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RESEARCH PAPER

Label-free peptide aptamer based impedimetric biosensor for highly sensitive detection of TNT with a ternary assembly layer

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Abstract We report a label-free peptide aptamer based biosensor for highly sensitive detection of TNT which was designed with a ternary assembly layer consisting of anti-TNT peptide aptamer (peptamer), dithiothreitol (DTT), and 6-mercaptohexanol (MCH), forming Au/peptamer–DTT/MCH. A linear relationship between the change in electron transfer resistance and the logarithm of the TNT concentration from 0.44 to 18.92 pM, with a detection limit of 0.15 pM, was obtained. In comparison, the detection limit of the aptasensor with a common binary assembly layer (Au/peptamer/MCH) was 0.15 nM. The remarkable improvement in the detection limit could be ascribed to the crucial role of the ternary assembly layer, providing an OH-richer hydrophilic environment and a highly compact surface layer with minimal surface defects, reducing the non-covalent binding (physisorption) of the peptamer and non-specific adsorption of TNT onto the electrode surface, leading to high sensitivity, and which can serve as a general sensing platform for the fabrication of other biosensors.

Keywords Label-free · Peptide aptamer · TNT · Ternary assembly layer

Introduction

Many kinds of biosensors have been developed that use electrical or optical techniques such as electrochemistry, fluorescence, surface plasmon resonance, and chemiluminescence. Among the biosensors developed, electrochemical biosensors have gained tremendous interest because of their unique advantages of high sensitivity, simplicity, rapid response and low cost [1–7]. Recently, much attention has been paid to aptamer-based biosensors, often termed *aptasensors* [8–14]. Aptamers are single-stranded oligonucleotides or peptides which exhibit superior merits over antibodies, the traditional recognition molecule, in the aspects of in vitro production, excellent thermal stability, and ease of synthesis [8–11]. They can be used as the recognition element for detecting various targets, such as ions, proteins, and cells [12–14]. Compared with oligonucleotide aptamers, peptide aptamers (peptamers) [15] are easier to assemble on a gold electrode via the Au–S bond because the peptide may contain a thiol group and the thiol modification of the peptide is easy. Moreover, the diverse chemistry of amino acids indicates that peptides are better suited as recognition elements against a wide range of target molecules [16, 17].

Most of the aptasensors developed are based on tagged aptamers, which need a complicated and time-consuming labelling procedure and will likely reduce the bioaffinity. Therefore, it is imperative to develop label-free aptasensors. Electrochemical impedance spectroscopy (EIS) has been extensively used as a "label-free" method in a wide range of biosensing applications, and it provides unique advantages, such as simplified assay design, ease of signal quantification, decreased assay time, reduction in reagent costs, and non-destructivity [18]. However, the sensitivity of EIS is limited by the inability to discriminate between specific recognition and non-specific adsorption. Therefore, effective removal of non-specific adsorption could enhance the sensitivity. It has been reported that a strategy using ternary surface monolayers consisting of a thiolated DNA probe, dithiothreitol (DTT), and 6-mercaptohexanol (MCH) could remarkably increase the sensitivity of amperometric detection of nucleic acid hybridization [19]. Inspired by that work, we extended the ternary sensing

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layers to the construction of label-free peptamer-based biosensors to improve the sensitivity.

We present the results of our use of 2,4,6-trinitrotoluene (TNT) as a model analyte. The detection of nitro explosives is important in the fight against terrorism and environmental contamination. TNT, with a low molar mass of 227.13 g/mol, is a widely available explosive both for military purposes and for industrial applications, and it may be abused in terrorist attacks, endangering public security. TNT enters the environment by industrial manufacture, destruction of bombs, and explosion of fireworks, causing environmental pollution. TNT is also a teratogen, and may cause mammalian cell mutation, hepatopathy, and cataract through inhalation or ingestion [20–29]. Several methods for detecting TNT, including high-performance liquid chromatography [20, 21], gas chromatography [22–26], electrochemiluminescence measurements [27], and fluorescence measurements [28, 29], have been developed. However, these techniques are time-consuming and require sophisticated equipment and professional operators. Therefore, there is a great need to develop a sensitive, simple, and low cost method for determination of TNT.

