Simultaneous Determination of Ascorbic Acid, Dopamine and Uric Acid with Chitosan-Graphene Modified Electrode

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Abstract
Chitosan functionalized graphene was synthesized by a together-blending and in situ chemical reduction method. UV-vis, FT-IR, Raman and SEM techniques were used to study the molecular structure and morphology of the resulting composite. The chitosan-graphene modified glassy carbon electrode showed high electrocatalytic activity towards oxidations of ascorbic acid (AA), dopamine (DA) and uric acid (UA). By using the differential pulse voltammetry (DPV) as the analytical technique the calibration curves for AA, DA and UA, determined simultaneously, were found linear with a concentration range of 50 – 1200 \(\mu\)M, 1.0 – 24 \(\mu\)M and 2.0 – 45 \(\mu\)M, respectively.

Keywords: Graphene, Chitosan, Electrocatalysis, Ascorbic acid, Dopamine, Uric acid, Chemical sensor

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1. Introduction
Graphene, a single-atom-thick nanostructured sheet arranged in a honeycomb two-dimensional (2D) lattice [1] is a member of the family of carbon nanoscaled materials. It is also used as material in a diverse range of applications due to its intrinsic unique mechanical and electronic properties [2 – 4]. Rapid developments have mainly been focused on exfoliations and chemically modifications of graphene in these years. Graphene exhibits unusual electronic conductivity, high specific surface area, high mechanical, thermal and chemical stabilities [4 – 6] properties that make it a suitable material for electrochemical catalysis and biosensing. To expand and optimize the use of graphene in electrochemical catalysis and in the different applications in bio-technology, it is necessary to functionalize graphene with biomaterials.

The biopolymer chitosan is a natural polysaccharide and is widely distributed in the exoskeleton of crustaceans, fungal cell wall, and other biological materials. Since there are primary amino groups in the polymer chain, solubility of chitosan in water can be easily controlled by adjusting pH. It is soluble in the protonated form and insoluble in the deprotonated form. The \(pK_a\) value of chitosan is approximately 6.5 [7, 8]. Scheme 1 shows the molecular structure and the protonation-deprotonation reaction of chitosan with different length of molecular chain. Due to the unique biodegradation, no toxicity and the excellent film-forming ability, chitosan is widely used in many applications in areas including biology, medicine, food, and electrochemistry [9 – 13]. It is also an appropriate material to immobilize enzymes and there are various bio-sensors based on the excellent biocompatibility of chitosan [7, 14 – 17].

Chitosan-carbon nanotubes (CCNT) composites have been used in fabrication of electrochemical and biosensors [18 – 21]. Investigations of chemical and biosensors based on chitosan-graphene composite materials, however, are still rather few. In a recent report, Li and coworkers developed a dopamine electrochemical sensor based on chemically synthesized graphene, where acidic aqueous solution of chitosan was used in fabrication of the modified electrode [22]. Lin et al. prepared glucose oxidase (GOD)/Pt/graphene-carbon nanotube composite material as a glucose biosensor. In another recent report, Li and coworkers developed a dopamine electrochemical sensor based on chemically synthesized graphene, where acidic aqueous solution of chitosan was used in fabrication of the modified electrode [22].

\[
R = \begin{array}{c}
\text{CH}_3 \text{OH} \\
\text{O} \\
\text{NH}_2
\end{array}
\]  

Scheme 1. Protonation–deprotonation reaction of chitosan.
phene/chitosan bionanocomposite film and used it for glucose sensing [23]. In their work, graphene sheets were first obtained through thermal exfoliation from graphene oxide. The sheets were then dispersed in aqueous acidic solution of chitosan to form the functionalized graphene. Further investigation of electrochemical properties of graphene based materials and their composites are necessary.

