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A regular nanostructured dithiolene metal complex film for ultrasensitive biosensing of liver enzyme

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Highlights

- A novel [TBA]$_2$[Ni(mnt)$_2$]/graphene composite film was first synthesized as an ultrasensitive biosensor.
- The nanostructure of [TBA]$_2$[Ni(mnt)$_2$] was in-situ controlled as the well-defined 500 nm cubic shape.
- The biosensor can perform both glutamate and ALT detections with high performance.

Abstract: A novel ultrasensitive biosensor was constructed by the [TBA]$_2$[Ni(mnt)$_2$]/graphene (mnt = maleonitriledithiolate) composite film for the detections of glutamate and alanine aminotransferase (ALT). The [TBA]$_2$[Ni(mnt)$_2$] nanocubes with a dimension of 500 nm were in-situ crystallized on the surface of Au electrode modified using thiol graphene in advance. Due to the synergistic effect
between the electrocatalysis of [Ni(mnt)₂]²⁻ anion and the excellent conductivity of graphene, the as-prepared biosensors showed remarkably reversible electrochemical redox and stability. It exhibited high sensitivities of 27.32 μA mM⁻¹ cm⁻² and 0.636 nA μM⁻¹ for the biosensing of glutamate and ALT with the corresponding wide linear ranges of 10-600 μM⁻¹ and 5-140 U L⁻¹, respectively. This high performance ALT biosensor is promising application in the trace analysis of more physiological substances, and the successful adoption of [TBA]₂[Ni(mnt)₂] material in the biosensor construction can arouse more dithiolene metal complexes in the further application of electrocatalysis.

**Keywords:** dithiolene metal complexes; thiol graphene; high sensitivity

1. Introduction

Alanine aminotransferase (ALT), one of hundreds of enzymes which maintain the body's digestive process and biogenic activities, has received enormous interests. It is because that the activity elevation of ALT is usually regarded as one of the most sensitive indicators for the damage of liver [1-4] which is the biggest apparatus of body which has the function of detox against harm. At present, a variety of analytical techniques have been reported for the ALT detection, such as colorimetry, chemoluminescence and spectrophotometry, but most of them require complex reagents, expensive instruments and time consumption with a weak ability in the real-time diagnosis [5]. Compared with these analytical techniques, the developing of electrochemical technique is desired for detecting ALT during the early diagnosis and treatment of severe liver diseases, such as hepatitis, liver intoxication and liver cancer [6-8], due to its advantages of simplicity, low cost, portability, and easy-to-use [9-12].
However, most of ALT electrochemical biosensors were aimed for point of care liver function testing [13,14] and the performance of currently produced electrochemical ALT biosensor cannot be satisfied. Although ferrocene carboxylic acid, bovine serum albumin, hydroquinone and horseradish peroxidase [1,7,15,16] have been reported as the biosensing materials, their redox properties were not strong. What's more, ALT electrocatalytic reaction steps are complex and multiple as follow:

\[
\text{L-Alanine} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-Glutamate} \quad (1)
\]

\[
\text{L-Glutamate} + \text{H}_2\text{O} + \text{GlutOx (FAD)} \rightarrow \\
\alpha\text{-Ketoglutarate} + \text{NH}_3 + \text{GlutOx (FADH}_2) \quad (2)
\]

\[
\text{GlutOx (FADH}_2) + \text{O}_2 \rightarrow \text{GlutOx (FAD)} + \text{H}_2\text{O}_2 \quad (3)
\]

\[
\text{H}_2\text{O}_2 \rightarrow 2\text{H}^+ + \text{O}_2 + 2e^- \quad (4)
\]

On the basis of above reaction mechanism, detection using electrochemical method will suffer the strong electric signal attenuation and the prolonging of response time due to the multiple enzyme reactions controlled by reaction kinetics. Therefore, in order to fulfil the sensitive detection, an advanced material with high electrocatalysis for signal producing is required to overcome these deficiencies.