In this work, a label-free peptamer-based biosensor for the detection of TNT is described. It was designed by our assembling anti-TNT peptamer, DTT, and MCH on a gold electrode surface, forming a ternary sensing layer (Au/peptamer–DTT/MCH). The change in the electron transfer resistance (ΔR_{et}) increased linearly with the logarithm of the TNT concentration in the range from 0.44 to 18.92 pM, with a detection limit of 0.15 pM, whereas the detection limit of the aptasensor with a common binary component (Au/peptamer/MCH) was 0.15 nM. The significant improvement in the detection limit could be ascribed to the ternary assembly layer, providing an OH-richer hydrophilic environment and a highly compact surface layer with minimal surface defects, reducing the non-covalent binding (physisorption) of the peptamer and non-specific adsorption of TNT onto the electrode surface, leading to high sensitivity, and which can serve as a general sensing platform for the fabrication of other biosensors.

Experimental

Reagents and apparatus

TNT standard solution (1.0 mg/mL), 2,4-dinitrotoluene, nitrotoluene, and nitrobenzene were purchased from Aladdin Reagent (Shanghai, China). The anti-TNT peptide aptamer was purchased from GL Biochem Co. (Shanghai, China), and the sequence of the aptamer is as follows: (N terminus) Trp–His–Trp–Gln–Arg–Pro–Leu–Met–Pro–Val–Ser–Ile–Lys–cysteamine (C terminus) [16]. DTT and MCH were obtained from J&K Chemical. Tween 20 was purchased from Alfa Aesar. Other chemicals were of analytical grade and used as received. Redistilled water was used throughout the experiments.

The electrochemical experiments were conducted with a CHI 760C electrochemical workstation (Shanghai, China) connected to the three-electrode system consisting of a gold disk working electrode (2 mm in diameter), a platinum wire counter electrode, and a Ag/AgCl (saturated KCl) reference electrode. EIS experiments were performed in 0.1 M KCl solution containing 5 mM $Fe(CN)_6^{3-/4-}$ (1:1) in the frequency range from 0.1 Hz to 10 kHz.

Fabrication of the aptasensors

The gold electrode was polished with 0.3- and 0.05-μm alumina slurry and washed thoroughly with water. It was electrochemically pretreated in 0.5 M H_2SO_4 by potential scanning between -0.2 and 1.6 V until a stable cyclic voltammogram was obtained. Then the electrode was washed by ultrasonication in water and ethanol, and dried by a nitrogen stream.

The aptasensor with a ternary sensing layer was fabricated as follows: Freshly prepared DTT (200 μM) was mixed with 10 μg/L anti-TNT peptamer solution (0.1 M $NaHCO_3$–Na_2CO_3 buffer, pH 9.6) [16]. Then 10 μL of the mixture solution was dropped on the electrode surface and incubated at 4 °C for 16 h. Then the Au/peptamer–DTT electrode was treated with 1 mM MCH for 1 h to block the non-specific s
ites, forming Au/peptamer–DTT/MCH. The aptasensor with a binary component, Au/peptamer/MCH, was prepared similarly to Au/peptamer–DTT/MCH except only the peptamer was used. Finally, the aptasensors were thoroughly rinsed with water and dried under nitrogen.

Preparation of soil samples and water samples

Soil samples were collected from the campus of Anhui Normal University (Wuhu) and Binjiang Park (Wuhu). All soil samples were dried naturally. For the measurement, 1.0 g of exactly weighed sample was dispersed in 2 mL methanol, sonicated for 5 min, extracted for 12 h, and filtered. After centrifugation for 5 min at 6000 rpm three times, the supernatant was diluted with water and used for detection. The ratio of methanol to water in the final extract was 1:27. Water samples were obtained from Jinghu Lake, Changjiang River, and tap water. All the water samples were filtered before detection.