Ascorbic acid, dopamine and uric acid are compounds of great biological and chemical interest and play a potential role in the metabolic system of human bodies. Ascorbic acid (AA) is important in health care of human beings. It is especially essential to the skin, connective tissues and immune system [24]. Dopamine (DA) is a monoamine neurotransmitter found in brain and is essential for the normal functions of the central nervous system. Uric acid (UA) is the final oxidation product of urine metabolism and is excreted in urine. Detection and quantification of AA, DA and UA are important in diagnoses, monitoring, prevention and treatments of some certain diseases such as HIV infections, schizophrenia, Parkinson, hyperuricaemia and a type of arthritis [25–29]. Therefore, simultaneous determination of these three species is of great significance not only in biomedical chemistry and neurochemistry but also for diagnostic and pathological investigations. It is well known that at traditional bare electrodes, AA, DA and UA exhibit oxidation peaks at potentials very close to each other resulting in an overlapping voltammetric response [30]. So far, various mediators have been developed in simultaneous determination of AA, DA UA including polymers [29, 31–33], noble metal-alloy nanoparticles [34], oxides [35, 36], carbon based materials [37–39] and composites based on the above materials [40–43]. However, no report is available concerning the use of graphene materials or their composites as electrode modifying material for simultaneous determination of biological compounds such as AA, DA and UA so far.

In this work, chitosan-graphene composite was applied for simultaneous determination of AA, DA and UA. During the synthesis process, a direct blending of chitosan with graphene oxide is performed followed by an in situ chemical reduction. Chitosan molecules are intercalated between graphene layers and act both as dispersant and stabilizer. The obtained chitosan-graphene composite can easily be purified, stored and re-dispersed in water by adjusting the pH value of the solution. Furthermore, the redispersed acidic chitosan-graphene solution depicts excellent dispersion and stability even after aging for a week. This material was found to exhibit both increased sensitivity and selectivity towards AA, DA and UA in presence of each others.

2. Experimental

2.1. Reagents and Instruments

Graphite powder (320 mesh, spectrographic grade) was obtained from Sinopharm Chemical Reagent Co., Ltd. Chitosan (low molecular weight), ascorbic acid (AA), 3-hydroxytyramine hydrochloride (dopamine, DA) and uric acid (99%, UA) were purchased from Sigma-Aldrich. Acetic acid, ammonia (NH₃, 25 wt% in H₂O) was obtained from E. Merck. All these chemicals were of analytical regent grade and used as received. Throughout all the experiments, ultra-pure water (18.2 MΩ cm from Purelab Ultra) were used.

Cyclic voltammetric (CV) and differential pulse voltammetric (DPV) measurements were carried out by using an Autolab PGSTAT100 electrochemical workstation. Glassy carbon electrode (GC) (d = 3 mm) and its modified electrodes were used as working electrodes. A Pt wire served as the counter electrode and an Ag/AgCl (in saturated KCl solution) was used as the reference electrode. UV-vis spectra were measured with Perkin Elmer Lambda 25 UV-vis Spectrometer. FT-IR spectra (KBr pellets) were recorded using a BRUKER IFS 66/S instrument. The Raman spectra were measured with the Renishaw Ramanscope instrument with λ_e = 780 nm diode laser excitation in 800–2000 cm⁻¹ region at room temperature. Leica Cambridge Ltd. Stereoscan 360 scanning electron microscope (SEM) was employed to characterize the morphology of chitosan-graphene composite.

2.2. Preparation of Chitosan-Graphene Composite

Graphene oxide (GO) was prepared by employing a modified Hummers method [44]. Preparation procedure for the chitosan-graphene composite was the following: typically, 5 mg chitosan was added into 20 mL GO aqueous solution (0.5 mg/mL) and the pH of the solution was adjusted to 3.5 with acetic acid. The obtained homogeneous chitosan-GO mixture was then stirred at 60 °C for 2 h. After that, 7 µL hydrazine and a certain amount of ammonia (25 wt% in H₂O) were added (adjusting pH to 8.5) followed by continuous stirring at 90 °C for 1 h. The dispersion was then centrifuged, washed with double-distilled water for several times and dried in air for 24 h.

2.3. Preparation of Modified Electrodes

A homogeneous dispersion of chitosan-graphene aqueous solution was prepared by dissolving 10 mg chitosan-graphene composite into 10 mL acetic acid (pH 3.5) with ultrasonic treatment for 30 min. Then, 10 µL of the chitosan-graphene solution (pH 3.5) was dropped onto the surface of the freshly polished glassy carbon (GC) electrode and dried in ambient air to get the chitosan-graphene modified GC electrode (chitosan-graphene-GC). For comparison, 10 µL of 1.0 mg/mL chitosan-acetic acid solution (pH 3.5) was also cast on bare GC to obtain the chitosan-GC electrode. Pristine graphene suspension solution was also prepared using a similar reflux method as used to make the chitosan-graphene composite. A dialysis process in deionized water was further used to neutralize the pH value of the
suspension solution. Then 10 μL of as-prepared graphene suspension was cast onto the bare GC and dried in room temperature to get the graphene modified GC electrode (graphene-GC).

