Ultrasensitive self-powered cytosensors based on exogenous redox-free enzyme biofuel cells as point-of-care tools for early cancer diagnosis†

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An exogenous redox-free, membrane-less enzyme biofuel cell-based ultrasensitive self-powered cytosensing platform was fabricated. With the ultrahigh sensitivity and the merits of not requiring external power sources or exogenous reagents, the device has great potential as a point-of-care tool for early diagnosis of cancer in vivo.

Enzyme biofuel cells (EBFCs) have attracted tremendous interest due to their moderate operating conditions and promising applications as implantable energy devices. Recently, the development of EBFCs has not only been focused on the fabrication of novel devices with high power output and long-term stability, but also been devoted to exploring their new applications, such as self-powered biosensors,1–4 drug-release systems,5 and miniaturization devices.6–8 In particular, the self-powered biosensors based on EBFCs, which themselves provided the power sources during sensing, were investigated recently as great promising candidates in human disease diagnosis.1,3,7 Compared with other counterparts, they possessed the merits of not requiring external power sources, good specificity, simple fabrication process, miniature size and low cost.10

Cancer is a major threat to human health and its morbidity has been increasing in recent years. It is reported that more than ten million people all over the world get cancer each year, causing serious illness and death.11 Within the medical profession, early cancer diagnosis has been regarded as the most effective route for curing the diseases and improving the survival rates. Therefore, it is imperative to develop a quantitative and reliable methodology for early diagnosis of cancer. To date, a variety of tools have been applied in cancer diagnosis, such as serum proteomic patterns,15–17 protein microarrays,18 computer tomography,19 and real-time-polymerase chain reaction (RT-PCR)-based DNA tests.20,21 However, these methods suffer from some drawbacks, such as complex sample treatment and expensive instrumentation. Recently, our group firstly reported an ultrasensitive and self-powered EBFC cytosensor, in which the aptamer-functionalized biocathode could specifically capture the target cancer cells and thereby cause a decrease in power output of the EBFC, reflecting the cell numbers.22 At present, constructing real-time, portable and implantable diagnostic devices has become the up-to-date demand of clinical cancer diagnosis. However, the developed cytosensors that relied on external power sources, complicated configuration, or exogenous reagents are incompatible with the above performance requirements. Thereby, to follow the trend of clinical application, there is a desperate need for a novel cytosensing platform without any external power source and reagents.

Herein, we proposed an exogenous redox-free, one-compartment EBFC-based-ultrasensitive self-powered cytosensing platform for point-of-care cancer diagnosis. At the biocathode, bilirubin oxidase (BOD)-catalyzed oxygen reduction played the role of accepting electrons, avoiding the introduction of other redox ions (e.g., [Fe(CN)]3−). At the bioanode, pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) was used for the catalytic oxidation of glucose. This biocatalytic reaction was independent of soluble cofactor and susceptible to dissolved oxygen, beneficial to configuring a membrane-less EBFC cytosensor. In addition, to accelerate the electron transfer and promote the electrical communication between the enzyme and electrode, a graphene/carbon nanotube/gold nanoparticle (G/CNT/Au NP) hybrid with a three-dimensional (3D) porous architecture was employed as a robust substrate electrode. Thiolated-aptamers Sgc8c and SYL3C with a specific sequence for recognizing target cancer cells were, respectively, assembled on the substrate electrode for capturing acute leukemia CCRF-CEM cells and circulating tumor cells MCF-7. As a signal probe, the BOD bioconjugate was obtained by linking BOD and the

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amino-modified partially complementary strand of aptamers (cs DNA) on carbon nanotube/gold nanoparticle (CNT/Au NP) scaffolds, which was subsequently immobilized onto the aptamer-functionalized substrate electrode through partial base pairing with the aptamer (Scheme 1A). The one-compartment EBFC self-powered cytosensor, composed of the BOD bioconjugate-aptamer-functionalized biocathode and the PQQ-GDH modified G/CNT/Au NP hybrid bioanode, was set up for detecting the cancer cells (Scheme 1B). The presence of target cancer cells induced the conformation change of the aptamer strand, capturing the cells and liberating the BOD bioconjugate from the biocathode. As a consequence, the reduced BOD loading affected the catalytic performance of the biocathode towards oxygen reduction and led to the decrease of open circuit voltage ($E_{OCV}$) of the cytosensor, thus realizing the determination of cancer cells. This effective strategy for cytosensing need not apply external power sources and introduce exogenous reagents, holding great promise as a point-of-care tool for early diagnosis of cancer in vivo.