Measurement procedure

TNT solution (6-μL drop) of different concentrations was deposited onto the aptasensor and incubated for an appropriate time. For the detection, the aptasensor was thoroughly rinsed with washing buffer [20 mM tris(hydroxymethyl)aminomethane hydrochloride, 0.1 M NaCl, 5 mM $MgCl_2$, 1.0% (v/v) Tween 20, pH 7.4] to remove unbound TNT. The concentration of TNT was

quantified by the change of the electron transfer resistance of the aptasensor.

Results and discussion

Fabrication of Au/peptamer–DTT/MCH

The fabrication procedure for the aptasensor with a ternary component is shown in Fig. 1. First, the anti-TNT peptamer and DTT were co-immobilized on a gold electrode surface via a classic Au–S bond. Next, the modified electrode was immersed in 1 mM MCH to further block the possible remaining active sites. On incubation with different concentrations of TNT, the peptamer on the electrode bound to TNT and folded, which hindered the electron transfer of the redox probe, $Fe(CN)_6^{3-/4-}$, leading to an increase in the electron transfer resistance.

Electrochemical properties of different aptasensors

Cyclic voltammetry were performed to investigate the electrochemical properties of the aptasensors during different fabrication steps. As shown in Fig. 2a and b, a well-defined reversible redox peak was observed at the bare gold electrode. When peptamer–DTT or peptamer was immobilized on the electrode surface, the peak current (I_p) decreased remarkably, indicating that the electron transfer of the redox probe was blocked by peptamer–DTT or peptamer, which formed an insulating layer on the electrode surface. The changes in the peak current ($\Delta I_p = I_{p,\text{modified electrode}} - I_{p,\text{bare Au electrode}}$) were 2.5 and 4.89 μA for the reduction peak and 1.79 and 5.86 μA for the oxidation peak for Au/peptamer and Au/peptamer–DTT respectively. The difference in ΔI_p reflected the compactness of the electrode surface. Obviously, the changes in the peak current before and after modification with peptamer–DTT were much larger than those with peptamer alone, indicating that a more compact layer was obtained when peptamer was co-immobilized with DTT. DTT was bound to the gold surface via two Au–S bonds with two hydroxyl groups exposed at the outer surface, providing an OH-richer hydrophilic environment, reducing the non-covalent binding (physisorption) of peptamer onto the electrode surface [14]. Subsequent assembly of MCH on the electrode surface resulted in a further decrease in I_p and an increase in the peak-to-peak potential separation (ΔE_p), suggesting that MCH could further reduce the surface defects, leading to greater compactness. From the cyclic voltammetry results, we can conclude that (1) the aptasensors were successfully prepared and (2) the ternary assembly layer can provide an OH-richer hydrophilic and more compact surface layer than the common binary layer, reducing the non-specific adsorption more effectively, leading to improved analytical performance.

EIS, a powerful tool to characterize the surface properties of modified electrodes, was also used to monitor the preparation of the aptasensors. The semicircular section in the electrochemical impedance plot is related to the electron transfer process, and the diameter of the semicircular section reflects the electron transfer resistance (R_{et}). Nyquist plots at different modified electrodes are shown in Fig. 2c and d. A small semicircular portion was obtained at the bare gold electrode, suggesting the excellent electron transfer capability of the electrode. The immobilization of peptamer–DTT or peptamer resulted in an obvious increase in R_{et}. Further treatment of the Au/peptamer–DTT-modified electrode or Au/peptamer-modified electrode with MCH led to a dramatic increase in R_{et}. The results are in agreement with

