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A highly selective and sensitive electrochemical sensor for tetracycline resistant genes detection based on the non-covalent interaction of graphene oxide and nucleobase

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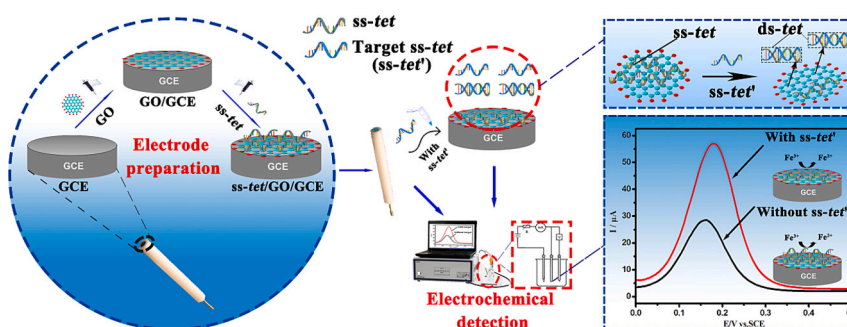
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HIGHLIGHTS

- The sensor was prepared based on the non-covalent interaction of GO and nucleobase.
- GO and nucleobase interaction energy calculated via PWmat.
- The sensor had exceptional 50 pM sensitivity and a rapid 35 min preparation time.
- The sensor exhibited outstanding accuracy comparable to RT-qPCR and PCR array.
- The sensor successfully achieved accurate detection of *tet* in actual water.

GRAPHICAL ABSTRACT



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ABSTRACT

Antibiotic resistance genes (ARGs) are causing worldwide environmental problems, however, the traditional analytical methods and test equipment for them are time-consuming and expensive. The electrochemical sensor using the non-covalent bond between graphene oxide (GO) and single-stranded *tet* (ss-*tet*) was established for specific tetracycline resistance genes (*tet*), composed of ss-*tet* and complementary ss-*tet'* (ss-*tet'*) in water) detection, which preparation time was only 35 min and far less than most reported sensors based on covalent bond. As the result of the detection for *tet*, the developed sensor not only had the low detection limit of 50.0 pM (8.1×10^2 copies·mL⁻¹), the short detection time within 42 min, but also had satisfactory stability, excellent reproducibility, and highly selectivity (RSD < 4.43 %). Besides, it also had acceptable accuracy comparing to the real-time quantitative polymerase chain reaction (RT-qPCR) and PCR array in *tet* detection. Noticeably, it also had been successfully applied to *tetA* detection in different water samples. In brief, the prepared non-covalent bond sensor is simple, rapid, and suitable for highly selective and sensitive detection of the ARGs in actual water.

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1. Introduction

Tetracycline is a broad-spectrum antibiotic and has been widely overused in agricultural purposes and human therapy, resulting in the production of tetracycline resistance genes (*tet*). Antibiotic resistance genes (ARGs, including *tet* etc.) are regarded as a novel pollutant, which can diffuse and transfer in different environmental media and organisms with a longer persistence (Tao et al., 2010; Chopra and Roberts, 2001; Zhou et al., 2021). At least an additional 10 million deaths a year stem from antibiotic resistance by 2050, according to the report commissioned by the UK government (Manaia, 2017). Therefore, ARGs may be potentially more toxic than antibiotics (Fan et al., 2021; Kim et al., 2018). At present, *tet* has been frequently detected in different water environments (Ushijima and Suzuki, 2016), such as the detected concentrations of *tet* in the water of the Yangtze River Estuary ranging from 8.48×10^2 to 2.23×10^4 copies·mL⁻¹ (Lu et al., 2019).

Molecular technologies, such as polymerase chain reaction (PCR) and next generation sequencing, are often used to detect the concentrations of ARGs in aqueous environments (Espulgar et al., 2021). However, they are laborious and need the long processing time (> 2 h), the expensive instrument, and the skilled technician (Chaibun et al., 2021; Peinetti et al., 2021; Pokhrel et al., 2020). Besides, fluorescence spectrometry has been used to detect the targeted single-stranded DNA (targeted ss-DNA, following as ss-DNA') such as the human immunodeficiency virus gene (Lu et al., 2009) etc. The detection is based on the signal of the modified fluorophore, the strong fluorescence quenching ability of carbon-based materials, and the affinity differences between ss-DNA and double-stranded DNA (ds-DNA). However, the labeling must be performed in ss-DNA' detection and can usually result in the low efficiency, the complicated operating procedure, and the sample contamination (Hu et al., 2012; Hu et al., 2011).

Compared with the above methods, electrochemical method for detection of DNA has the advantages of high sensitivity, good selectivity, rapidity, low cost, and not requiring labeling (Chuljin et al., 2021). Additionally, the method also has the characteristics of easy detection, suitable for miniaturization, flexible size, and controllable parameters (Xu et al., 2014). Despite the significant advantages of the electrochemical method, it is still further improving sensor performance. Recently, the scientists have devoted to prepare the sensing materials, design DNA probes, and develop new sensing mechanisms (Chen et al., 2021).

Currently, the nucleobases in ss-DNA probes can be attached with the surface of materials on electrode by the covalent or the non-covalent bond, which can be fabricated the electrochemical sensor for ss-DNA' detection. Using the covalent bond to attach the ss-DNA probes can avoid steric hindrances to hybridization and increase the stability of the electrode. In addition, the ss-DNA probe also can hybrid with ss-DNA' in solution, and the formed ds-DNA must remain on the electrode surface. However, it usually requires more preparation steps and incubation time for the immobilization of ss-DNA probes (Xu et al., 2020; Richard and Venkataraman, 2021; Guo et al., 2020), such as capture probe/hexanedithiol/mercaptohexanol/self-assembled mixed monolayers/screen-printed Au electrode, capture probe/Au nanoparticles/electropolymerization of aniline/chitosan-graphene sheets/glassy carbon electrode (GCE), and ss-DNA/Ni-organic framework composite/Au nanoparticles/carbon nanotubes/polyvinyl alcohol electrode, the preparation processes were complex and all of the preparation time was >17 h (Meng et al., 2020; Lu et al., 2021; Sánchez-Paniagua et al., 2020).

Nevertheless, using non-covalent bond to attach ss-DNA probes has the advantages of easy operation, time-saving, and without label (Wang et al., 2014; Liu et al., 2013). The physical adsorption, formed by non-covalent bond, between miRNA and the modification materials on electrode only required incubation 20 min with continuous shaking (Tang, 2011). The bonding energy of hydrogen bonds ($25\text{--}40\text{ kJ}\cdot\text{mol}^{-1}$) between nucleobases pairs was much greater than the π -stacking effect between GO and nucleobases (Wan et al., 2020). In addition, compared

with ss-*tet*, the affinity between double-stranded *tet* (ds-*tet*) and GO was weaker in that the nucleobases of ds-*tet* were shielded inside the double helix structure, breaking the π -stacking between GO and nucleobase (Li et al., 2014). The ss-*tet* probes adsorbed on the GO/GCE with negative charge prevented the transfer of electrons and caused the decreasing of the peak current. As the ss-*tet* probes on the GO/GCE hybridized with the ss-*tet*' in phosphate buffer solution (PBS, 0.1 M and pH at 7.4), the ss-*tet* probes released from GO/GCE surface, causing the peak current to increase. Naturally, the electrochemical sensor based on the non-covalent interaction of GO and nucleobases is expected to be widely used in the quantitative analysis of ARGs.

