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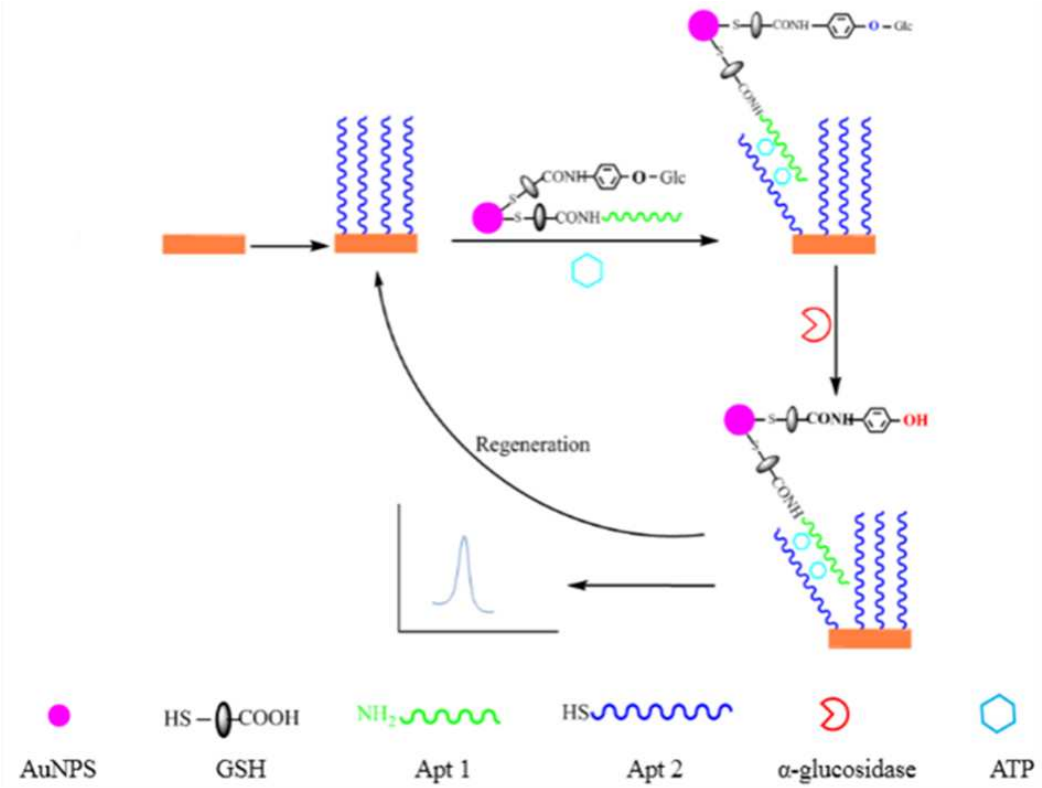

Fabrication of reusable electrochemical biosensor and its application for the assay of α-glucosidase activity

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Abstract:

A reusable biosensor has been fabricated in this work for the assay of α-glucosidase activity and the inhibitor screening. In this design, the aptamer of ATP is split as split aptamer 1 (Apt 1) and split aptamer 2 (Apt 2), and Apt 2 can link gold nanoparticles (AuNPs) modified with Apt 1 and 4-aminophenyl-α-D-glucopyranoside (pAPG). Consequently, the functional AuNPs can be immobilized onto the surface of gold electrode, allowing for salt-induced regeneration. In the presence of α-glucosidase, the glycosyl of pAPG is cut off, and the electroactive phenolic hydroxyls appear to give a

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strong current signal. Furthermore, the biosensor can be recovered very easily by incubating it in water to dissociate the AuNPs modified with Apt 1 and pAPG. So, a new biosensor for α-glucosidase activity detection and inhibitor screening is developed based on enzyme-activated signal generation and recovery. The biosensor may also exhibit good sensitivity for α-glucosidase determination with the detection limit 0.005 U/mL and can be reused by water-washing regeneration with good repeatability. Meanwhile this biosensor can also be utilized for inhibitor screening, which may have potential for clinical applications.