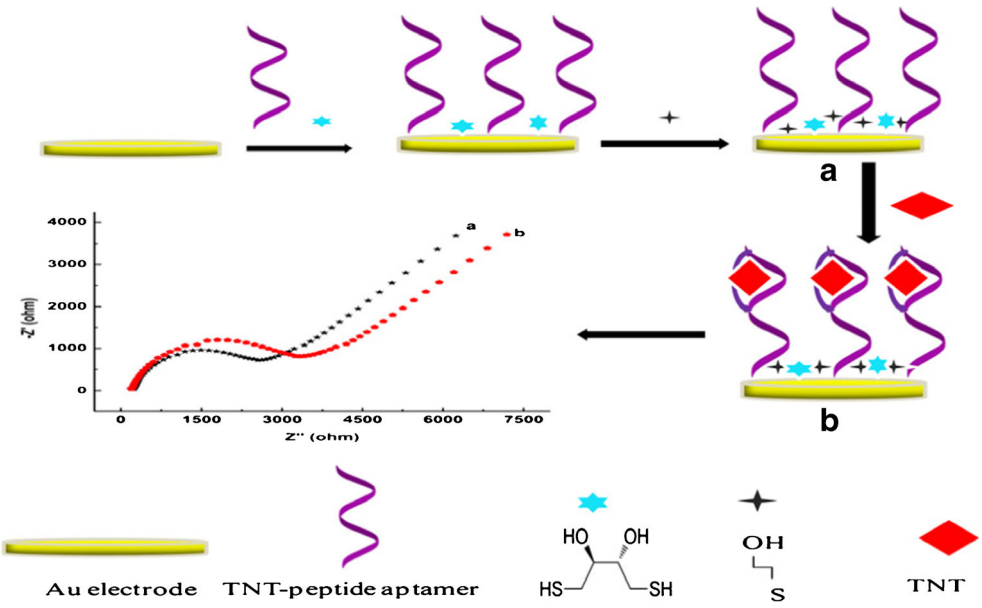

Fig. 1 Principle of fabrication of the aptasensor with a ternary component

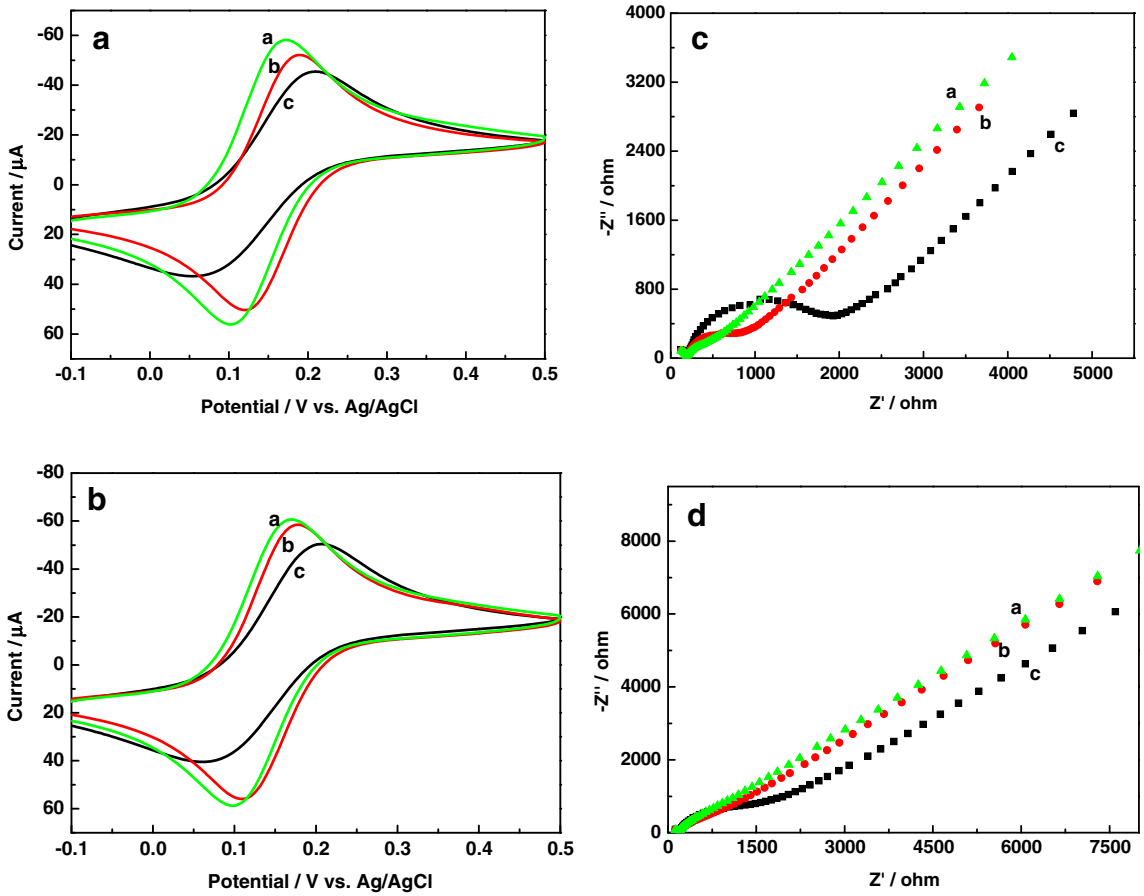

Fig. 2 **a** Cyclic voltammograms and **c** electrochemical impedance plots of Au (*a*), Au/peptamer–dithiothreitol (DTT) (*b*), and Au/peptamer–DTT/6-mercaptohexanol (MCH) (*c*) in 0.1 M KCl solution containing 5.0 mM $Fe(CN)_6^{3-/4-}$ (1:1). **b** Cyclic voltammograms and **d** electrochemical impedance plots of Au (*a*), Au/peptamer (*b*), and Au/peptamer/MCH (*c*) in 0.1 M KCl solution containing 5.0 mM $Fe(CN)_6^{3-/4-}$ (1:1)

the results of the cyclic voltammetry experiment, further demonstrating the successful construction of the aptasensors.

The changes in R_{et} obtained at the bare gold electrode and at gold electrodes modified with peptamer, peptamer–DTT, peptamer/MCH, and peptamer–DTT/MCH are illustrated in Table 1. R_{et} of the electrode increased after modification because peptamer, DTT, and MCH form an insulating layer on the electrode, which hindered the electron transfer of $Fe(CN)_6^{3-/4-}$. Moreover, the ΔR_{et} values ($\Delta R_{et} = R_{et,\text{modified electrode}} - R_{et,\text{bare Au electrode}}$) reflected the compactness of the electrode surface. As shown in Table 