The ds-*tet* had been proven to exist in actual water (Bonanni, 2012), and it could be unwound to ss-*tet* and ss-*tet*' by pretreatment of water, the amount of ss-*tet*' could represent the *tet* at this moment. Herein, an electrochemical DNA sensor based on the non-covalent interaction of graphene oxide (GO) and nucleobases had been prepared, which could specifically detect *tet* in actual water. The principle and experimental procedure of sensing are as follows: (1) ss-*tet* probes could be effectively and conveniently adsorbed on the GO/GCE surface by π -stacking (Ling et al., 2013) to prepare for the developed sensor (ss-*tet*/GO/GCE) sensor successfully, which simplified the sensor preparation steps and saved time compared to preparation of the covalent immobilization probe (Park et al., 2014); (2) by immersing the ss-*tet*/GO/GCE in PBS (0.1 M and pH at 7.4) containing the targeted of ss-*tet* (ss-*tet*'), the ss-*tet* probes on the GO/GCE were hybridized with ss-*tet*' to form ds-*tet*, and accompanied by release from the GO/GCE surface; (3) the number of ss-*tet* probes and the concentration of ss-*tet*' in solution could be monitored by differential pulse voltammetry (DPV) in the presence of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox system, and the above system was often used in electrochemical analysis and which electrochemical behavior in solution was a reversible process with single electron transfer. Thus, the developed sensor with excellent electrochemical performance could be used to accurately, specifically and selectively detect *tet* in actual water, and had broad application prospects in gene-sensing and enrichment gene detection methods.

2. Materials and methods

2.1. Reagents, solutions and apparatus

Information on the used reagents, solutions and apparatus is provided in Text S1 in the Supporting Information (SI).

2.2. Preparation and characterization of ss-*tet*/GO/GCE

The bare working electrode (GCE, with a diameter of 3 mm) was washed with water and kept at room temperature after being polished using $0.05\text{ }\mu\text{m}$ alumina powder. The GO dispersion was sonicated for 5 min to achieve uniform dispersion before being used. The $5\text{ }\mu\text{L}$ GO suspension ($1.0\text{ mg}\cdot\text{mL}^{-1}$) was dropped onto the treated GCE, transferred to an infrared lamp (40 W), and irradiated for 2 min to obtain the GO/GCE. Afterwards, $10\text{ }\mu\text{L}$ phosphate buffer solution (PBS, 0.1 M and pH at 7.4) containing $1\text{ }\mu\text{M}$ (equaling to 1.62×10^7 copies·mL⁻¹) ss-*tet* (including five subtypes: ss-*tetA*, -*tetD*, -*tetE*, -*tetW*, and -*tetX*, respectively, 20-mer) was dropped onto the GO/GCE surface and oscillated for 30 min in a thermostatic orbit incubator at 180 rpm to adsorb the ss-*tet* probes. The obtained ss-*tet* (ss-*tetA*, -*tetD*, -*tetE*, -*tetW*, and -*tetX*) /GO/GCE was then washed with PBS (0.1 M and pH at 7.4) to remove excess non-adsorbed ss-*tet*. The adsorption conditions of ss-*tet* probes on the GO/GCE were optimized as shown in Fig. S1 (details described in Text S2 in the SI). The oligonucleotide sequences of used ss-*tet* and ss-*tet*' are shown in Table S1 of SI.

The GO and ss-*tetA*/GO were characterized by atomic force microscopy (AFM), X-ray diffraction (XRD), Raman spectra, scanning electron microscopy (SEM), and energy dispersive X-ray spectroscopy (EDS) spectrometer. UV visible (UV-vis) absorption spectrum was applied to

characterize GO, *ss-tetA*, and *ss-tetA*/GO. Fluorescence emission spectrum was used to characterize GO, *ss-tetA*, *ss-tetA*/GO, *ds-tetA*, and mixed PBS (0.1 M and pH at 7.4) containing GO and *ds-tetA*. The modified electrodes were characterized by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS).

2.3. Electrochemical detection mechanism of *tet*

The adsorption energies between adenine, thymine, guanine, cytosine and GO were calculated by the PWmat. The exchange-correlation interaction was described by generalized gradient approximation (GGA) with the Perdew-Burke-Ernzerhof (PBE) functional. The cutoff energy was adopted to be 60 Ryd, the vacuum interval was set as 15 Å, and the k-point was chosen as $1 \times 1 \times 1$ for the energy of substrate a (E_a), substrate b (E_b), and system ab after the adsorption between E_a and E_b (E_{ab}) calculation.

The E_{ab} , removing the energies of E_a and E_b , was the adsorption energy (E_{ads}). The E_{ads} was calculated by the following Eq. (1):

$$E_{ads} = E_{ab} - E_a - E_b \quad (1)$$

2.4. Electrochemical analysis

2.4.1. Quantitative detection of *ss-tet*

The prepared *ss-tet*/GO/GCE was immersed in the PBS (0.1 M and pH at 7.4) containing different concentrations ($50.0 \text{ pM} - 1.0 \text{ nM}$, equaling to $8.10 \times 10^2 - 1.62 \times 10^4 \text{ copies} \cdot \text{mL}^{-1}$) of *ss-tet'* (*ss-tetA'*, *-tetD'*, *-tetE'*, *-tetW'*, and *-tetX'*, 20-mer) for hybridization for 40 min. DPV experiments were conducted in a $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox system in the range from 0 to +1.0 V, with a pulse amplitude of 50 mV and a pulse width of 50 ms to monitor the change of peak current before and after hybridization. The above processes were performed at room temperature. The hybridization conditions between *ss-tet* on GO/GCE and *ss-tet'* in solution were optimized as shown in Fig. S2 (details described in Text S2 in the SI).

2.4.2. Detection of *tet* in actual water samples

The inlet and outlet of Sewage Treatment Plant (Changchun, China), the water of Yitong River (Changchun, China), the sewage from primary sedimentation pond and secondary sedimentation tank of Jilin Petrochemical Company Sewage Treatment Plant, and domestic sewage from a community (Changchun, China) were collected and filtered through a $0.45 \mu\text{m}$ filter. Then, the pH was adjusted to 7.4 using 0.1 M HCl and 0.1 M NaOH. The prepared *ss-tet*/GO/GCE were used to detect *tet* in the above water samples. Before electrochemical measurement, all the water samples were placed at 94°C for 40 min to denature and unwinding the existing ds-DNA (including ds-*tet*) and rapidly cooled to 4°C for 10 min to prevent DNA renaturation. In addition, the standard interior label experiment was also used to analyze *tet* in the above water samples. The recovery was calculated by means of the following Eq. (2):

$$\text{Recovery\%} = \frac{C_{\text{Found}} - C_{\text{Actual}}}{C_{\text{Added}}} \times 100\% \quad (2)$$

where C_{Found} was the concentration of *ss-tet'* calculated by the linear equations, C_{Actual} was the actual concentration of *ss-tet'* in the water sample and C_{Added} was the concentration of *ss-tet'* added into the water samples. All experiments were repeated three times. PCR array was also used to quantitatively detect *tet* in the above water samples. Verification principle is that relative standard deviation (RSD) is $<5.00\%$ to prove the accuracy of the method according to the regulations of the Food and Drug Administration (FDA).

2.5. Accuracy analysis

Real-time quantitative PCR (RT-qPCR) was applied to quantitatively detect *tetA* ($R^2 > 0.9900$), which process was consistent with previous

reports (Zhang et al., 2020) (details described in Text S3 in the SI). The required primers are listed in Table S1. The standard curve of *tetA* by RT-qPCR was shown in Table S3.

2.6. Statistical analysis

SPSS 22.0 and Origin 8.0 were used for statistical analysis. The data reported are the mean, and the error bars represent the standard deviation of three repeated experiments.