The properties of the substrate electrode play an important role in improving the performance of EBFCs. The preparation of the substrate material G/CNT/Au NP hybrid is stated in the ESL†. The morphology of the G/CNT/Au NP hybrid was characterized by field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM). As shown in Fig. 1A and B, the G/CNT/Au NP hybrid exhibited a well-defined and highly porous 3D network structure, in which CNTs and G were interconnected with each other and decorated with uniform gold nanoparticles (Fig. 1C and D). In addition, electrochemical impedance spectroscopy (EIS) was performed to examine the conductivity of the substrate electrode. The charge-transfer resistance ($R_{ct}$) of the $[\text{Fe(CN)}_6]^{3^-/4^-}$ probe at the G/CNT/Au NP hybrid modified carbon cloth electrode (CCE) decreased only about 8 Ω compared to the bare electrode (Fig. S1, ESI†), indicating the excellent conductive capability of the substrate electrode. The highly porous 3D architectures and excellent conductivity of the substrate electrode would remarkably enhance the electron transport and further facilitate the bioelectrocatalysis of enzymes.23 Furthermore, the uniform distribution of the biocompatible Au NPs all over the 3D network provided abundant anchor sites for the thiolated-aptamer. On the other hand, the secondary electrode interface, the BOD bioconjugate, was elaborately constructed and characterized in our study (Fig. S2, ESI†). With their large surface for enzyme immobilization and high electrical conductivity for electron transfer, carboxyl-functionalized CNTs/Au NPs were adopted as carriers to accommodate a high amount of BOD and csDNA. In the obtained BOD bioconjugate, the high loading amount of BOD on the CNT/Au NP scaffolds greatly fuelled the oxygen reduction reaction and magnified the response signal for ultrasensitive cytosensing.

With the CCRF-CEM cells as the representative model, the assembly process of the BOD bioconjugate-aptamer-functionalized biocathode was firstly monitored by EIS. Compared to the G/CNT/Au NP substrate electrode (Fig. S1, curve b, ESI†), $R_{ct}$ of the $[\text{Fe(CN)}_6]^{3^-/4^-}$ probe significantly increased after Sgc8c aptamer modification and MCH block, probably owing to the inert blocking layer formed by biomolecules that hindered the electron transfer (Fig. 2A, curve a). Upon binding of the BOD bioconjugate, $R_{ct}$ of the probe decreased correspondingly (Fig. 2A, curve b), which was attributed to the accessibility of the redox probe to the conductive secondary electrode interface. The subsequent capture of the CCRF-CEM cells onto BOD bioconjugate-aptamer-functionalized biocathode obstructed the approach of the redox species to the biocathode surface due to the steric hindrance effect, and thus exported an elevated $R_{ct}$ (curve c in Fig. 2A). The above $R_{ct}$ response to each modification step demonstrated the successful assembly and effective recognition to the cancer cells. Cyclic voltammetry (CV) was performed to investigate the bioelectrocatalysis behaviour towards oxygen reduction and the capture of cancer cells (Fig. 2B and Fig. S3, ESI†).
Captured by the biocathode, we stained the captured MCF-7 cells for feasibility for cytosensing based on the BOD bioconjugate-cell selective detection and quantification, and confirmed the response to the cell number, proving that it was binding of cells that drew the BOD bioconjugate away from the electrode surface.

An absorption peak at 260 nm was found after incubating biocathode in the supernatant of the cell suspension (a), the supernatant of the cell suspension incubation in the 5000 cell suspension. (C) UV-Vis spectra of the blank and the biocathode after incubation in the 5000 cell suspension. (D) CVs of the G/CNT/Au NP hybrid substrate electrode (a), the G/CNT/Au NP/PQQ-GDH bioanode (b) in PBS (pH = 7.4) containing 5 mM of glucose.

Fig. 2 EIS of the aptamer-functionalized G/CNT/Au NP hybrid substrate electrode (a), the BOD bioconjugate-aptamer-functionalized biocathode (b) and the biocathode after incubation in the 5000 cell suspension (c). Insets show the enlarged views of curves a and b (top), and the Randles equivalent circuit used to fit the EIS data (below). (B) CVs of the BOD bioconjugate-aptamer-functionalized biocathode in PBS (pH = 7.4) saturated with N2 (a) and O2 (b). (c) CVs of the biocathode in PBS (pH = 7.4) saturated with O2 after incubation in the 5000 cell suspension. (C) UV-Vis spectra of the blank supernatant of the cell suspension (a), the supernatant of the cell suspension with a cell number of 5000 (b) and 50 000 (c) after reacting with the BOD bioconjugate-aptamer-functionalized biocathode. The inset shows the UV-Vis spectrum of the BOD bioconjugate. (D) CVs of the G/CNT/Au NP hybrid substrate electrode (a), G/CNT/Au NP/PQQ-GDH bioanode (b) in PBS (pH = 7.4) containing 5 mM of glucose.