1, the ΔR_{et} values were 20.2, 300.1, and 1184.6 for Au/peptamer, Au/peptamer–DTT, and Au/peptamer–DTT/MCH respectively, indicating that the aptasensors with a ternary component exhibited a highly compact surface with minimal surface defects.

Analytical performance of Au/peptamer–DTT/MCH

The control of the surface properties of the electrode was critical to obtain high sensitivity of the label-free aptasensor, and DTT can effectively reduce the surface defects, providing more compactness. Therefore, the influence of DTT

Table 1 Comparison of ΔR_{et} for the fabrication of different aptasensors

Aptasensor	Surface	R_{et}	ΔR_{et}	Equivalent circuit
Au/peptamer/MCH	Bare Au	144.2	20.2	$,p) = '' : 1 :: pdgts11747057600216 - 017 - 0576 - 3Fmba.tif''$
	Au/peptamer	164.4		
	Au/peptamer/MCH	962.8	818.6	
Au/peptamer–DTT/MCH	Bare Au	187.4	300.1	
	Au/peptamer–DTT	487.5		
	Au/peptamer–DTT/MCH	1372	1184.6	

DTT dithiothreitol, *MCH* 6-mercaptohexanol

concentration on the response of Au/peptamer–DTT/MCH was investigated. Figure 3a shows that ΔR_{et} of the aptasensor increased with increasing DTT concentration in the range from 100 to 200 μM and decreased when the DTT concentration was higher than 200 μM. Thus, 200 μM DTT was selected to fabricate Au/peptamer–DTT/MCH.