3. Results and discussion

3.1. Characterization of *ss-tet*/GO/GCE

The main control parameters of the developed sensor based on non-covalent bond are the adsorption and release of the *ss-tet* probes from the GO surface, which were analyzed by AFM (a), XRD (b-I), Raman spectroscopy (b-II), UV-vis (b-III), fluorescence emission spectrum (b-IV), SEM (c), CV (d-I), and EIS (d-II) as shown in Fig. S3 (details described in Text S4 in the SI). The obtained results were consistent with previous findings on the non-covalent interactions between GO and five nucleobases using molecular dynamics (MD), density functional theory (DFT), and isothermal titration calorimetry (ITC) (He et al., 2010; Varghese et al., 2010; Antony and Grimme, 2008), and illustrated that: (1) the ss-DNA (such as *ss-tetA*) probes could be physically adsorbed on the surface of GO by the non-covalent bond between nucleobases and GO (Xu et al., 2010; Giovanni et al., 2012); (2) the non-covalent bond between the nucleobases and the GO could be destroyed and led to the release of ss-DNA probes from GO/GCE after being formed the ds-DNA (such as ds-*tet*) (Li et al., 2010).

3.2. Interaction energy of GO with nucleobases

GO was proposed as a substrate on the GCE for ss-DNA adsorption. The adsorption energy of a single DNA base on GO was calculated based on DFT via the PWmat code (Fig. 1). The obtained adsorption energies between adenine (A), thymine (T), guanine (G), and cytosine (C) with GO were -0.48 , -0.61 , -0.54 , and -0.52 eV (equaling to -7.68×10^{-23} , -9.76×10^{-23} , -8.64×10^{-23} , and $-8.32 \times 10^{-23} \text{ kJ}$), respectively, and less than bonding energy of hydrogen bonds between A and T ($50-80 \text{ kJ} \cdot \text{mol}^{-1}$, equaling to $3.10 \times 10^{23} - 4.98 \times 10^{23} \text{ eV} \cdot \text{mol}^{-1}$), and also less than G and C ($75-120 \text{ kJ} \cdot \text{mol}^{-1}$, equaling to $4.65 \times 10^{23} - 7.47 \times 10^{23} \text{ eV} \cdot \text{mol}^{-1}$). Therefore, it was convinced that the interaction between nucleobases and GO belonged to physical adsorption, which had the following advantages: (1) the adsorption process was simple and time-saving; (2) its desorption process was relatively easy and fast compared to chemisorption (Song et al., 2009).

3.3. DPV sensing of *tet*

The conditions for GO adsorption *ss-tet* probes and hybridization between *ss-tet* probes and *ss-tet'* were optimized as shown in Figs. S1 and S2 (details described in Text S2 in the SI) in order to develop a high-performance sensor. Under the optimal experimental conditions, the sensitive detection of *ss-tet'* (*ss-tetA'*, *-tetD'*, *-tetE'*, *-tetW'*, and *-tetX'*, 20-mer) in PBS (0.1 M and pH at 7.4) was performed by DPV using the prepared *ss-tet* (*ss-tetA*, *-tetD*, *-tetE*, *-tetW*, and *tetX*) /GO/GCE. Subsequently, the linear regression equations and limit of detections (LODs) for the *ss-tet'* were also fitted. As shown in Fig. 2, the peak currents increased linearly with the logarithmic concentrations of the *ss-tet'*, and the linearity range was from 50.0 pM to 1.0 nM . The above linear response range was similar to the concentration range of *tet* in most actual water environments at recent years. For example, the concentration range of *tet* in the water sample from Yangtze River Estuary was $8.48 \times 10^2 - 2.23 \times 10^4 \text{ copies} \cdot \text{mL}^{-1}$ (Guo et al., 2018). The abundance of *tetX* in the coastal waters of the Bohai and Yellow Seas was as high as

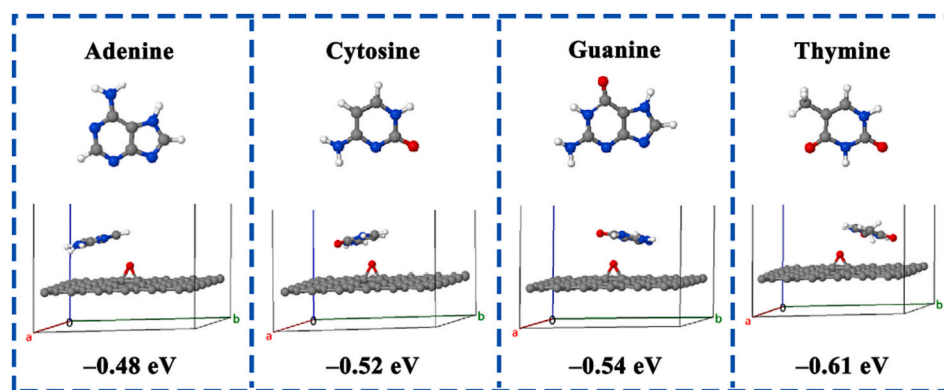


Fig. 1. The chemical structures of adenine, thymine, cytosine, guanine and their adsorption on GO.

5.05×10^4 copies·mL⁻¹ (Lu et al., 2019). In addition, dilution could solve the problem of *tet* concentration exceeding the linear range. As the result, the prepared *ss-tet*/GO/GCE for detection of *ss-tet'* showed the lower LODs of 50.0 pM. As shown in Table S2, the sensitivity of the prepared *ss-tet*/GO/GCE was higher than other sensors ($100\text{--}1.2 \times 10^5$ pM, equaling to $1.62 \times 10^3\text{--}2.13 \times 10^5$ copies·mL⁻¹), owing to the following reasons: (1) electrochemical sensor could be more suitable for DNA detection than fluorescent sensor (Hu et al., 2012; Hu et al., 2011); (2) DPV with smooth background current had higher detection sensitivity because the current difference could eliminate the background currents generated by the oxidation and reduction of impurities (Batchelor-Mcauley et al., 2009a; Vilas-Boas et al., 2019).

The preparation time and electrochemical performance of the prepared *ss-tet*/GO/GCE were compared with other DNA sensors with the immobilization probe by covalent bond and the results are shown in Table 1 (Lu et al., 2021; Sánchez-Paniagua et al., 2020; Wang et al., 2014; Jaber et al., 2019).

The fabrication process of ssDNA/CCP (Lu et al., 2021), CP/HDT/MCH-SAM/SPEAu (Sánchez-Paniagua et al., 2020), CP/AuNPs/PANI/CS-GS/GCE (Wang et al., 2014), ss-DNA/GS/rGO-Au-aptamer-MU (Jaber et al., 2019), ss-DNA/AuNPs/rGO/SPCE (Nagar et al., 2019), CS/Apt/SWCNT/SPGE (Abnous et al., 2017), TOX/MCH/GDE (Marchlewicz et al., 2021), ss-DNA/MCH/Au (Sun et al., 2022), PNA/ePAD (Lomae et al., 2023), and H1-2/C1/H2-2/Au (Wang et al., 2022), demanded over 18 h, along with intricate surface coating procedures. For ss-DNA/PANI/GP/SPCE (Mohamad et al., 2017), cDNA/MCH/MB-Apt/Au (Wang et al., 2019), ss-DNA/rGO/Au/PGE (Moattari et al., 2021), and DNA-Au@Nb₄C₃T_x/GCE (Rasheed et al., 2022), the preparation process involved intricate steps and necessitated a duration exceeding 4 h. Compared with the above electrodes, the prepared *ss-tet*/GO/GCE based on non-covalent bond need a shorter time (< 35 min) and easier preparation process, due to the non-covalent bond could be easier and faster to form than covalent bonds (Pumera, 2011). Additionally, the ss-DNA/polypyrrole modified electrode had been successfully prepared via entrapping method, which had simple preparation process and could sensitive detect bovine leukaemia virus after PCR amplification (Ramanaviciene and Ramanavicius, 2004). In comparison, the preparation of *ss-tet*/GO/GCE was not only simpler and more time-efficient, but it also eliminated the need for PCR amplification in the *tet* sensing process. Prior studies utilized pyrrole as a monomer and ss-DNA' as a template molecule to create a molecularly imprinted sensor for ss-DNA' detection (Ratautaite et al., 2013). In contrast to this molecularly imprinted sensor, the prepared *ss-tet*/GO/GCE demonstrated the capability to detect *ss-tet'* molecules of varying lengths.