The dithiolene-metal complex [TBA]_2[Ni(mnt)_2] (mnt = maleonitriledithiolate) possesses an excellent reversible electrochemical redox nature which is promising application in the electrochemical biosensor. Albeit the optical and optoelectrical properties have been wide studied [17], to the best of our knowledge, the work has never been investigated for the fabrication of [TBA]_2[Ni(mnt)_2] film using for the biosensor construction. The main challenge is the weak conductivity for the electrocatalytic electron transfer, which may lead to the response attenuation of electrochemical reaction. How to establish a channel to transport the electrons from the electrocatalytic reaction of [TBA]_2[Ni(mnt)_2] with physiological substances is a
key to realize its application in the biosensor. Graphene has been widely applied in sensing fields owing to its outstanding conductivity, environment stability and biocompatibility in biomaterial fabrication [18-21]. However, as reported, pure graphene film does not exhibit the obvious advance in terms of sensitivity and detection limit for biosensing due to its weak catalysis [22,23]. If both advantages of \([\text{TBA}]_2[\text{Ni(mnt)}]_2\) and graphene are coupled together in a composite film, a superior ALT biosensor is expectable.

In this work, we have successfully designed and fabricated a new typed biosensor to realize the rapid and accurate detection of glutamate and ALT with a \([\text{TBA}]_2[\text{Ni(mnt)}]_2\)/graphene composite film. Through a facile preparation approach, \([\text{TBA}]_2[\text{Ni(mnt)}]_2\) nanocubes with a diameter of 500 nm were uniformly formed on a thiol graphene modified Au substrate. The introduction of the thiol graphene cannot only function as the linking bridge between \([\text{TBA}]_2[\text{Ni(mnt)}]_2\) and Au substrate, but also remarkably promote the conductivity and electrocatalysis of \([\text{TBA}]_2[\text{Ni(mnt)}]_2\) film due to the existence of \([\text{Ni(mnt)}]_2^{2-}\). The as-prepared glutamate and ALT biosensors can both exhibit ultra-sensitivities, wide linear range, as well as good selectivity and stability in case of synergic effects from the composited \([\text{TBA}]_2[\text{Ni(mnt)}]_2\) and graphene layers.

2. Experimental

2.1. Reagents and apparatus

Thiol graphene (thGP, 4.0 wt% hydroxyl ratio) was synthesized by Jcnano Technology Co. Glutamate oxidase (GMOx) from Streptomyces sp. (EC1.4.3.11, 5 U mg\(^{-1}\)) and ALT (from porcine heart) were purchased from Sigma–Aldrich. Glutaraldehyde 25% (v/v) was obtained from Shanghai Lingfeng Chemical Reagent
Sodium glutamic acid monosodium salt monohydrate was purchased from Alfa-Aesar. Uric acid (UA), ascorbic acid (AA), L-alanine and α-ketoglutarate were received from Sinopharm Chemical Reagent Co. Ltd. (China).

The morphology of [TBA][Ni(mnt)$_2$] surface was observed by field emission scanning electron microscope (FESEM) (Hitachi, ModelS-4800II, Japan). The spectroscopy of thiol graphene modified with Au substrate was investigated with Fourier-transform infrared (FTIR) (AVATAR-FT-IR-360, Thermo Nicolet, USA) through Attenuated Total Reflection (ATR) spectra. The UV-visible diffuse reflectance spectra (UV-vis DRS) were obtained on a PerkinElmer Lambda 950 UV-Vis Spectrometer. Atomic force microscopy (AFM) characterization was relied on the XE100. The X-ray diffraction (XRD) of [TBA]$_2$[Ni(mnt)$_2$] was measured on an X-ray diffractometer (D/MAX 2500 V/PC) with a Cu-Ka line (0.15419 nm). All of electrochemical measurements were carried out by using electrochemical workstation (CHI 660E, Shanghai Chenhua Instrument Co.Ltd., China). Among, the cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were operated in a 0.1 M phosphate buffered saline (PBS, pH 7.0) containing 0.1 M KCl and electrochemical impedance spectroscopy (EIS) measurements were performed in the presence of a 5mM K$_3$Fe(CN)$_6$/K$_4$Fe(CN)$_6$ (molar ratio is 1:1)-mixture as a redox probe solution with the frequency changed from 0.1 Hz to 1000 kHz with signal amplitude of 5 mV at the 0.05 V potential. A Pt wire and Ag/AgCl (saturated KCl) were respectively used as the counter and reference electrodes.