Keywords: Electrochemical biosensor; α-glucosidase; inhibitor screening; split aptamer.

1. Introduction

Electrochemical biosensors have been considered as the most promising devices for biological studies and clinical diagnostics due to their inherent advantages, such as cost-effective for use and sensitive for measurement [1-5]. Nevertheless, electrochemical biosensors also suffer from some drawbacks. One main problem is that the biosensing layer is usually required to be reconstructed after it has been used. Therefore, tedious and time-consuming procedures for the reconstruction of the biosensing layer have to be conducted for the continuous measurements, which makes the use of these biosensors inconvenient. So, it is very important to develop some strategies for introducing gentle regeneration of the surface architectures.

Diabetes is one of the most serious chronic diseases with high incidence around the world [6-8]. In particular, diabetes has shown a new change trend that it increases

exponentially and sickens younger people[9, 10]. α-glucosidase is the main target in the early treatment and prevention of diabetes[11-13]. Meanwhile, α-glucosidase inhibitors (AGIs) play a very important role in controlling the patients' postprandial blood glucose levels and keeping glucose levels within a relatively normal range [14-18]. Therefore, it is of great importance to develop easy-to-use methods to assay the activity of α-glucosidase and to screen its inhibitors. Up to now, hyperglycemic animal model for in vivo screening and enzyme-inhibitor model for in vitro screening have been developed for the determination of α-glucosidase activity and its inhibitors screening[16, 19]. However, the in vivo screening involves long-term lasting experiments and is relatively expensive, and the in vitro enzyme-inhibitor model requires the use of para-nitrophenyla-D-glucopyranoside (pNPG) as artificial substrate. Thus, its sensitivity is limited to some extent. Another drawback is the assessment will be interfered when the overlap of absorption happens between AGIs and 4-nitrophenol[20, 21]. To overcome the drawbacks of the above methods, much effort has been made to detect α-glucosidase activity using fluorescence[22], colorimetric[23], and electrochemical methods[18]. Among them, electrochemical methods display unique merits of high sensitivity and simplicity of operation, which have attracted much attention in the field of α-glucosidase activity detection. However, most of available electrochemical sensors are not reused, which restricts the application of these biosensors. So, it is highly required to make extensive efforts as to develop a reused, sensitive and effective method.

Aptamers are specific nucleic acids selected from random sequence libraries. And

the advantages of the oligonucleotides are that they can be chemically synthesized at very low cost and show good stability during long-term storage. Small molecules triggered linkage of split-aptamer fragments has been introduced to construct biosensors[24]. Herein we have fabricated a reusable biosensor in this work for α-glucosidase activity assay and its inhibitors screening. In this design, ATP aptamer is split into two flexible single strand deoxyribo-nucleic acid (ssDNA) fragments: Apt 1 and Apt 2[25-27]. The Apt 1 and 4-aminophenyl-α-D-glucopyranoside (pAPG) are immobilized on gold nanoparticles (AuNPs). At the same time, the Apt 2 is modified on gold electrode by self-assembly via Au-S bonds. In the presence of ATP, the "sandwich-type" construction is formed at a suitable salt concentration. Then the electroactive phenolic hydroxyls appear when the pAPG is cut by α-glucosidase. Thus, the redox response can be generated on gold electrode surface with a strong current signal, which is direct related to the amount of α-glucosidase. Furthermore, the biosensor can be recovered very easily by incubating the working electrode in water to dissociate the AuNPs modified with Apt 1 and pAPG. Experiments show that the biosensor can be reused for at least five times and the electrochemical signal is not significantly attenuated. By taking the enzyme-activated signal strategy, the biosensor shows high sensitivity toward the assay of α-glucosidase activity with the detection limit of 0.005 U/mL. This work may provide a novel strategy for the design of reusable electrochemical biosensors and a new method for the detection of α-glucosidase activity and its inhabitor screening.