3. Results and Discussion

3.1. Preparation and Characterization of Chitosan-Graphene Composite

Scheme 2 illustrates the synthetic process to make chitosan-graphene composite material and the snapshots of the outcomes at different stages of the process and at different pH values. In Scheme 2a, it is observed that when GO and chitosan were mixed at pH 3.5, the solution is homogeneous and is yellow-brown in color. After it was reduced with hydrazine in an alkaline solution (pH 8.5), the chitosan-graphene composite turned its color to dark and slowly sedimented. It can be seen in the snapshot in Scheme 2b, that after freely standing for 2 hours after reduction, the upper aqueous layer is completely transparent and colorless revealing extensive interaction between chitosan and the graphene sheets. After the precipitate was washed and dried and were redispersed into aqueous solution with pH 3.5, a stable and homogeneous chitosan-graphene dispersion was obtained (shown in Scheme 2c).

Results of UV-vis, FTIR, Raman and SEM measurements for characterization of the studied materials are shown in Figure 1. The UV-vis spectra of chitosan-acetic acid solution (pH 3.5, spectrum a), GO aqueous solution (spectrum b), and chitosan-graphene-acetic acid solution (pH 3.5, spectrum c) are presented in Figure 1A. Ultrapure water was used as the reference solution in taking the spectra of GO solution. In measurements with the chitosan and chitosan-graphene solutions acetic acid (pH 3.5) was used as the reference solution. As can be seen in spectrum (a) the chitosan solutions shows no absorptions in the whole studied wavelength region: from 200 to 600 nm. Spectrum (b) has a distinct absorption peak at 233 nm which is attributed to the characteristic absorption of graphene oxide [6]. After reduction of GO and combination with chitosan, the chitosan-graphene composite depicts a maximum absorption peak at ~270 nm (spectrum c), indicating formation of restored electronic conjugation structure within the graphene sheets [6].

FT-IR spectra of GO, chitosan and chitosan-graphene are shown in Figure 1B. As can be seen in the spectrum (a), pristine chitosan exhibits characteristic peaks at 3412 ($\nu$O/C0H), 2878 ($\nu$C/C0H), 1659 (Amide II), 1553 ($\delta$N/C0H), 1423 ($\delta$C-CH, Amide I), 1377 ($\nu$C-OH), 1158 and 1084 ($\nu$C-O) cm$^{-1}$ [45, 46]. Spectrum of GO (b) shows the peaks of $\nu$O/C0H at 3376, $\nu$C-O at 1054, $\nu$C-O/C at 1227, $\nu$C-OH at 1367, and $\nu$C=O stretch in the carboxylic group at 1731 cm$^{-1}$. The peak at 1618 cm$^{-1}$ may be attributed to skeletal vibrations of unoxidized graphitic domains [47, 48]. It is observed in the spectrum (c) that the chitosan-graphene composite demonstrates similar absorption bands as the pristine chitosan. No peaks, however, can be seen around 1731 cm$^{-1}$ indicating complete reduction of GO in the composite.

Scheme 2. Synthesize of chitosan-graphene composite and snapshots of the outcomes at different stages of the procedure.
The Raman spectra are shown in Figure 1C. Chitosan exhibits a broad band at 1399 cm$^{-1}$ and a small but broad band at ~1898 cm$^{-1}$, spectrum (a). GO has (spectrum b) the characteristic peaks at 1567 cm$^{-1}$ and at 1310 cm$^{-1}$ corresponding to the G and the D bands of the graphene structure, respectively. Generally stating the G band is considered arising from the zone center E$_{2g}$ mode, which corresponds to the ordered sp$^2$ bonded carbon, while the D band is deemed arising from sp$^3$-hybridized carbon and is an indication of disorder in the structure [49]. It is observed that the obtained chitosan-graphene composite exhibits a similar Raman behavior as GO with the G band at 1570 cm$^{-1}$ and D band shifted to 1282 cm$^{-1}$ indicating that the main graphene structure is conserved even in the composite. However, the increasing value of the D/G intensity ratio indicates a decrease in the average size of the sp$^2$ domains upon reduction and combination with chitosan [50].

