feasibility of fabricating flexible paper-based self-powered biosensors.

4. Conclusion

Herein, a 'signal-on' EBFC-based self-powered aptasensor was developed for the sensitive determination of E2 using a sandwich structure of the aptamer-target-aptamer bioconjugate for signal amplification. Cu-MOF@AuNPs with peroxidase mimicking activities were prepared as a supporting substrate, which enhanced biomolecule loading, facilitated glucose oxidation and improved the power density of EBFCs under neutral conditions. The aptasensor demonstrated a broad linear range (1.0 pM to 1.0 nM) and an impressive detection limit (0.13 pM). Moreover, the sensor response remained unaffected in the presence of numerous co-existing interferents. In addition, the proposed aptasensor was successfully employed in detecting E2 in real samples without any special pre-treatment, owing to its exceptional anti-interference capability and high sensitivity. The device is self-powered and cost-effective and serves as a viable alternative to conventional E2 detection approaches, making significant contributions to ongoing efforts in food safety and environmental monitoring. As production technologies are developed to produce nanoparticles and immobilise biocomponents, the relative complexity of the sensing structures and procedures should not constitute an obstacle. The integration of the sensor in portable low-cost and wearable substrates can be combined with additive manufacturing methods such as printing, enabling large-scale production with increased reproducibility. Further developments in flexible electronics using of additive manufacturing methods, and appropriate tools are envisioned to improve fabrication and commercialisation of sensors on a large scale.

CRediT authorship contribution statement

Kangping Ning: Writing – original draft, Methodology, Formal analysis, Data curation. **Zheng Wang:** Validation, Investigation, Formal analysis. **Honglan Qin:** Methodology, Formal analysis, Data curation. **Maruf Ahmed:** Writing – review & editing, Methodology, Investigation. **Seitkhan Azat:** Investigation. **Xiaoya Hu:** Investigation. **Qin Xu:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Dandan Yang:** Writing – review & editing, Supervision.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

Acknowledgments

We gratefully thank the foundations including the National Natural Science Foundation of China (22076161, 21675140 and 21705141), Yangzhou University Interdisciplinary Research Foundation for Chemistry Discipline of Targeted Support (yzuxk202009), Yangzhou Science and Technology Public Service Platform (YZ2022150), the projects supported by PAPD and TAPP. The Test Center of Yangzhou University was also thanked for the SEM and TEM characterizations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2024.140780>.

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