2. Materials and Methods

2.1. Materials and reagents

Chloroauric acid ($HAuCl_4 \cdot 3H_2O$), trisodium citrate, adenosine triphosphate (ATP) glutathione (GSH), and α-glucosidase were purchased from Sigma (Shanghai, China). 4-Aminophenyl-α-D-glucopyranoside (pAPG) was purchased from Beijing Chemsynlab Pharmaceutical Co. Ltd (Beijing, China). IPEC-J2 cells obtained from Guang Zhou Jennio Biotech Co. Ltd. All buffers and aqueous solutions were prepared with ultrapure water, which was purified with a Millipore Milli-Q water purification system (Barnstead, USA) to a specific resistance of 18 MΩ·cm. The solution of α-glucosidase was obtained by dissolving at different concentrations in 10 mM phosphate buffer solution (PBS) (pH 6.8). The split aptamer sequences of ATP were all synthesized by Sangon (Shanghai, China). Their sequences were as below: Aptamer-1 (Apt 1):5′-NH_2-$(CH_2)_6$-ACC TGG GGG ATA AT-3′, Aptamer-2 (Apt 2):5′-TGC GGA GGA AGG TAA AAA AAA AA- $(CH_2)_6$-SH-3′.

2.2. Apparatus

Electrochemical experiments, including cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and differential pulse voltammetry (DPV) were performed with a CHI660D electrochemical workstation (Shanghai CH Instruments Co.). A conventional three-electrode system comprised of a Pt wire auxiliary electrode, a saturated calomel reference electrode (SCE) and an Au working electrode. All potentials herein were referenced to the SCE. UV-vis absorption spectra were acquired on a TU-1901 UV-vis spectrometer (Beijing Purkinje General Instrument Co. Ltd). The zeta potential values were acquired by a Brookhaven Zeta 90Plus analyzer.

2.3. Preparation of gold nanoparticle modified with GSH

AuNPs were synthesized according to a published procedure[28-30]. In brief, trisodium citrate (10 mL, 38.8 mM) was added to a boiling solution of $HAuCl_4$ (100 mL, 1 mM), and the resulting solution was kept continuously boiling for another 30 min to give a wine red mixture. The mixture was cooled to room temperature and then filtered through a Millipore syringe (0.45 μm) to remove the precipitate, and the filtrate was stored in a refrigerator at 4 °C before use. 10 μL concentrated aqueous solution of glutathione (100 μM) was added dropwise to the 1 mL gold colloidal solution and left to stir about 2 h in dark to ensure the self-assembly of the glutathione onto the surface of AuNPs. The mixture containing GSH/AuNPs was centrifuged at 12000 rpm for 20 min at room temperature and the mixture was redispersed in MES buffer (10 mM, pH 6.0) three times.

2.4. Preparation of gold nanoparticle modified with pAPG and Apt 1

The solution of EDC (20 mM) and NHS (50 mM) were added into 500 μL GSH/AuNPs solution. After 15 minutes, 25 μL solution containing pAPG (20 mM) and 50 μL solution containing Apt 1 (10 μM) were added. Then the mixed solution was stirred at room temperature for 12 h to give the final product (Apt 1/pAPG/GSH/AuNPs). The mixture was centrifuged at 12000 rpm for 20 min at room temperature and the resulting Apt 1/pAPG/GSH/AuNPs conjugates were redispersed in PB buffer (10 mM, pH 6.8) three times and stored at 4 °C before use.

2.5. Fabrication of biosensor

The gold electrode was treated according to the previous literature[31, 32]. For

probe immobilization, 10 μL of probe immobilization buffer containing 1.0 μM thiol-capped Apt 2 was dripped on gold electrode surface and maintained for 12 h at humid condition. Then, the Apt 2 modified electrode was rinsed three times with probe immobilization buffer. After that, 5 μL of Tris-HCl (10 mM) containing 1 mM MCH was dripped on the electrode surface and kept for 15 min to hold a good orientation of Apt 2 for its good recognition ability.