The TNT incubation time plays an important role in the sensitivity of the aptasensor. The ΔR_{et} values of the aptasensor at different incubation times were recorded. As shown in Fig. 3b, ΔR_{et} increased with increasing incubation time from 50 to 70 min and then decreased after 70 min. The results demonstrated that the optimal incubation time was 70 min, which was chosen for the subsequent experiments.

The analytical performances of aptasensors with a ternary or a binary component were investigated. As shown in Fig. 4a, for the aptasensor with a binary assembly layer, Au/peptamer/MCH, ΔR_{et} increased linearly with the logarithm of the TNT concentration in the range from 0.44 to 10.12 nM (ΔR_{et} = 873.8 + 1824.3 log$C$, R = 0.994), with a detection limit of 0.15 nM. In comparison, for the aptasensor with a ternary assembly layer, Au/peptamer–DTT/MCH, the linear range for ΔR_{et} and the logarithm of the TNT concentration was from 0.44 to 18.92 pM (ΔR_{et} = 1156 + 2373 log$C$, R = 0.992), with a detection limit of 0.15 pM (Fig. 4b). Apparently, the detection limit of the aptasensor with the ternary layer was much lower than that of the aptasensor with the binary layer, suggesting that the ternary assembly layer could be a valuable and effective tool to improve the sensitivity of label-free biosensors. Moreover, compared with the previously reported methods for detection of TNT, the detection limit of the ternary model aptasensor was better than that of other methods [28–30] (Table 2).

The sensitivity of the aptasensors with common binary layers was low because the surface blocked with MCH

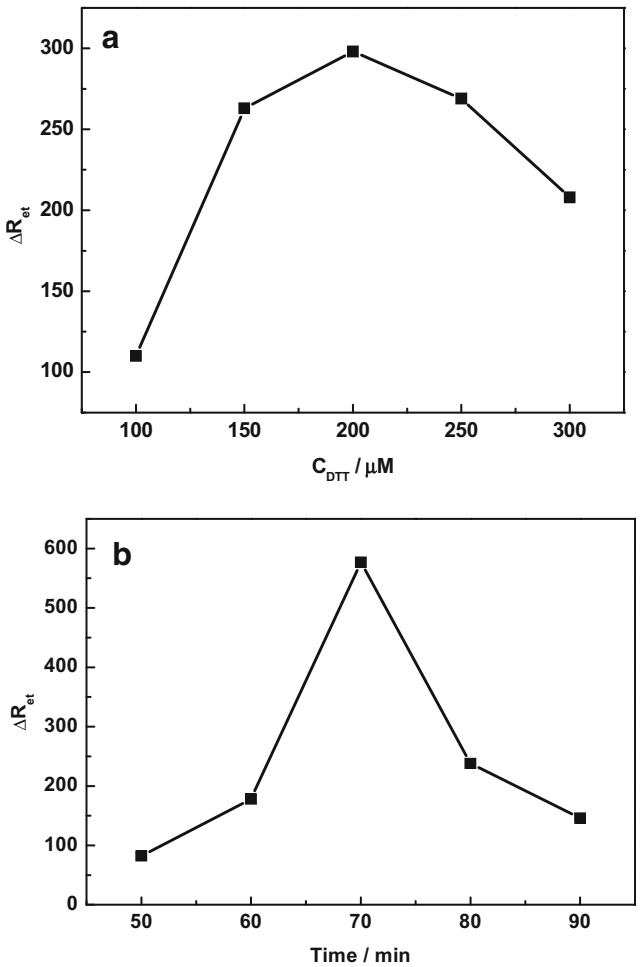

Fig. 3 Effects of **a** DTT concentration and **b** TNT incubation time on the response of Au/peptamer–DTT/MCH.