3.4. Stability, reproducibility, and selectivity

Stability is one of the most important aspects of electrochemical sensors. The detection experiments were performed using the *ss-tet* (*ss-*

tetA, *-tetD*, *-tetE*, *-tetW*, and *-tetX*)/GO/GCE, prepared under optimized experimental conditions, after being aged for 5, 10, and 15 h, respectively. As shown in Fig. 3(a, b, c, d, e-I), the peak currents were all less reduced, and the obtained RSD values were all <4.43 %. The reproducibility of prepared *ss-tet*/GO/GCE was assessed with 15 measurements for the same concentration of *ss-tet'* (*ss-tetA'*, *-tetD'*, *-tetE'*, *-tetW'*, and *-tetX'*, 20-mer, 1 nM) under the same conditions. As can be seen from Fig. 3(a, b, c, d, e-II), there were few changes in the peak currents. The obtained RSD values for *ss-tet'* (*ss-tetA'*, *-tetD'*, *-tetE'*, *-tetW'*, and *-tetX'*, 20-mer, 1 nM) detection were 2.31, 1.91, 1.83, 1.89, and 1.85 %, respectively. These results implied that the developed sensor had exceptional stability and reproducibility, which was ascribed to the hydrophilicity and chemical stability of GO (Compton and Nguyen, 2010; Song et al., 2021), as well as its ability to stably adsorb bases (Zhu et al., 2016).

The major advantage of the developed sensor in this work was the capability to selectively detect *ss-tet'*, the changing of peak currents owing to that *ss-tet* probes on the GO surface could be released from GO in the presence of *ss-tet'* in PBS (0.1 M and pH at 7.4) (Zhao et al., 2011). Besides, the non-complementary sequences in PBS (0.1 M and pH at 7.4) could not lead to an changing of peak current, due to not disrupt the interactions of the *ss-tet* probes and GO (Bi et al., 2011; Wen et al., 2010). The *ss-tetA*/GO/GCE was also immersed in the mixed PBS (0.1 M and pH at 7.4) containing five subtypes of *ss-tet'* (*ss-tetA'*, *-tetD'*, *-tetE'*, *-tetW'*, and *-tetX'*, 20-mer, 1 nM) for the *ss-tetA'* detection to investigate the selectivity. Meanwhile, the control experiment was performed by immersing *ss-tetA*/GO/GCE in the PBS (0.1 M and pH at 7.4) containing only *ss-tetA'* (20-mer, 1 nM). The above operations were also conducted for *ss-tetD*, *-tetE*, *-tetW*, *-tetX*/GO/GCE, respectively, to investigate their selectivity. Compared with the control, the RSD values for current responses of *ss-* (*tetA'*, *tetD'*, *tetE'*, *tetW'*, and *tetX'*) (20-mer) in the mixed PBS (0.1 M and pH at 7.4) were not >3.54 % (shown in Fig. 4a). The results implied a limited influence of non-complementary sequences on the detection of *tet* in PBS (0.1 M and pH at 7.4) using the developed sensors. In order to further confirm the selectivity of the prepared *ss-tetA*/GO/GCE, it was immersed in the mixed PBS (0.1 M and pH at 7.4) containing *ss-tetA'* (20-mer, 1.0 nM) and interference sequences (*ss-tetD'*, *-tetE'*, *-tetW'*, and *-tetX'*, 20-mer) with different concentrations (1.0×10^3 , 5.0×10^2 , 1.0×10^2 , and 10.0 pM, equaling to 1.62×10^4 , 8.1×10^3 , 1.62×10^3 , and 162 copies·mL⁻¹) for *ss-tetA'* detection (Fig. 4b). The selectivities of *ss-tetD*, *-tetE*, *-tetW*, and *-tetX*/GO/GCE (Fig. 4(c-f)) in the detection of *ss-* (*tetD'*, *tetE'*, *tetW'*, and *tetX'*, 20-mer) in PBS (0.1 M and pH at 7.4) were also investigated as the above method. In the range of 10.0– 1.0×10^3 pM, peak currents did not change (RSD values < 2.11 %), suggesting that the quantitative detection of *ss-* (*tetA'*, *tetD'*, *tetE'*, *tetW'*, and *tetX'*, 20-mer) was almost unaffected by non-complementary sequences in the PBS (0.1 M and pH at 7.4) and the developed sensor had excellent selectivity.