2.6. α-glucosidase activity assay

The α-glucosidase activity assay was performed using the following procedure. The binding assay was carried out by dripping 10 μL of the above mixture containing 150 mM NaCl and 2.5 mM ATP on the electrode surface at 37 °C for 60 min. After that, the electrode was rinsed three times with PBS to remove the unligated Apt 1/pAPG/GSH/AuNPs and dried with nitrogen blowing. 10 μL of enzyme solution dissolved in PB (10 mM, pH 6.8) with different concentrations ranging from 0.01 to 1.3 U/mL was dripped on above electrode and incubated at 37 °C for 20 min. The modified electrode was finally ready for measurement.

2.7. Electrochemical measurements

Differential pulse voltammetry (DPV) was performed in a certain volume of 0.1 M PB (pH 6.8) containing 0.5 M NaCl with the scan range from -0.25 - 0.75 V. Electrochemical impedance spectra (EIS) was performed in 5.0 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl with the frequency from 10 to 10^5 Hz. The electrochemical measurements were all carried out at room temperature.

3. Results and discussion

3.1. The principle of the biosensor

Please insert Scheme 1 here.

The principle of the biosensor is shown in Scheme 1. Firstly, GSH is linked to AuNPs to form GSH/AuNPs through Au-S bonds. Then, Apt 1 and pAPG are conjugated to the GSH/AuNPs surface through chemical reaction between the amine groups and the carboxylic acid groups to give the functionalized AuNPs (Apt 1/pAPG/GSH/AuNPs). Meanwhile, Apt 2 is immobilized on gold electrode through Au-S bonds. Subsequently, the addition of ATP as a linker can trigger the combination of Apt 2 and Apt 1/pAPG/GSH/AuNPs. Then the electroactive phenolic hydroxyls appear when pAPG is cut by α-glucosidase. Thus, the current will occur as a result of the specific cleavage of pAPG into 4-aminophenol. Since the degree of current is related to the concentration of α-glucosidase, a new method for monitoring α-glucosidase activity can be developed. And the biosensor can be regenerated very easily by incubating the working electrode in water to dissociate the Apt 1/pAPG/GSH/AuNPs.

3.2. Synthesis and characterization of Apt 1 /pAPG/GSH/AuNPs

Please insert Fig. 1 here.

UV-vis spectrophotometry has been used to validate the successful coupling of Apt 1 and pAPG to AuNPs modified with GSH. As shown in Fig. 1A, compared to the UV-vis spectra of AuNPs and GSH-AuNPs, strong absorbance peaks at 260 nm and 280 nm are detected for Apt 1/pAPG/GSH/AuNPs, which shows an obvious change in the shape, position and symmetry of the absorption peak. The result demonstrates that

Apt 1/pAPG/GSH/AuNPs complexes have been successfully synthesized.

Zeta potentials of AuNPs averaged in Fig. 1B further verify the modification process of Apt 1 /pAPG/GSH/AuNPs. The samples have all been centrifuged and suspended in Tris-HCl buffer to the same concentration in advance. The zeta potentials of AuNPs, GSH/AuNPs, and Apt 1 /pAPG/GSH/AuNPs are -35.70 mV ± 3.75 mV, -21.24 mV ± 6.11 mV, and -2.78 mV ± 2.37 mV, respectively. When AuNPs are modified successively with Apt 1 and pAPG, the zeta potential values are obviously increased, which also clearly indicates that Apt 1 /pAPG/GSH/AuNPs *have been proven to be successfully synthesized.*

3.3. Characterization of the modified electrode

Please insert Fig. 2 here.

Electrochemical impedance spectroscopy (EIS) has been adopted to characterize the step by step modification of the gold electrode surface. As shown in Fig. 2, firstly, no impedance can be observed on the bare gold electrode, indicating the electrochemical active molecules $[Fe(CN)_6]^{3-/4-}$ is free to the electrode surface (curve a). Subsequently, after the self assembly of Apt 2 on the electrode surface, an increase of interfacial electron resistance can be observed (curve b). Then, MCH is immobilized on the electrode surface, thus the interfacial electron resistance increases obviously (curve c), which is attributed to that MCH may obstruct the electron transfer. Furthermore, when the AuNPs modified with Apt 1/pAPG/GSH have been immobilized on the electrode surface, there is a much bigger interfacial electron resistance, due to the fact that the immobilized compounds have largely increased the

steric hindrance on the electrode surface (curve d). Therefore, the results have revealed the progressively assembling processes on the electrode surface.