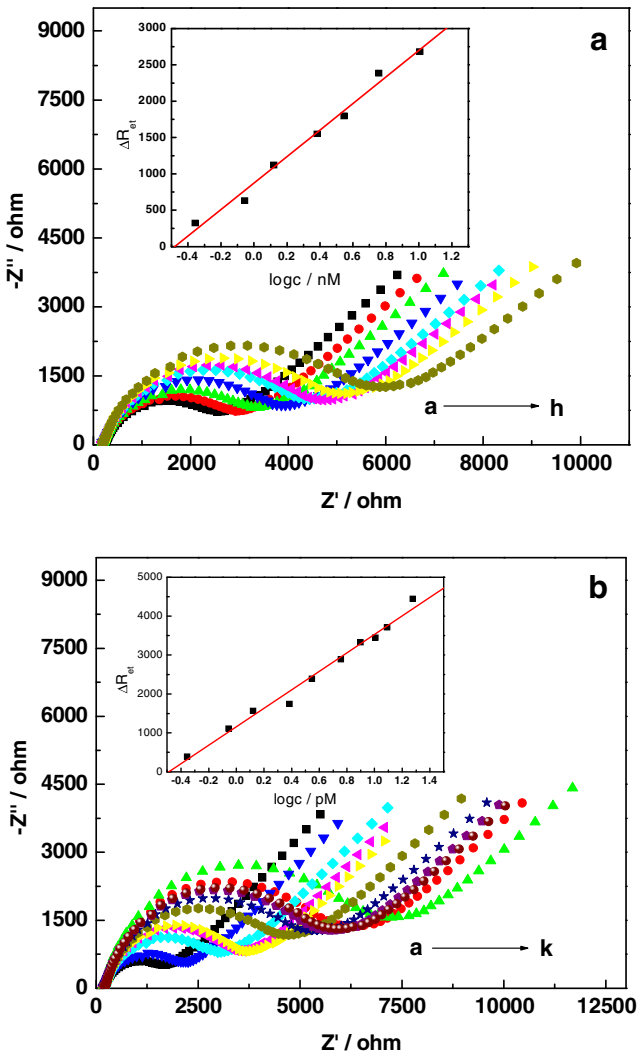

Fig. 4 Nyquist plots of different aptasensors with diverse concentrations of TNT: **a** Au/peptamer/MCH, (from *a* to *h*, 0, 0.44, 0.88, 1.32, 2.42, 3.52, 5.72, 10.12 nM); **b** Au/peptamer–DTT/MCH, (from *a* to *k*, 0, 0.44, 0.88, 1.32, 2.42, 3.52, 5.72, 7.92, 10.12, 12.32, 18.92 pM). *Inset* corresponding calibration curve

Table 2 Comparison of the methods for detection of TNT

Method	Linear range	Detection limit	Reference
FRET	0–6.8 μM	0.4 nM	[28]
ECL	4.4×10^{-12}–4.4×10^{-9} M	2.8×10^{-12} M	[29]
FL	1.0×10^{-7}–5.0×10^{-5} M	2.1×10^{-8} M	[30]
EIS	0.44–18.92 pM	0.15 pM	This work

ECL electrochemiluminescence, *EIS* electrochemical impedance spectroscopy, *FL* fluorescence, *FRET* fluorescence resonance energy transfer

alone was incomplete and still displayed non-specific background contributions. The sensitivity can be significantly enhanced by use of a ternary assembly layer. MCH is a linear-chain compound and is often used to block non-specific adsorption, whereas DTT has a cyclic configuration and id known to chemisorb onto a gold surface via two Au–S bonds, with the two hydroxyl groups exposed at the outer surface [31, 32]. The coupling of DTT and MCH could effectively reduce the pinhole defects and minimize the non-specific adsorption, resulting in lower detection limits. In addition, DTT and MCH provided an OH-richer hydrophilic environment, leading to a greater resistance to non-specific adsorption and hence minimization of the related background contributions.