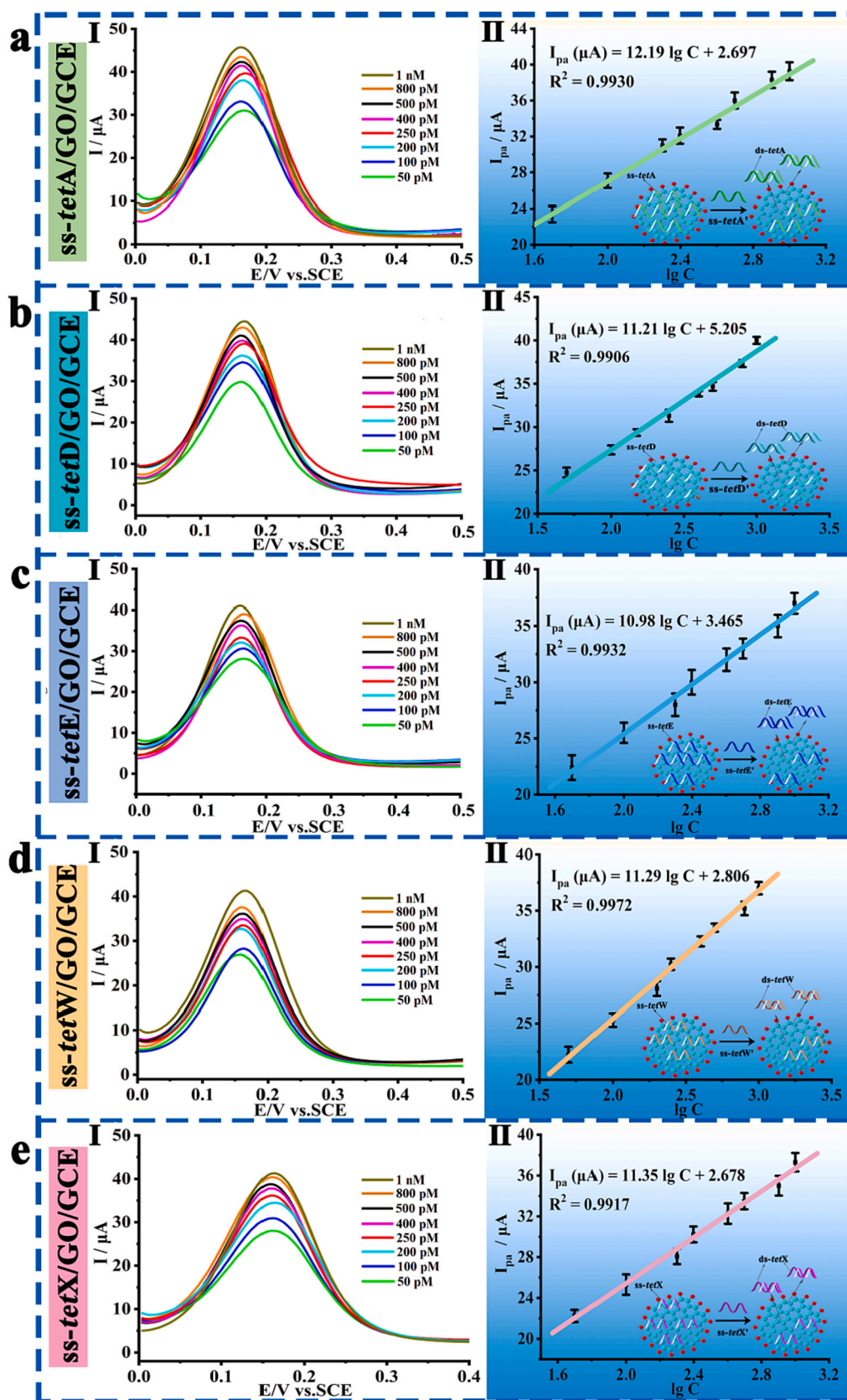


Fig. 2. DPVs recorded at the ss-tet (ss-tetA, -tetD, -tetE, -tetW, and tetX) /GO/GCE in PBS (0.1 M and pH at 7.4) containing ss-tetA' (a-I), ss-tetD' (b-I), ss-tetE' (c-I), ss-tetW' (d-I), ss-tetX' (e-I) of different concentrations (a-h: 50.0, 100.0, 200.0, 250.0, 400.0, 500.0, 800.0 pM, 1.0 nM); the related calibration curves of the logarithm of concentrations (pM) of ss-tetA' (a-II), ss-tetD' (b-II), ss-tetE' (c-II), ss-tetW' (d-II), ss-tetX' (e-II) and the peak currents.

Table 1Comparison of the prepared ss-*tet*/GO/GCE to other electrochemical DNA sensors based on the covalent immobilization strategy.

Num.	Electrode	Preparation time	Linear range (M)	LOD (pM)	Ref.	Method
1	ssDNA/CCP	> 24 h	1.00×10^{-8} – 1.00×10^{-6}	130.0	(Lu et al., 2021)	DPV
2	CP/HDT/MCH SAM/SPEAu	> 19 h	5.00×10^{-1} – 5.00×10^{-8}	500.0	(Sánchez-Paniagua et al., 2020)	DPV
3	CP/AuNPs/PANI/CS-GS/GCE	> 18 h	1.00×10^{-11} – 1.00×10^{-9}	2.1	(Wang et al., 2014)	DPV
4	ss-DNA/GS/rGO-Au-aptamer-MU	overnight	1.00×10^{-9} – 13.83×10^{-6}	1.0×10^3	(Jaberi et al., 2019)	DPV
5	ss-DNA/PANI/GP/SPCE	> 6 h	1.00×10^{-6} – 1.00×10^{-9}	7.8×10^5	(Mohamad et al., 2017)	DPV
6	ss-DNA/AuNPs/rGO/SPCE	> 65 h	1.00×10^{-8} – 2.00×10^{-5}	–	(Nagar et al., 2019)	EIS
7	cDNA/MCH/MB-Apt/Au	> 4 h	2.00×10^{-9} – 4.00×10^{-6}	2.0×10^3	(Wang et al., 2019)	DPV
8	CS/Apt/SWCNT/SPGE	overnight	1.80×10^{-10} – 3.00×10^{-8}	52.0	(Abnous et al., 2017)	DPV
9	TOX/MCH/GDE	overnight	1.35×10^{-8} – 6.0×10^{-8}	500.0	(Marchlewicz et al., 2021)	DPV
10	ss-DNA/rGO/Au/PGE	> 4 h	1.00×10^{-4} – 1.00×10^{-10}	125.0	(Moattari et al., 2021)	EIS
11	DNA – Au@Nb ₄ C ₃ T _x /GCE	> 4 h	5.00×10^{-6} – 1.00×10^{-8}	4.0×10^3	(Rasheed et al., 2022)	DPV
12	ss-DNA/MCH/Au	> 18 h	1.00×10^{-6} – 1.00×10^{-8}	1.0×10^4	(Sun et al., 2022)	DPV
13	PNA/ePAD	> 13 h	1.00×10^{-10} – 2.00×10^{-7}	1.0	(Lomae et al., 2023)	EIS
14	H1–2/C1/H2–2/Au	> 20 h	1.00×10^{-13} – 2.50×10^{-9}	0.085	(Wang et al., 2022)	SWV
15	ss- <i>tet</i> /GO/GCE	< 35 min	5.00×10^{-11} – 1.00×10^{-9}	50.0	This work	DPV

*CCP: composite/CNT/PVA; CP: capture probe; HDT: hexanedithiol; MCH: mercaptohexanol; SAM: self-assembled mixed monolayer; PANI: polyaniline; CS: chitosan; GS: graphene sheets; PVA: polyvinyl alcohol; MU: 11-mercapto-1-undecanol; PANI: polyaniline; GP: graphene; SPCE: screen printed carbon electrode; cDNA: capture DNA; MB: methylene blue; Apt: aptamer; CS: complementary strands of aptamer; SWCNT: single-walled carbon nanotubes; SPGE: screen printed gold electrodes; TOX: gene fragments of toxigenic *Corynebacterium diphtheriae* strain; GDE: gold disc electrode; PGE: pencil graphite electrode; PNA: peptide nucleic acid; ePAD: electrochemical paper-based analytical device.

3.5. The accuracy analysis

The prepared ss-*tet*/GO/GCE was used to detect ss-*tet* with the known concentration in order to verify its accuracy. The obtained peak current was used to calculate the concentration of ss-*tet* by the obtained linear equation. As shown in Table 2, the RSD values between actual and detected concentrations of ss-*tet* (ss-*tetA**, -*tetD**, -*tetE**, -*tetW**, and -*tetX**, 20-mer) ranged from 0.74 to 4.48 %, implying that the developed sensor had the high accuracy for ss-*tet* detection in PBS (0.1 M and pH at 7.4).

Previous studies have shown that RT-qPCR can be used for *tet* detection with high accuracy (Zhang et al., 2022). Here, RT-qPCR was used to detect the ds-*tetA* with different concentrations (5.08×10^4 , 2.54×10^4 , 1.27×10^4 , and 6.35×10^3 pM, equaling to 8.23×10^5 , 4.12×10^5 , 2.06×10^5 , 1.03×10^5 copies·mL⁻¹), which linear range was 3.48×10^2 – 3.48×10^6 pM (equaling to 5.64×10^3 – 5.64×10^7 copies·mL⁻¹) with LOD of 3.48×10^2 pM. As shown in Table 3, the detection concentrations of ds-*tetA* were closed to the actual concentrations, and the RSD values were all <2.78 %. The ss-*tetA*/GO/GCE with a LOD of 50.0 pM and a linear range of 50.0 pM–1 nM were used to detect ss-*tetA* for the above ds-*tetA* solutions diluted by 100 times and pretreated by denaturation (details shown in Detection of *tet* in actual water samples, the amount of ss-*tetA* could represent the *tetA* at this moment). The obtained RSD values were all <3.36 %. These results not only indicated that the developed sensors had a lower LOD (50.0 pM, equaling to 8.01×10^2 copies·mL⁻¹) than RT-qPCR (3.48×10^2 pM, equaling to 5.64×10^3 copies·mL⁻¹) and could be used for the accurate quantification of *tetA*, but also proved that the high-temperature denaturation and rapidly cooling were the accurate pretreatment methods in order to form ss-*tetA* and -*tetA* for effective *tetA* detection (Andersen et al., 1992; Sturtevant and Geiduschek, 1958). Significantly, the developed electrochemical sensor would be the more suitable choice for gene sensing than RT-qPCR, given its time-saving, miniaturization capability, user-friendly, low-cost and high sensitivity.