3.4. Feasibility investigation for enzyme activity assay

Please insert Fig. 3 here.

To investigate the feasibility of the proposed strategy for α-glucosidase activity assay, differential pulse voltammetric (DPV) is employed to investigate the electrochemical behaviors of different electrodes in 0.1 M PB (pH 7.4) containing 0.5 M NaCl from -0.25 to 0.75 V. As shown in Fig. 3, in the presence of α-glucosidase, a significant current signal is observed indicating the occurrence of cleavage of pAPG by α-glucosidase on the gold electrode (curve a). However, in the absence of α-glucosidase, there is a smaller current signal response since the signalling molecule can not occur (curve b). Furthermore, in the absence of Apt 1 (curve c), Apt 2 (curve d) or pAPG (curve e), small electrochemical signals are observed, which clearly demonstrates that Apt 1, Apt 2, and pAPG are necessary to achieve significantly amplified signals. Therefore, the above results indicate the feasibility of this method for the detection of α-glucosidase activity.

3.5. Optimization of assay conditions

In order to provide the biosensor with a good performance, different parameters such as the concentration of ATP, and incubation time between Apt 2 and Apt 1 /pAPG/GSH/AuNPs have been optimized. The effect of ATP concentration on the biosensor response has been examined. As shown in Fig. S1A, with the increasing concentrations of ATP, the effect of the biosensor response increases and trends to a

constant value at 2.5 mM. Therefore, the optimal ATP concentration is 2.5 mM.

Incubation time between Apt 2 and Apt 1 /pAPG/GSH/AuNPs is another important factor to the sensitivity of the biosensor. So the effect of the incubation time on the biosensor response has also been investigated. For this purpose, Apt 2 and Apt 1 /pAPG/GSH/AuNPs are incubated for different time (10 - 80 min). As shown in Fig.S1B, the biosensor response enhances with increasing incubation time and then reaches to level-off after 60 min. Therefore, 60 min is used for measuring α-glucosidase in the assay.

3.6. α-glucosidase activity assay

Please insert Fig. 4 here.

The activity of α-glucosidase is investigated by DPV in 0.1 M PB containing 0.5 M NaCl. As can be seen in Fig.4, the peak current magnifies with increasing α-glucosidase concentration ranged between 0.01-1.3 U/mL. The linear relationship between the logarithm of α-glucosidase concentration and the peak current is obtained as $y=1.61\ x+ 4.68$ ($R^2=0.994$) and the detection limit is 0.005 U/mL (S/N=3). So the biosensor has a good sensitivity for α-glucosidase determination.

3.7. Selectivity of the biosensor

Please insert Fig. 5 here.

To investigate the selectivity of the method, alkaline phosphatase (ALP) is selected as an interference enzyme to estimate the selectivity of this method, which is also rich in small intestine. Due to the specific site recognition of α-glucosidase toward its substrate, the method can easily discriminate α-glucosidase from ALP. As shown in

Fig.5, significant peak current is observed in the presence of α-glucosidase. In contrast, only small peak current is observed in the presence of ALP, which is similar to the blank. These results demonstrate that the assay method for α-glucosidase activity exhibits a good selectivity.

3.8. Reusability investigation for α-glucosidase activity assay

Please insert Fig. 6 here.

To investigate whether the biosensor can be reused, the working electrode modified with Apt 2 and Apt 1/pAPG/GSH/AuNPs is rinsed with water for three times and then used again for measurement. As shown in Fig. 6, at first, a small electrochemical signal is observed. Then, after the electrode is rinsed and used for the detection of the α-glucosidase activity, an obvious electrochemical signal is observed. The biosensor has been reused for at least five times, and electrochemical signal is not significantly attenuated. These results have well demonstrated the reusability of this biosensor for the detection of α-glucosidase activity.