Selectivity of Au/peptamer–DTT/MCH

The specificity was important in detecting real samples in situ without separation. To evaluate the selectivity of Au/peptamer–DTT/MCH, control experiments were conducted with 10-fold (50 pM) DNT, nitrotoluene, and nitrobenzene, as well as Fe^{3+}, Cu^{2+}, Pb^{2+}, Hg^{2+}, Ag^{+}, Cd^{2+}, Al^{3+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, and Cr^{3+}, to replace 5 pM TNT; the results are shown in Fig. 5. It can be seen that little changes in R_{et} were observed for the interfering molecules, whereas addition of 5 pM TNT produced a substantial increase in R_{et}, indicating excellent specificity of the aptasensor with a ternary assembly layer, which hinted that Au/peptamer–DTT/MCH was promising for the detection of TNT in real samples.

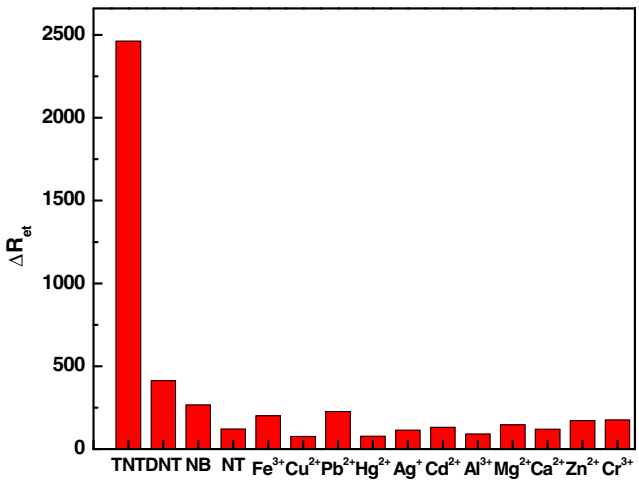

Fig. 5 Selectivity of Au/peptamer–DTT/MCH. *DNT* 2,4-dinitrotoluene, *NB* nitrobenzene, *NT* nitrotoluene

To test its practical application, Au/peptamer–DTT/MCH was used to determine TNT in soil and water samples. Soil samples were collected from the campus of Anhui Normal University (Wuhu) and Binjiang Park (Wuhu). Water samples were obtained from Jinghu Lake, Changjiang River, and tap water. The samples were diluted properly and were spiked with a given amount of TNT. The results are summarized in Table 3; the recoveries ranged from 94% to 104%, with relative standard deviation less than 5%, indicating that the aptasensor with a ternary assembly layer could detect TNT in real samples.

Conclusion

In summary, a label-free EIS aptasensor for TNT detection was fabricated by assembly of a peptide aptamer, DTT, and MCH on a gold electrode, forming a ternary assembly layer aptasensor, Au/peptamer–DTT/MCH. The linear range for ΔR_{et} and the logarithm of the TNT concentration was from 0.44 to 18.92 pM, with a detection limit of 0.15 pM. Compared with the common binary model aptasensor, Au/peptamer/MCH, the detection limit was significantly improved, which can be attributed to the ternary assembly layer.

Table 3 Recovery tests for TNT in real samples

Sample	Added (pM)	Found (pM)	Recovery (%)	RSD (%)
Binjiang Park soil	1.1	1.14	104	5.1
	1.1	1.11	101	
	1.1	1.03	94	
Campus soil	1.1	1.10	100	3.5
	1.1	1.04	94	
	1.1	1.10	100	
Jinghu Lake	1.1	1.11	101	2.6
	1.1	1.05	96	
	1.1	1.08	99	
Changjiang River	1.1	1.04	95	4.0
	1.1	1.09	99	
	1.1	1.13	103	
Tap water	1.1	1.09	99	3.7
	1.1	1.04	94	
	1.1	1.11	101	

RSD relative standard deviation

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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