2.2. Pretreatment of the Au electrode

In order to satisfy the clean and plane demands to substrate, Au electrode (2 mm diameter) were sequentially polished on a polishing doth with slurries of 0.5, 0.1 and 0.05 mm alumina particles, and followed by thorough washing with deionized water.
Then the electrode was dipped into a Piranha solution (7:3 mixture of H$_2$SO$_4$ and H$_2$O$_2$, v/v) for 30 min and rinsed with water. Finally, it was washed in ultrasonic environment for 30 min for ready usage.

2.3. Preparation of graphene film on Au electrode

20 mg thiol graphene powder was added into 40 ml deionized water to form 0.5 mg/ml graphene solution. In order to make graphene thoroughly dispersed evenly, this suspension liquid was then vibrated by a probe-type sonicator (KBS-650, Kunshan Shumei, China) with a 20 KHz of ultrasonic frequency for 30 min. Subsequently, Au electrode was vertically immersed in the graphene solution. After 30 min, the graphene film has been already modified on the surface of electrode and deionized water was used to wash electrode's surface for eliminating extra graphene which was not adsorbed weekly.

2.4. Preparation of [TBA]$_2$[Ni(mnt)$_2$] film on Au electrode

10 mg [TBA]$_2$[Ni(mnt)$_2$] powder, prepared following the published literature [24] and 40 ml ethanol were ultrasonically mixed for 15 min to make sure it completely dissolved. 5 μL of the above solution was coated on the Au electrode and let it dry at the room temperature. Once dried, the next 5μL solution was continuously coated until the total volume of solution dipped was 100μL. Finally, the electrode was cleaned by deionized water and dried at room temperature.

2.5. Construction of glutamate biosensor

In order to immobilize enzyme, the enzyme solution contained 0.25 % glutaraldehyde (v/v), in which 1 U/100 μL GMOx were included. Then, 5 μL of enzyme solutions were dropped evenly onto the center of the surface of electrode, followed by drying at 4 °C. The interaction produced by the -S-S- from protein and -SH from graphene can make the enzyme directly adhere on the film. When detected,
4 µL of 1 M glutamate was successively injected into the electrolytic cell with working, counter and reference electrodes, in which 0.1 M PBS was used as electrolyte.

3. Results and discussion

3.1. Structure and UV-vis spectroscopy of \([\text{TBA}]_2[\text{Ni(mnt)}_2]\)

The hybrid material, with a formula of \([\text{TBA}]_2[\text{Ni(mnt)}_2]\), is comprised of the ion-pairs. Fig. 1a shows an asymmetric unit of \([\text{TBA}]_2[\text{Ni(mnt)}_2]\), which contains one half of planar \([\text{Ni(mnt)}_2]^{2-}\) dianion and one \(\text{TBA}^+\) monocation [25]. The planar \([\text{Ni(mnt)}_2]^{2-}\) dianion possesses a center of inversion symmetry, which coincides with the Ni atom. The HOMO orbital of planar \([\text{Ni(mnt)}_2]^{2-}\) dianion mainly consists of the orbitals of two \(\text{mnt}^{2-}\) ligands together with a little amount of 3d orbits of Ni ion, and the electrons are delocalized in the whole molecular framework. The reversible redox feature of \([\text{Ni(mnt)}_2]^{2-}\) dianion is as a result of its unique electron structure. The XRD (Fig. 1b) pattern of our own homemade \([\text{TBA}]_2[\text{Ni(mnt)}_2]\) powder is consistent well with the simulated one of \([\text{TBA}]_2[\text{Ni(mnt)}_2]\) from the single crystal data [24]. The UV-vis spectroscopy of \([\text{TBA}]_2[\text{Ni(mnt)}_2]\) dissolved in the ethanol was prepared to estimate the band gap. As shown in Fig. 1c, the lowest energy absorption band falls within the wavelength ranges of 430-600 nm with a maximum of ca. 480 nm. The band gap of \([\text{TBA}]_2[\text{Ni(mnt)}_2]\) was calculated as ca. 2.25 eV, being indicative of the semiconductor behavior. Although the electrochemical redox of this material is competent, the low conductivity may block the transfer of electrocatalytic electrons, which affects the biosensing performance of the prepared film. This deficiency is required to address during the preparation of \([\text{TBA}]_2[\text{Ni(mnt)}_2]\) film for the further application.
3.2. Morphology evolution of [TBA]$_2$[Ni(mnt)$_2$]/graphene film