3.9. Electrochemical detection of α-glucosidase activity in the cell medium

Please insert Fig. 7 here.

Human α-glucosidases are membrane bound enzymes presenting in the epithelium of the small intestine. The α-glucosidase activity in cell lysates is subsequently evaluated by the biosensor. The DPV peak currents upon analyzing different numbers of IPEC-J2 cell are illustrated in Fig. 7. The peak currents increase with the number of IPEC-J2 cells. This result is in accordance with the fact that, with increasing cell number, more pAPG is hydrolyzed by α-glucosidases, resulting in a strong peak

current.

3.10. Inhibition assay for enzymatic activity

Please insert Fig. 8 and Table 1 here.

It has been reported that specific α-glucosidase inhibitors may decrease postprandial hyperglycemia and improve impaired glucose tolerance. In order to verify the developed biosensor can be employed for the screening of α-glucosidase inhibitors, 3-propylidenephthalide has been selected for this study. As exhibited in Fig. 8, with increasing 3-propylidenephthalide concentration from 0.0 to 0.8 mM, the peak currents gradually decrease. The 3-propylidenephthalide is found to inhibit 50% α-glucosidase activity with 0.35 mM. This result is consistent with the phenomenon reported by others and demonstrates that the method can also be used for inhibitor screening.

4. Conclusions

In this study, we have fabricated a reusable biosensor to detect α-glucosidase. In this design, the aptamer of ATP is split and the linkage and separation of the split-aptamer may facilitate regeneration of the sensing interface by simply varying the salt concentration. And the electrode can be conveniently reused for the next detection. These findings provide a reusable, stable, and simple amperometric method to detect α-glucosidase compared to existing approaches. By using pAPG as substrate, the proposed biosensor can detect α-glucosidase activity sensitively with a low detection limit of 0.005 U/mL, which is similar to previously reported (Table 1). The results have well demonstrated the biosensor can be reused for at least five times for

the detection of α-glucosidase activity. Moreover, the biosensor can be used to screen its inhibitor. Overall, this novel strategy would have widespread applications in the detection of α-glucosidase activity and diabetes drug development in the future.

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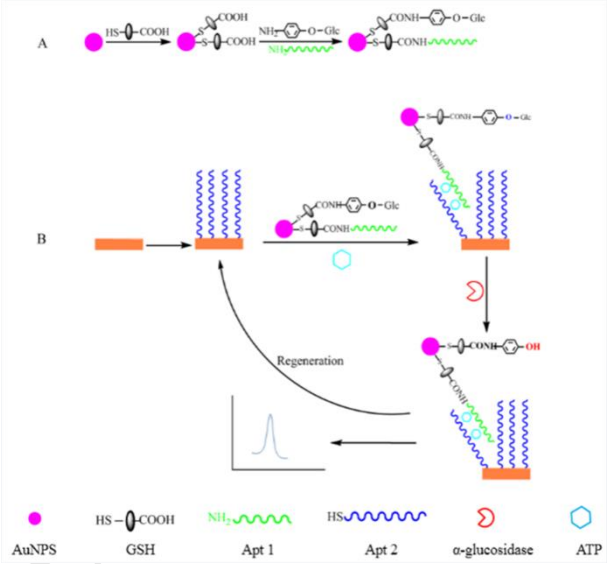
Figures and Captions