PCR array is also an accurate and sensitive gene detection method (Huang et al., 2021; Weber et al., 2021). The ss-*tet*/GO/GCE and PCR array were applied to detect *tet* in the pretreated river, inlet and outlet of sewage treatment plant. As shown in Table 4, the above methods measured *tetX* in inlet and outlet, and two subtypes (*tetA* and *tetX*) of *tet* in river, which determined concentrations were almost identical (RSD < 4.85 %). These results indicated that the ss-*tet*/GO/GCE could achieve accurate detection of *tet* in actual water. In addition, the ss-*tet*/GO/GCE was more specific and cost-effective than PCR array. Thus, it was a good

alternative to PCR array.

In addition, the feasibility and practicability of the prepared ss-*tet*/GO/GCE for ss-*tet* detection in actual water were also investigated by standard additions method. Firstly, the ss-*tetA*/GO/GCE was used to electrochemically quantitatively detect ss-*tetA* in the pretreated domestic sewage, primary and secondary sedimentation tank of sewage treatment plant. Secondly, it was applied for ss-*tetA* detection in the above water samples after adding different concentrations (0.0 – 7.0×10^2 pM, equaling to 0.00 – 1.13×10^4 copies·mL⁻¹) of ss-*tetA**, respectively. As shown in Table 5, all detected concentrations of ss-*tetA* were close to the real values, and the recovery ranged from 95.14 to 104.02 %, indicating that the developed sensor enabled the efficient determination of ss-*tet* in water samples with excellent reliability and practicability.

There were not only ss-*tet* but also ss-*tet* in the actual water. Therefore, it was necessary to analyze the influence of the existence of ss-*tet* with different lengths and concentrations on the ss-*tet* detection. The ss-*tetA* detection was carried out in the mixed PBS (0.1 M and pH at 7.4) containing the ss-*tetA* and ss-*tetA** at equal concentrations (50.0, 100.0, and 1.0×10^3 pM, equaling to 8.10×10^2 , 1.62×10^3 , and 1.62×10^4 copies·mL⁻¹ for 20-mer, 2.99×10^2 , 5.98×10^2 , and 5.98×10^3 copies·mL⁻¹ for 50-mer, 1.495×10^2 , 2.99×10^2 , and 2.99×10^3 copies·mL⁻¹ for 100-mer, respectively). In the control experiment, ss-*tetA*/GO/GCE was immersed in the PBS (0.1 M and pH at 7.4) containing only ss-*tetA** (50.0, 100.0, and 1.0×10^3 pM). As shown in Table 6, the current responses of the detection for ss-*tetA** in the mixed PBS (0.1 M and pH at 7.4, containing ss-*tetA** and ss-*tetA*) were similar to the results of the control, and the RSD values were all <4.98 %. The above results indicated that the concentrations (50.0 – 1.0×10^3 pM) and the different lengths (20, 50, and, 100-mer) of ss-*tetA* in PBS (0.1 M and pH at 7.4) containing ss-*tetA** had little interference for the ss-*tetA** quantitative detection. The reasons for the above results were as follow. Firstly, the specific binding of DNA was determined by the random collision of ss-DNA and ss-DNA*, following the second-order reaction kinetics, which reaction rate could be expressed as $-dC/dt = kC^2$ (C represented the concentration of ss-DNA per unit time, and k was the DNA specific binding rate constant) (Batchelor-McAuley et al., 2009b). In general, the specific binding was accelerated with the increasing of ss-DNA and ss-DNA* concentration, which could be attributed to the increasing probability of collision and binding of complementary sequences (Wetmur and Davidson, 1968). The density of ss-*tet* probes adsorbed on the GO/GCE was higher than the concentration of *tet* (including ss-*tet** and ss-*tet*) in actual water. Besides, dilution was useful to solve the problem of high concentration of the *tet* (> 1.0 nM) in actual water, which broadened the

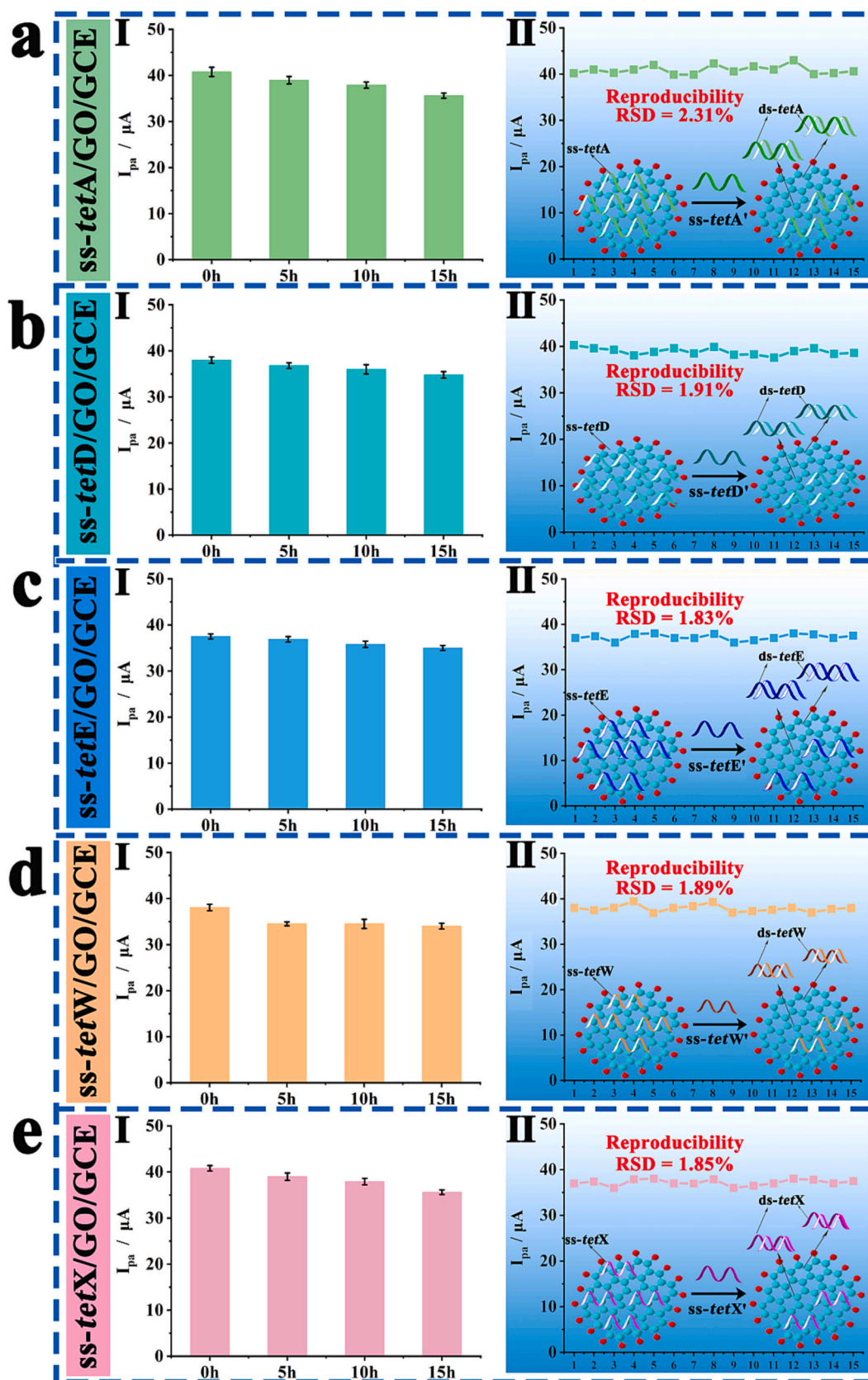


Fig. 3. The contradistinction of current responses of detection experiments for five subtypes of ss-tet (1 nM) after aging 0 h, 5 h, 10 h, 15 h: ss-tetA' (a-I), -tetD' (b-I), -tetE' (c-I), -tetW' (d-I), and -tetX' (e-I); The comparison of current responses of 15 measurements for the detection of 1 nM ss-tetA' (a-II), -tetD' (b-II), -tetE' (c-II), -tetW' (d-II), and -tetX' (e-II) under the same experimental conditions.