In order to enhance the conductivity, multi layers of thiol graphene was introduced as the ground film for the crystallization of [TBA]$_2$[Ni(mnt)$_2$]. To be confirmed the successful introduction of sulfhydryl group, graphene film modified the Au substrate was characterized by FTIR. As shown in the Fig. 2a, the substrate modified with thiol graphene exhibited a strong absorption peak at 804 cm$^{-1}$ [26] which belonged to the stretching absorption band of the -SH- group. Besides, an adsorption peak at 1100 cm$^{-1}$ illustrated the already combination of thiol graphene and Au substrate [27]. Fig. 2b showed the UV-vis spectra of [TBA]$_2$[Ni(mnt)$_2$] and [TBA]$_2$[Ni(mnt)$_2$]/graphene films modified the supports respectively. The two spectra showed almost the same adsorption peaks except at about 232nm in [TBA]$_2$[Ni(mnt)$_2$]/graphene film compared to the only [TBA]$_2$[Ni(mnt)$_2$], which could be the adsorption peak of graphene attributed to π–π* transitions of the aromatic C=C band. Therefore, it can be verified that [TBA]$_2$[Ni(mnt)$_2$] tightly adsorbed on graphene.

According to the Fig. 3a and b, neglection of the height difference caused by the support, the thickness of modified multi-layer thiol graphene can be calculated to be 41.5 nm. The in-situ growth of [TBA]$_2$[Ni(mnt)$_2$] crystals on this graphene surface obviously affected the crystal morphology. As shown in Fig. 3c, nanocubic [TBA]$_2$[Ni(mnt)$_2$] crystals were evenly covered on the surface of graphene, benefitting for decreasing the electrochemical resistance due to the uniform surface state. The dimensions of crystals were about 500 nm. This isolated distribution and regular nanostructure of [TBA]$_2$[Ni(mnt)$_2$] crystals is beneficial to the electrocatalysis of the prepared film due to the existence of more catalytic active sites in the edges of the cube shape [28]. For comparisons, Fig. 3d and e respectively show the
morphologies of free crystallized \([\text{TBA}]_2[\text{Ni(mnt)}_2]\) powers and only \([\text{TBA}]_2[\text{Ni(mnt)}_2]\) modified electrode. It is obviously that the size of each crystal can reach more than 10 \(\mu\text{m}\) when free crystallization was allowed. Moreover, if \([\text{TBA}]_2[\text{Ni(mnt)}_2]\) was the only material modified on the substrate without the thiol graphene film, its morphology was disorderly and the diameter of crystal was varied in size. The results demonstrate that the introduction of graphene can guide the growth of \([\text{TBA}]_2[\text{Ni(mnt)}_2]\) to small and regular crystals. This phenomenon may be attributed to the negatively charge of thiol graphene, which was tested as -14mV. After the graphene film touched the mother solution, TBA cations may easily be attracted on its surface by the electrostatic interaction. When the crystallization began, \([\text{Ni(mnt)}_2]^{2-}\) anions would pair the TBA cations combined on the graphene layer, which produced numerous nucleuses for the further growth. In this case, the dimension of crystal can be well controlled to nanoscale.