Scheme 1. Illustration of the α-glucosidase activity assay principle

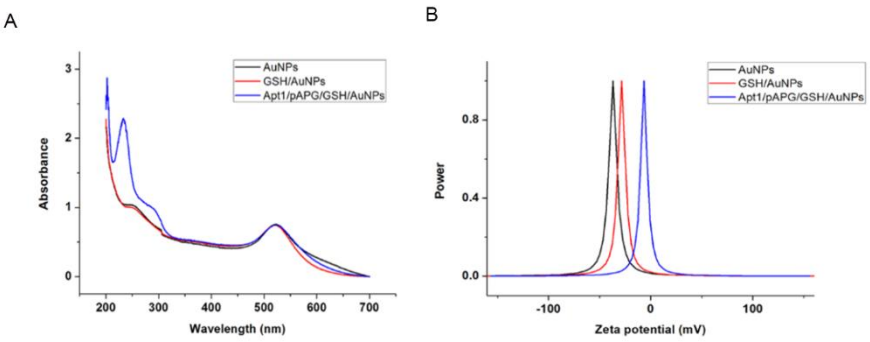


Fig.1. (A) UV-vis spectra of AuNPs, GSH/AuNPs and Apt1/pAPG/AuNPs. (B) Zeta

potentials of AuNPs, GSH/AuNPs and Apt1/pAPG/AuNPs.

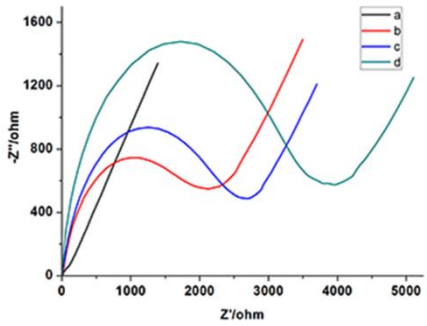


Fig.2. Electrochemical impedance spectra of (a) the bare gold electrode, (b) the electrode treated with Apt 2, (c) for the treated with MCH, (d) for the treated with AuNPs modified with Apt 1/pAPG/GSH.

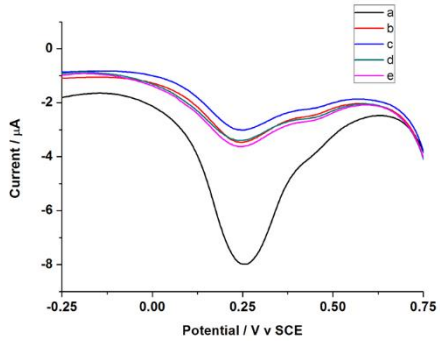


Fig.3. DPV curves obtained at the gold electrode treated successively with (a) Apt 2, ATP, AuNPs modified with Apt 1/pAPG/GSH, and α-glucosidase, (b) Apt 2, ATP, and AuNPs modified with Apt 1/pAPG/GSH, (c) Apt 2, ATP, AuNPs modified with pAPG/GSH, and α-glucosidase, (d) ATP, AuNPs modified with Apt 1/pAPG/GSH, and α-glucosidase, (e) ATP, AuNPs modified with Apt 1/GSH, and α-glucosidase for 0.1 M PB (pH 7.4) containing 0.5 M NaCl.

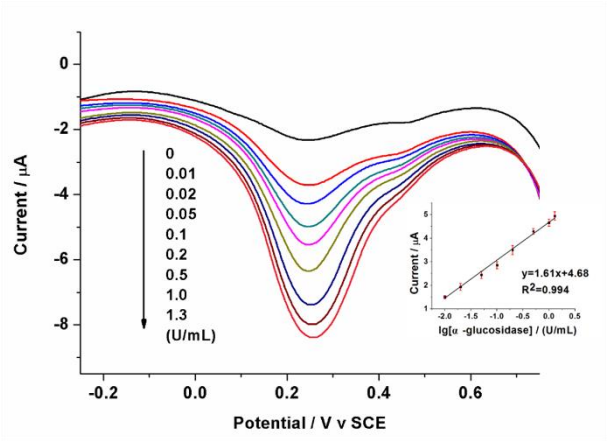


Fig. 4. DPV curves for different concentrations of α-glucosidase (U/mL): (a) 0, (b) 0.01, (c) 0.02, (d)0.05, (e)0.1, (f)0.2, (g)0.5, (h)1.0, and (i)1.3 U/mL in 0.1 M PB containing 0.5 M NaCl. The inset is the calibration curve. Error bars indicate standard deviation of three independent experiments.