Morphology of the chitosan-graphene composite was also investigated by the SEM technique. Figure 1D shows that the composite is formed of nanosheets with the size of ~300 nm $\times$ 400 nm in plane (Top right inset) and ~30 nm in thickness (Down left inset). The figure may also be used as the proof of the proposed mechanism for formation of the chitosan-graphene composite according to the Scheme 2 where chitosan molecules are tightly attached onto the surface of the GO nanosheets and after reduction by hydrazine, the chitosan-graphene composite with nanosheets structure is formed.

3.2. Voltammetric Responses at Different Electrodes

Figure 2 shows the cyclic voltammetric responses at bare GC (A), chitosan-GC (B), graphene-GC (C) and chitosan-
graphene-GC (D) electrodes in 0.05 M PBS solution (pH 7.0) containing 2 mM AA (black line), 1 mM DA (red line) and 0.5 mM UA (blue line). As can be seen in Figure 2A, AA and UA show irreversible oxidation peaks at 312 and 516 mV, respectively. DA depicts a reversible redox couple with anodic and cathodic peaks at 284 and 113 mV at the bare GC electrode. For chitosan-GC electrode (Figure 2B), the corresponding oxidation peak potentials of AA, DA and UA are shifted to 514, 319 and 408 mV. The currents observed at graphene-GC electrode (Figure 2C) are approximately twice the values observed at the bare GC and chitosan-GC electrodes (Figures 2A and 2B). This may be due to the increased surface area and/or the catalytic activity of the graphene-GC electrode. The oxidation peaks of DA and UA are also negatively shifted to 238 and 382 mV indicating a catalytic activity of the electrode material. The oxidation peak of AA in Figure 2C is not well-defined and the CV shows two indistinctive peaks around 70 and 324 mV. At the chitosan-graphene-GC electrode AA, DA and UA show well-defined oxidation peaks at 85, 213 and 296 mV, respectively (Figure 2D). The peak currents are also approximately 1.5 times higher than at bare GC and chitosan-GC electrodes. The peak potentials are also more negative than the peak potentials observed at the other electrodes studied. Still only DA show clear anodic and cathodic current peaks.

Cyclic voltammograms with different scan rates at the chitosan-graphene-GC electrode in the same solution as in Figure 2 are shown for AA in Figure 3A, for DA in Figure 3B and for UA in Figure 3C. The plots of the oxidation peak current as function of the scan rate are shown as an inset in each Figure. In all three cases a good linear relationships of current response against the scan rate was observed indicating an adsorption-controlled process for all three spices as was also observed at another modified electrode surface [43].

3.3. Simultaneous Determination of AA, DA and UA

Figure 4A shows CVs of bare GC (a, black line), chitosan-GC (b, green line), graphene-GC (c, blue line) and chitosan-graphene-GC (d, red line) electrodes in 0.05 M PBS solution (pH 7.0) containing the mixture of 5 mM AA, 0.5 mM DA and 2 mM UA. As shown in curves a–c in Figure 4A, only one distinct anodic peak is observed for bare GC at around 488 mV, at 484 mV for chitosan-GC and at 400 mV for graphene-GC electrode. However, curve (d) shows that modification of GC electrode with the chitosan-graphene composite film can effectively resolve the merged voltammetric peak into three well-defined oxidation peaks at potentials around 118, 243 and 332 mV for AA, DA and UA, respectively. Similar peak resolution was also observed and shown in Figure 4B when differential pulse voltammetric technique, DPV, was used with the four different electrodes in 0.05 M PBS solution (pH 7.0) containing the mixture of 200 μM AA, 100 μM DA and 120 μM UA. As shown in curves (a) and (b) in Figure 4B, the voltammetric response at bare GC and chitosan-GC electrodes were observed as only one weak and broad peak, which suggested that both of the two electrodes were very poor in selectivity and sensitivity. In curve (c), when the graphene-GC electrode was used, the current increased as found with the CV technique in Figure 2C, but simultaneous detection of AA, DA and UA is not still possible. While in curve (d) in Figure 4B, three distinct and well-defined voltammetric peaks at ca. 20, 185 and 275 mV for AA, DA and UA, respectively, were observed at the chitosan-graphene-GC electrode.
electrode. Separation of the DPV peak potentials of the voltammetric responses between AA-DA, DA-UA and AA-UA was calculated to be 165, 90 and 255 mV, respectively. These separations are large enough to allow simultaneous determination of these three species in the same solution. Due to the found selectivity and sensitivity, chitosan-graphene composite is a good candidate for simultaneous electrochemical determination of AA, DA and UA.