3.3. Electrocatalytic behavior of the \([\text{TBA}]_2[\text{Ni(mnt)}_2]/\) graphene film

As our design, the main purpose of graphene introduction is the promotion of electron transfer. EIS technique was applied to confirm the interface conductivity of \([\text{TBA}]_2[\text{Ni(mnt)}_2]\) films assisted with or without graphene. Fig. 4a shows the Nyquist plots for \([\text{TBA}]_2[\text{Ni(mnt)}_2]\) and \([\text{TBA}]_2[\text{Ni(mnt)}_2]/\)graphene films in 0.1 M KCl solution containing 5 mM \(\text{K}_3\text{Fe(CN)}_6/\text{K}_4\text{Fe(CN)}_6\) (molar ratio is 1:1) with a frequency ranging from 0.1 Hz to 1000 kHz. The standard Nyquist curve of EIS possesses a semicircle portion at high frequency and a linear portion at low frequency. According to the classical Randles circuit, the exact data of \(R_{ct}\) which represents the charge transfer resistance have been respectively calculated as 600 and 2500 -\(\Omega\), illustrating that the transfer rate of reacted electrons from the film to the electrode surface was faster when graphene was modified on the electrode. Direct electrochemical behaviors of these electrodes were compared by using a differential pulse voltammetry (DPV)
technology. As shown in Fig. 4b, none of evident redox peaks appeared for the only thiol graphene modified gold electrode, indicating that the electrode cannot undergo redox reactions and hardly catalyze glutamate in the investigated potential window. However, it should be noticed that the as-prepared [TBA]$_2$[Ni(mnt)$_2$] film exhibited a wide oxidation peak between 0.1 and 0.2 V which indicated the strong conductive resistance for its electrochemical reaction. The [TBA]$_2$[Ni(mnt)$_2$]/graphene film produced the sharp peak at 0.15 V, well catalyzing glutamate. Therefore, it is concluded that although graphene layer cannot provide any redox function, its superior conductivity can benefit the transfer of electrocatalytic electrons generated from the [TBA]$_2$[Ni(mnt)$_2$] film. The catalytic effect for oxidation of glutamate to α-Ketoglutarate was mainly caused by [Ni(mnt)$_2$]$^{2-}$/[Ni(mnt)$_2$]$^{1-}$ redox couple and valence variation was resulted from delocalized electron of anionic groups. The reaction mechanism can be illustrated as the following:

$$\text{L-Glutamate} + O_2 \overset{\text{GMOx}}{\longrightarrow} \alpha\text{-Ketoglutarate} + H_2O_2 \quad (5)$$

$$2[Ni(mnt)_2]^{2-} + H_2O_2 \longrightarrow 2[Ni(mnt)_2]^{1-} + 2OH^- \quad (6)$$

$$[Ni(mnt)_2]^{1-} + e^- \longrightarrow [Ni(mnt)_2]^{2-} \quad (7)$$

The electrons transfer from solution to the surface of electrodes. Therefore, the integration of [TBA]$_2$[Ni(mnt)$_2$] and graphene enhanced the electronic transport capacity of the electrode and synergistic effects.

CV technique was applied to evaluate the electrochemical properties of the prepared films. As shown in Fig. 5a, a quasi-reversible electrochemical process can be proposed according to the linear increase of the peak potential separation ($\Delta$Ep) with the scan rate. Furthermore, it showed that the observed anodic and cathodic peak currents were directly proportional to square root of the scan rate ($\nu^{1/2}$). Such a behavior implied that the redox process is controlled by linear diffusion of the electroactive species [29,30]. The electrocatalytic behaviors of the as-prepared composite film before/after the immobilization of GMOx were also investigated by
CVs (Fig. 6). After the immobilization of GMOx on the [TBA]$_2$[Ni(mnt)$_2$]/graphene film, both currents of redox peaks showed an obvious decline. This may be attributed to the poor conductivity of enzyme layer, which hindered the electron transfer towards the electrode surface. Furthermore, after the addition of glutamate, the oxidation current increased obviously, as well as the decrease of reduction current.