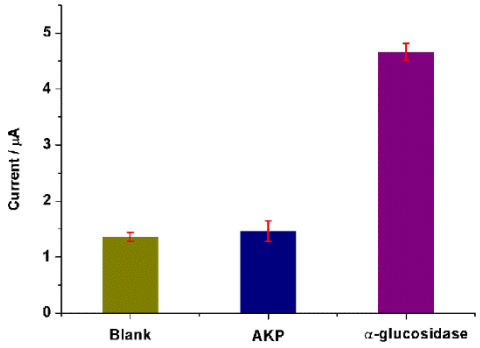


Fig.5. Comparison of the DPV peak currents in the presence of 1.0 U/mL α-glucosidase and 1 U/mL AKP respectively, in which blank indicates the condition in the absence of AKP as well as α-glucosidase. The error bars represent the standard deviation of three measurements.

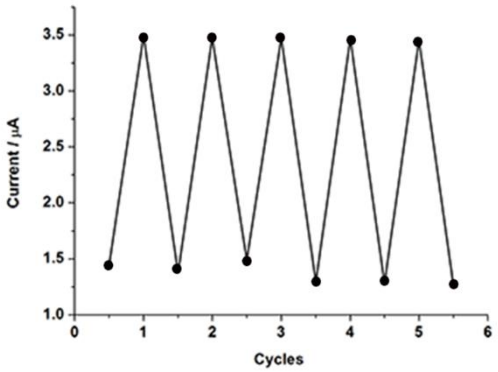


Fig.6. The reusability of the biosensor. The concentration of α-glucosidase is 0.2 U/mL.

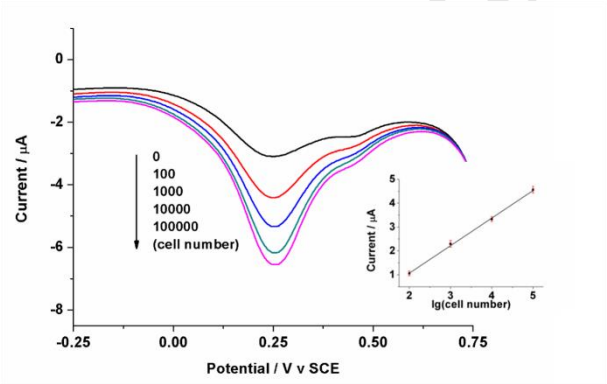


Fig.7. DPV obtained in detecting IPEC-J2 cells (0, 100,1000,10000,100000 cells). The inset is relationship between the signal response and IPEC-J2 cells, with error bars representing standard deviation (n=3).

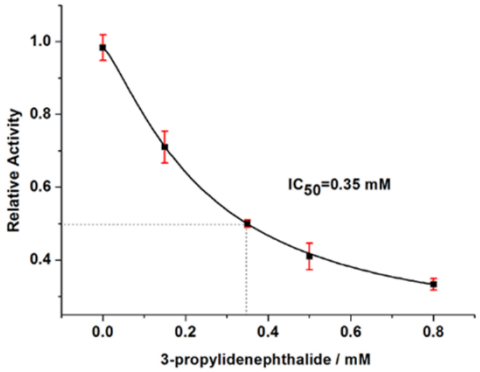


Fig.8. The inhibition effect of 3-propylidenephthalide on α-glucosidase activity.

Table 1. A comparison of the method with previously reported ones.

System	Detection method	Detection limit (U/ mL)	Reusability	Reference
Conjugated polymer and PNPG	Fluorescence	0.01	No	[22]
pAPG-functionalized AuNPs	Absorption	0.004	No	[14]
Unmodified gold nanoparticles	Absorption	0.001	No	[23]
AgNPs/DA and MNPs/ pAPG with PBA/GE	Electrochemical	0.1	No	[18]
AuNPs modified with ATP aptamer and pAPG	Electrochemical	0.005	Yes	This study

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Highlights:

- A reusable biosensor has been fabricated for the assay of α-glucosidase activity and the inhibitor screening.
- This biosensor is on the basis of split aptamer of ATP.
- The gold nanoparticles modified here is multifunctional.
- The biosensor can be reused by water-washing regeneration with good repeatability.