Determination of AA, DA and UA in their mixtures is performed by employing the DPV technique and using the chitosan-graphene-GC electrode in 0.05 M PBS (pH 7.0). In the experiments performed concentration of one component was continuously increased with the successive addition of the standard sample solution, while the concentrations of the other two species were kept constant. In the first experiment increasing amounts of AA were added to a solution of 5 mM DA and 20 mM UA. The results are shown in Figure 5A. In the second experiment increasing additions of DA were added to a solution containing 100 mM AA and 20 mM UA. Those results are shown in Figure 5B. In the third experiment increasing additions of UA are added to a solution containing 100 mM AA and 5 mM DA. Those results are shown in Figure 5C. All the results are summarized in Table 1 where also limit of detection (LOD = 3 S/N) and limit of quantification (LOQ = 10 S/N) are given. It should be pointed out that the values given in Table 1 are practical values. Linear response ranges and the LOD and LOQ values may certainly be further improved by optimization of the experimental parameters. The catalytic properties of graphene are mainly affecting separation of the redox peaks of the analytes thus enabling their determination in a mixture rather than influencing the sensitivity of the method.

4. Conclusions

In this work we have used a method where by together-blending and in situ chemical reduction of chitosan and graphene a new composite was synthesized. Chitosan played a key role both as dispersant and stabilizer in the composite. By adjusting the solution pH value, a well dispersed and stabilized chitosan-graphene dispersion was obtained and therefore the easy drop-casting procedure could be used to make the chitosan-graphene modified
glassy carbon electrodes. In the electrochemical investigations the chitosan-graphene modified GC electrode showed excellent electrochemical catalytic activities towards AA, DA and UA compared with the other electrodes studied. Further more, well-defined voltammetric responses were observed in simultaneous determination of AA, DA and UA in mixtures. The chitosan-graphene composite shows to have good electrochemical catalytic activity to the reactions studied by considerably degreasing the over-potential of the oxidation reactions studied. The excellent electrocatalytic properties of the composite may also be used in other electrochemical studies.

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References


Table 1. Practical values of simultaneous determination of AA, DA and UA on chitosan-graphene modified electrode in 0.05 M PBS solution (pH 7.0).

<table>
<thead>
<tr>
<th>Solution: (0.05 M PBS, pH 7.0) including Additions</th>
<th>Linear range</th>
<th>Limit of detection (LOD)</th>
<th>Limit of quantification (LOQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μM DA and 20 μM UA AA, 50 – 1500 μM</td>
<td>50 – 1200 μM</td>
<td>50 μM</td>
<td>166 μM</td>
</tr>
<tr>
<td>100 μM AA and 20 μM U DA, 1 – 32 μM</td>
<td>1.0 – 24 μM</td>
<td>1.0 μM</td>
<td>3.3 μM</td>
</tr>
<tr>
<td>100 μM AA and 5 μM DA UA, 2 – 65 μM</td>
<td>2.0 – 45 μM</td>
<td>2.0 μM</td>
<td>6.6 μM</td>
</tr>
</tbody>
</table>

Fig. 5. DPVs at chitosan-graphene-GC electrode in 0.05 M PBS (pH 7.0). A) 5 μM DA, 20 μM UA and different concentrations of AA; B) 100 μM AA, 20 μM UA and different concentrations of DA; C) 100 μM AA, 5 μM DA and different concentrations of UA. Scan rate: 5 mV/s pulse amplitude 25 mV. Inset: Plot of peak current vs. sample concentrations.