Based on above confirmations, this enzyme loaded film was capable for the biosensor construction. Differential pulse voltammetry (DPV) curves of GMOx/[TBA]$_2$[Ni(mnt)$_2$]/graphene electrodes in Fig. 7a show the current responses with the increasing glutamate concentrations. It can be seen that with the increasing concentration of glutamate, the current intensity increase correspondingly. The peak current is fitted to be linearly correlated with glutamate concentration between the ranges of 10-600 µM in Fig. 7b. The linear regression equation is expressed as $\Delta I_p$ ($\mu$A)$= 0.561+ 0.858c$ (mM) with a correlation coefficient of 0.991. The sensitivity was calculated as 27.32$\mu$A mM$^{-1}$ cm$^{-2}$ for the detection of glutamate, and the limit of detection is 5µM. Compared with the performance of the only [TBA]$_2$[Ni(mnt)$_2$] film without graphene layer (Fig. 7c), the sensitivity was about 25 times. Besides, also compared with the already reported glutamate biosensors, the sensitivity was not higher than the others with a more wider range (Table 1). The excellent redox ability [TBA]$_2$[Ni(mnt)$_2$] is attributed to its anion group of [Ni(mnt)$_2$]$^{2-}$. Under the excitation of the certain scanning potential, this group will lose its electrons to being an oxidation state during the enzyme oxidation of glutamate. The electrocatalytic electron can fast transfer from the film to substrate electrode due to the graphene layer, which formed a sharp oxidation peak at 0.16V and the linear relation between peak
current and glutamate concentration. Therefore, the high biosensing performance is mainly attributed to the synergetic effects of [TBA]_2[Ni(mnt)_2] and graphene layers which one produced the electrocatalysis and another fulfilled the signal transfer.

Because L-glutamate is a common product generated from a reaction between α-ketoglutarate and L-alanine in the presence of ALT, the as-prepared film can also be promising in the detection of ALT which is a very important physiological index for the evaluation of liver damage. Due to the existence of α-ketoglutarate inhibiting the activities of GMOx, an optimum concentration of α-ketoglutarate was 10 mM when L-alanine concentration was 100 mM [15]. Fig. 8a show the typical DPV response for ALT detection, demonstrating that a steady current increase with each 20 U L^{-1} addition. Fig. 8b exhibits the calibration curve which shows the relationship between peak current of biosensors and ALT concentrations. It can be seen that the peak current is proportion to ALT concentration over a range of 5-140 U L^{-1}. The linear regression equation is expressed as ΔIp (nA) = 570.33+ 0.636с (U L^{-1}). Meanwhile, the achieved sensitivity is 0.636 nA U L^{-1}, which is higher than the most previously reported biosensors. Although the sensitivity of the prepared biosensor was not the highest, its using content of GMOx per electrode was only 0.05 U.

3.4. Selectivity and stability of the [TBA]_2[Ni(mnt)_2]/graphene electrode

Selectivity is a crucial character which affects the biosensing performance for the real application. Ascorbic acid (AA) and uric acid (UA), which are usually considered as the most common electrochemical interfering species for producing redox reaction and releasing interference signal, were introduced to investigate the selectivity of the as-prepared electrodes. As shown in Fig. 9, 0.1 mM AA and 0.1 mM UA were
respectively added into the system after 0.1 mM glutamate was injected. It can be clearly seen that the DPV response produced obvious variations in the presence of glutamate. But when AA and UA were existed, it showed no evident variations. Hence, the results suggested that the proposed biosensors possessed good selectivity ability.

Subsequently, the stability of the electrode was determined after 3 weeks, which was stored at 4°C in the refrigerator. From the experiment result, it is showed that [TBA][Ni(mnt)]/graphene still retained 81.4% of their initial sensitivities in glutamate, indicating an excellent usage stability. Consideration of the simple storage method, this performance stability can be accepted.

4. Conclusions

In this work, a novel [TBA][Ni(mnt)]/graphene composite film was fabricated on the gold electrode for the detection of glutamate and ALT. The [TBA][Ni(mnt)] with nanocubic structure were uniformly distributed on the surface of electrodes modified with thiol graphene in advance. Due to the synergistic effect of the uniform isolated distribution and regular nanostructure, the [TBA][Ni(mnt)]/graphene electrode showed an excellent electrocatalysis to glutamate and the sensitivities of biosensors were respectively 27.32 μA mM⁻¹ cm⁻² and 0.636 nA μM⁻¹ for glutamate and ALT. Moreover, the prepared biosensors possessed good selectivity and stability. We believe that the proposed [TBA][Ni(mnt)]/graphene electrode will possess a great promise in constructing more sensitive and selective biosensors.