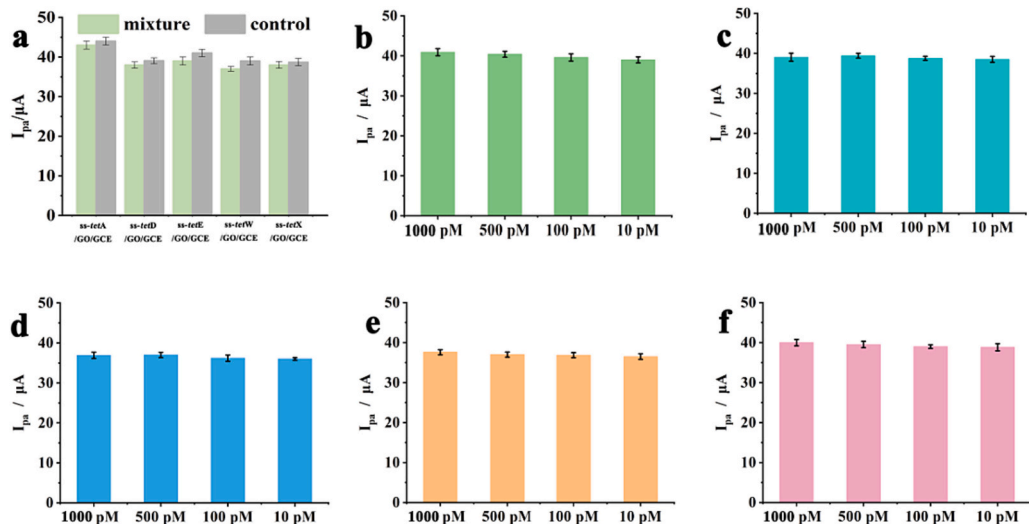


Fig. 4. (a) Comparison of the current responses recorded after hybridization in the mixed PBS (0.1 M and pH at 7.4) containing ss-*tet*' (ss-*tetA*', -*tetD*', -*tetE*', -*tetW*', and -*tetX*', 20-mer, 1 nM) using ss-*tetA*', -*tetD*', -*tetE*', -*tetW*', -*tetX*'/GO/GCE, respectively; Control: The current responses recorded after hybridization in PBS (0.1 M and pH at 7.4) only containing ss-*tetA*', ss-*tetD*', ss-*tetE*', ss-*tetW*', ss-*tetX*' (20-mer, 1 nM), respectively; Comparison of the current responses recorded after hybridization with interference sequences of different concentrations (1.0×10^3 –10 pM) on the ss-*tetA* (b), -*tetD* (c); -*tetE* (d); -*tetW* (e); -*tetX*'/GO/GCE (f).

Table 2
Comparison of the actual concentrations and detected concentrations of ss- (*tetA*', *tetD*', *tetE*', *tetW*', and *tetX*').

Type	Actual (pM/copies·mL ⁻¹)	I _{pa} (μA)	Found (pM/copies·mL ⁻¹)	Equation	RSD (%)
ss- <i>tetA</i> '	700.0/11340.00	37.48	713.5/11559.01	I _{pa} (μA) = 12.19 lg C (pM) + 2.697	1.35
	450.0/7290.00	35.16	460.4/7457.66	I _{pa} (μA) = 12.19 lg C (copies·mL ⁻¹) - 12.04	1.61
	150.0/2430.00	28.60	133.3/2159.94		0.83
	80.0/1296.00	25.71	77.2/1251.29		2.48
ss- <i>tetD</i> '	700.0/11760.00	36.79	657.0/11037.77	I _{pa} (μA) = 11.21 lg C (pM) + 5.205	4.48
	200.0/3360.00	30.84	193.6/3251.64	I _{pa} (μA) = 11.21 lg C (copies·mL ⁻¹) - 9.244	2.32
	150.0/2520.00	29.65	151.6/2546.54		0.74
	120.0/2016.00	28.26	113.9/1914.02		3.67
ss- <i>tetE</i> '	700.0/11774.00	34.79	712.7/11987.78	I _{pa} (μA) = 10.98 lg C (pM) + 3.465	1.27
	180.0/3027.60	28.32	183.5/3086.64	I _{pa} (μA) = 10.98 lg C (copies·mL ⁻¹) - 9.812	1.37
	150.0/2523.00	27.52	155.2/2609.96		2.34
	120.0/2018.40	26.16	116.7/1962.22		2.00
ss- <i>tetW</i> '	650.0/10725.00	34.51	642.9/10608.02	I _{pa} (μA) = 11.29 lg C (pM) + 2.806	0.78
	350.0/5775.00	31.73	364.7/6017.22	I _{pa} (μA) = 11.29 lg C (copies·mL ⁻¹) - 10.85	2.90
	380.0/6270.00	32.06	390.1/6436.16		1.85
	180.0/2970.00	28.41	185.3/3057.29		2.05
ss- <i>tetX</i> '	700.0/11564.00	34.80	676.3/11172.31	I _{pa} (μA) = 11.35 lg C (pM) + 2.678	2.44
	180.0/2973.60	28.46	186.9/3087.09	I _{pa} (μA) = 11.35 lg C (copies·mL ⁻¹) - 11.05	2.65
	150.0/2478.00	27.21	145.0/2395.63		2.39
	120.0/1982.40	26.46	124.5/2056.74		2.60

Table 3
Comparison of the RT-qPCR to the developed sensor based on non-covalent adsorption strategy for the determination of ds-*tetA*.

Actual concentration (dilute by 100 times) (pM/ copies·mL ⁻¹)	RT-qPCR		Electrochemical detection	
	Detection concentration (dilute by 100 times) (pM/ copies·mL ⁻¹)	RSD (%)	Detection concentration (pM/ copies·mL ⁻¹)	RSD (%)
508.0/8229.60	524.0/8488.80	2.19	506.0/8197.04	2.80
254.0/4115.00	260.5/4220.10	1.79	245.0/3968.35	2.56
127.0/2057.40	132.1/2140.02	2.78	133.2/2157.68	3.36
63.5/1028.70	65.1/1054.62	1.76	65.4/1058.83	2.04

application range of the developed sensor. Secondly, according to the formula of $C/C_0 = 1/(1 + kC_0t)$ (C_0 was the starting concentration of ss-DNA, and t was time in seconds), C_0t value, at the halfway of DNA specific binding ($C_0t_{1/2} = 1/k$), represented the relationship between

Table 4
Comparison of the PCR array to the developed sensors for ss- (*tetA*', *tetD*', *tetE*', *tetW*', and *tetX*') determination in actual water samples.