Compared with deoxygenated electrolyte solution, a bioelectrocatalytic current emerged approximately at 0.6 V for the oxygen-saturated solution, close to the redox potential of the T1 Cu site of BOD, suggesting that the BOD bioconjugate-aptamer-functionalized biocathode possessed high-efficient catalytic activity for oxygen reduction. After incubation in the CCRF-CEM cell suspension, the biocathode witnessed a remarkable decrease in the reduction current and an increase in the over-potential of oxygen reduction, resulting from the dissociation of the BOD bioconjugate from the electrode interface along with the aptamer specific capture of CCRF-CEM cells. To verify the reason for \( R_{ct} \) and CV signal change, UV-Vis spectra were used to monitor the liberation of the BOD bioconjugate caused by target cell recognition (Fig. 2C). As shown in the Fig. 2C inset, an absorption peak at 260 nm was detected for the BOD bioconjugate, corresponding to the DNA characteristic UV absorption peak. There was no species featuring 260 nm absorption in the blank supernatant of the cell suspension, while a new absorption located at 260 nm was found after incubating biocathode in the solution. Moreover, the absorption intensity exhibited a positive response to the cell number, proving that it was binding of cells that drew the BOD bioconjugate away from the electrode surface. This cell-related signal response lays a solid foundation for cell selective detection and quantification, and confirmed the feasibility for cytosensing based on the BOD bioconjugate-aptamer-functionalized biocathode. To examine the cell viability captured by the biocathode, we stained the captured MCF-7 cells with calcein-AM, a widely used cell viability indicator that only stained living cells. As shown in Fig. S4, ESI† the strong fluorescence signal of the cells on the electrode surface demonstrated that the cells were alive during the whole sensing process.

Meanwhile, as the electron generator in EBFCs, the bioanode was also a highly determining component for the performance. Aiming at devising in vivo sensors, we were pursuing a membrane-less electrolytic cell. In this case, PQQ-GDH was selected as the enzyme for catalyzing anodic glucose oxidation, furthermore, no crossover reactions occurred between the bioanode and biocathode, thereby suitable for the construction of one-compartment EBFC. To simulate the blood environment, the concentration of glucose in the system was set as 5 mM, identical to the glucose level in human blood. Given the high surface area and electron transfer efficiency, the G/CNT/Au NP hybrid acted as the anodic substrate material for PQQ-GDH attachment. CVs in Fig. 2D show that compared with the G/CNT/Au NP hybrid substrate electrode, the PQQ-GDH modified bioanode generated a remarkable anodic current, which suggested its exceptional capability for catalyzing glucose oxidation and provided the prerequisite for fabricating high-performance EBFCs and ultrasensitive self-powered sensors.

Coupling the BOD bioconjugate-aptamer-functionalized bioanode with the G/CNT/Au NP/PQQ-GDH bioanode, a self-powered cytosensor based on the exogenous redox-free, one-compartment glucose/O2 EBFC was constructed. As shown in Fig. 3, \( E_{OCV} \) of the assembled EBFC cytosensor reached 0.54 V (curve a). With the increase of cell numbers \( N(\text{cells}) \) bound to the biocathode, the \( E_{OCV} \) of the EBFC cytosensor gradually decreased. It was noted that the signal of the cytosensor to MCF-7 cells (Fig. 3B) displayed a sharper downward trend than that of CCRF-CEM cells (Fig. 3A), probably ascribed to the bigger size of MCF-7 cells that exerted a greater block influence. As shown in Fig. S3, the OCV of the EBFC cytosensor gradually decreased over a linear range of 5–50 000 cells. The linear equations for CCRF-CEM cells and MCF-7 cells were \( E_{OCV} = 0.529 - 0.040 \log N(\text{cells}) \) (correlation coefficient \( R^2 = 0.9994 \)) and \( E_{OCV} = 0.523 - 0.059 \log N(\text{cells}) \) (\( R^2 = 0.9954 \)), with the limits of detection of 3 cells and 2 cells (S/N = 3), respectively, comparable to the most sensitive acute leukemia and circulating tumor cell cytosensing approaches.

Fig. 3 \( E_{OCV} \) of the cytosensor incubated with (A) CCRF-CEM and (B) MCF-7 cells with a cell number of 0, 5, 50, 500, 5000 and 50 000 (curves a–f). Insets show the plot of \( E_{OCV} \) vs. the logarithm of the numbers of cancer cells. Error bars represent the standard deviation of an average value from independent measurements of three cytosensors.
The specificity of the cytosensors is also a key issue that determines the performance in analysing real samples. In this case, K562 and HL-60 cells at 10 times concentration of CCRF-CEM cells were selected as negative controls to evaluate the selectivity of the cytosensors. As depicted in Fig. 4, despite introducing the predominant amount of K562 and HL-60 cells, $E_{OCV}$ of the CCRF-CEM cytosensors output an identical signal at around 0.38 V for each test (bar b, c and d). Similarly, under the interference of 50 000 HeLa cells (g) and 50 000 293T cells (h), respectively. The error bars represent the standard deviation obtained from measurements performed on three cytosensors.

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Notes and references