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Biographies

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Fig. 1. (a) Schematic illustration of molecular structure of $[\text{TBA}]_2[\text{Ni(mnt)}_2]$; (b) XRD patterns of simulated and homemade; (c) UV-vis spectroscopy of $[\text{TBA}]_2[\text{Ni(mnt)}_2]$. 
Fig. 2. (a) FTIR spectroscopy diagram of graphene modified Au substrate; (b) UV–vis absorption spectra of $[\text{TBA}_2\text{Ni(mnt)}_2]$ and $[\text{TBA}_2\text{Ni(mnt)}_2]/$graphene modified the supports.
Fig. 3. (a) AFM image and (b) line height scanning of thiol graphene deposited surface; (c) FESEM images of $[\text{TBA}]_2[\text{Ni(mnt)}_2]$/graphene modified electrode, (d) powder of $[\text{TBA}]_2[\text{Ni(mnt)}_2]$ and (e) $[\text{TBA}]_2[\text{Ni(mnt)}_2]$ modified electrode.
Fig. 4. (a) EIS diagrams of [TBA]$_2$[Ni(mnt)$_2$] and [TBA]$_2$[Ni(mnt)$_2$]/graphene modified electrode in 5 mM [Fe(CN)$_6$]$_{4/3}$ (1:1) containing 0.1 M KCl solution across the frequency range from 1000 kHz to 0.1 Hz; (b) DPVs of [TBA]$_2$[Ni(mnt)$_2$], thGP and [TBA]$_2$[Ni(mnt)$_2$]/graphene modified electrodes in 0.1 M PBS containing 0.1 M KCl.
Fig. 5. (a) CV diagrams of the prepared biosensor at scan rate of 10-100 mV s\(^{-1}\) (from curves a to j). (b) Linear correlation between current and scan rate from 20 to 100 mV s\(^{-1}\).
Fig. 6. CV curves of electrodes modified with (a) [TBA]$_2$[Ni(mnt)$_2$], (b) GMOx/[TBA]$_2$[Ni(mnt)$_2$] and (c) the addition of 0.1 mM target in 0.1 M PBS containing 0.1 M KCl. Scan rate of CV was 0.05 V s$^{-1}$. 
Fig. 7. DPVs of the as-prepared biosensors modified with graphene (a) and without graphene (c) in various concentration of glutamate in 0.1 M PBS (pH 7.0). The concentration of glutamate from bottom to top: 0 (curve a), 0.1 (curve b), 0.2 (curve c), 0.3 (curve d), 0.4 (curve e), 0.5 (curve f) and 0.6 mM (curve g); (b) and (d) Linear calibration curves for the current response of glutamate.
Fig. 8. (a) DPVs of the as-prepared biosensors for successive additions of 20 U L$^{-1}$ ALT and (b) the corresponding calibration curve. Background electrolyte 0.1 M PBS (pH 7.0), in the presence of 100mM 1-alanine and 10 mM $\alpha$-ketoglutarate.
Fig. 9. The selectivity test of AA and UA on the responses of the prepared biosensor in 0.1 M PBS.
**Tab. 1.** Performance comparisons of reported glutamate and ALT biosensors.

<table>
<thead>
<tr>
<th>Glut sensitivity (nA μM$^{-1}$)</th>
<th>Glut range (μM)</th>
<th>ALT sensitivity (nA U$^{-1}$ L$^{-1}$)</th>
<th>ALT range (U L$^{-1}$)</th>
<th>Ref.</th>
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<tbody>
<tr>
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<td>0.636</td>
<td>5-140</td>
<td>This work</td>
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<td>0.845</td>
<td>10-1000</td>
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<td>1.6-60</td>
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<td>0.15(calculated)</td>
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<td>-</td>
<td>[33]</td>
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