Name	Detection	PCR array (pM/copies·mL ⁻¹)	Electrochemical detection (pM/copies·mL ⁻¹)	RSD (%)
Inlet	<i>tetX</i>	910.0/14741.42	887.6/14378.36	1.76
Outlet	<i>tetX</i>	195.2/3163.04	198.2/3210.76	1.06
River	<i>tetA</i>	83.8/1355.43	89.5/1448.06	4.67
	<i>tetX</i>	95.5/1565.59	89.2/1461.80	4.85

DNA specific binding rate and DNA sequence complexity, which was inversely proportional to the k and proportional to the DNA sequence complexity (Thrower and Peacocke, 1966). The ss-*tet* in the actual water was generally more complex than the used ss-*tet* probe (20-mer) on the GO/GCE. Therefore, the ss-*tet*' in actual water tended to bind the ss-*tet* probes (20-mer) adsorbed on GO/GCE than the ss-*tet* in actual water.

The surface area, not covered by the ss-*tet* probes on the GO/GCE,

Table 5

Recovery results for *ss-tetA* determination in actual water samples (Added *ss-tetA* with 20-mer).

Sample	Actual (pM/copies-mL ⁻¹)	Added (pM/copies-mL ⁻¹)	Found (pM/copies-mL ⁻¹)	Recovery (%)	RSD(%)
Domestic sewage	119.1/1928.61	700.0/11340.00	834.6/13520.68	102.22	1.33
		500.0/8100.00	627.3/10161.77	101.64	0.93
		100.0/1620.00	223.1/3613.73	104.02	1.29
		0.0/0.00	121.4/1965.87	101.93	1.35
Primary sedimentation tank	238.5/3864.35	700.0/11340.00	913.1/14791.39	96.36	1.95
		500.0/8100.00	728.2/11796.67	97.93	1.00
		100.0/1620.00	333.7/5405.61	95.14	1.02
		0.0/0.00	229.6/3719.84	96.26	2.69
Secondary sedimentation tank	98.4/1594.57	700.0/11340.00	781.6/12662.23	97.60	1.50
		500.0/8100.00	579.9/9394.05	96.29	2.23
		100.0/1620.00	194.0/3142.96	95.58	1.59
		0.0/0.00	93.8/1519.56	95.30	3.41

Table 6

Comparison of influences of length and concentration of *ss-tetA* and *-tetA* in PBS (0.1 M and pH at 7.4) for *ss-tetA* detection.

Length (mer)	Added concentration (pM/copies-mL ⁻¹)	Control			Experiment		
		I _{pa} (μA)	Concentration (pM/copies-mL ⁻¹)	RSD (%)	I _{pa} (μA)	Concentration (pM/copies-mL ⁻¹)	RSD (%)
20	1000.0/16200.00	39.01	952.6/15432.28	3.43	38.93	941.7/15255.05	4.25
	100.0/1620.00	27.40	106.3/1721.57	4.30	27.31	107.1/1734.21	4.81
	50.0/810.00	23.53	51.2/828.95	1.64	23.22	48.3/781.81	2.50
50	1000.0/5980.00	38.97	945.4/5653.73	3.97	38.93	938.7/5613.25	4.47
	100.0/598.00	27.44	107.1/630.64	4.83	27.44	107.1/640.58	4.86
	50.0/299.00	23.58	51.7/309.11	2.35	23.63	52.2/311.86	2.98
100	1000.0/2990.00	38.92	936.2/2799.21	4.66	38.89	932.0/2786.56	4.98
	100.0/299.00	27.44	107.1/320.32	4.87	27.45	107.2/320.53	4.91
	50.0/149.50	23.17	47.8/142.95	3.17	23.12	47.4/141.67	3.80

was increasing as the hybridization reaction progress. And the GO/GCE could adsorb any ss-DNA in real water samples (Liu et al., 2012). Thus, it was necessary to analyze the possibility of adsorption between the ss-DNA in actual water and the area obtained by releasing *ss-tet* probes. Firstly, the *ss-tet* in solution was more likely to bind specifically to the *ss-tet* probes than to be adsorbed by GO, because the energy of hydrogen bond (details shown in Interaction energy of GO with nucleobases) formation by the specific binding of *ss-tet* and *ss-tet'* was much larger than the π -stacking between GO and *ss-tet'*. Secondly, the *ss-tet* probes releasing into the solution existed in the form of a double helix, with little interaction with GO (He et al., 2010). Thirdly, during the hybridization process, the adsorption of ss-DNA, at the sites generated by the *ss-tet* probes release from the GO/GCE, could be ignored, because of the low concentration of ss-DNA in solution, and the electrostatic repulsion of ss-DNA (electronegativity) in solution by the adsorbed *ss-tet* probes (electronegativity) on the GO/GCE.

4. Conclusion

A highly sensitive electrochemical DNA sensor (*ss-tet*/GO/GCE) based on π -stacking between GO and nucleobase as well as specific hybridization of probe (*ss-tet*) on GO surface and complementary sequence (*ss-tet'*) in PBS (0.1 M and pH at 7.4) was developed for *tet* quantitative detection. Compared with the conventional electrochemical DNA sensors based on covalent bond and fluorescent sensors, the prepared *ss-tet*/GO/GCE required only a simple preparation process with a shorter preparation time (<35 min) and had no disadvantages such as low labeling efficiency. Besides, the prepared *ss-tet*/GO/GCE with superior specificity, stability, and reproducibility (RSD < 4.43 %) displayed a wider linear range from 50 pM (equaling to 8.1×10^2 copies-mL⁻¹) to 1 nM (equaling to 1.62×10^4 copies-mL⁻¹) and a lower LOD of 50 pM ($R^2 \geq 0.9906$) for *tet* (including five subtypes: *tetA*, *tetD*, *tetE*, *tetW*, and *tetX*) detection. Significantly, it had an excellent accuracy for *tet* determination by comparing with RT-qPCR (RSD < 3.36 %) and PCR array (RSD < 4.85 %). And, it not only could detect *tet* with concentrations as low as

83.8 pM (equaling to 1355.43 copies-mL⁻¹) in different water samples, but also could detect added *ss-tetA* in the above water samples with recovery efficiencies ranging from 95.14 to 104.02 %. We also proved that the presence of *ss-tetA* with different lengths and concentrations in PBS (0.1 M and pH at 7.4) containing *ss-tetA* induced little interference for *tetA* detection. In conclusion, we can thus conclude that the developed sensor based on non-covalent adsorption has great prospects for application in the efficient, sensitive and convenient detection of ARGs in actual water.

CRediT authorship contribution statement

Yi Xing: Conceptualization, Methodology, Investigation, WritingOriginal Draft, Writing Reviewing and Editing. **Yanan Zhang:** Conceptualization, Methodology, Writing - review and editing. **Xiaolin Zhu:** Conceptualization, Methodology, Writing - review and editing, Funding acquisition. **Chengzhi Wang:** Data curation, Investigation, Writing - review and editing. **Tingting Zhang:** Data curation, Investigation, Writing - review and editing. **Fangyuan Cheng:** Data curation, Investigation, Writing - review and editing. **Jiao Qu:** Methodology, Funding acquisition. **Willie J.G.M. Peijnenburg:** Writing - review and editing.

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.

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Appendix A. Supplementary data

Additional reagents (Text S1), solutions (Text S1), apparatus (Text S1), optimization of experimental conditions (Text S2), detailed procedure of RT-qPCR (Text S3), characterization of developed sensor (Text S4), and references. Figs. S1, optimization of adsorption conditions; Figs. S2, optimization of hybridization conditions; Figs. S3, characterization of electrode modification materials; Table 1, DNA sequences used in experiments; Table 2, Comparison of the developed sensor to the other electrochemical DNA sensors; Table 3, standard curve of *tetA* by RT-qPCR (DOC). Supplementary data to this article can be found online at doi:<https://doi.org/10.1016/j.scitotenv.2023.167615>.

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