Reduced Graphene Oxide-Based Covalent Hybrid Film Electrode Self-Assembled with Gold Nanoparticles for the Enzyme-Free Amperometric Sensing of Serum Uric Acid

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Abstract

Purine metabolism in human body leads to the production of uric acid (UA) at the end. But abnormal level of UA in human body creates health problems. The sensing and quantification of UA is essentially required to prevent and diagnose hypertension, arthritis, gout, hyperuricemia or Lesch-Nyhan syndrome, etc. Herein, the development of sensing platform for measurement of UA using Au nanoparticle-based hybrid self-assembly has been described. Self-assembling of thiol-terminated silicate network functionalized graphene oxide hybrid on polycrystalline Au surface yields three-dimensional assembly. The oxygen functionalities of the self-assembly were partially reduced by NaBH₄ treatment. The free –SH groups of the self-assembly were successfully used for the immobilization of Au nanoparticle by chemisorption. The nanoparticle-based hybrid self-assembly is highly sensitive toward UA and shows wide linear response with a detection limit of 40 nM UA (S/N=7) without interference from co-exiting ascorbic acid. The practical application is demonstrated using human serum samples.

Keywords: Hybrid material, Silylation, Graphene oxide, Au nanoparticles, Uric acid
Introduction

Purine metabolism in human body produces uric acid (UA) as an end product. The detection and quantification of serum UA are important for the prevention and diagnosis of gout, arthritis, hypertension, hyperuricemia or Lesch-Nyhan syndrome, etc.\textsuperscript{1-3} The normal concentration\textsuperscript{4} of UA in human serum is 2.18–7.7 mg dL\textsuperscript{-1} and the elevated level of UA is also a risk factor for cardiovascular disease.\textsuperscript{5} The development of highly selective and sensitive detection of UA is of significant interest in clinical analysis. Several methods such as chromatographic, colorimetric, fluorimetric, chemiluminescence, electrophoretic analysis have been reported for UA sensing.\textsuperscript{6-9} However, these methods are relatively time consuming and they require laborious sample pretreatment, tedious procedures and expensive equipment. The amperometric technique has received significant interest due to the fact that it does not require expensive instruments.\textsuperscript{10, 11} The concentration of UA can be electrochemically monitored either by its direct oxidation at the electrode surface or by the enzymatic procedures involving the oxidation of enzymatically generated H\textsubscript{2}O\textsubscript{2}. Although the enzyme-based biosensors show high selectivity, they are very delicate and suffer from lack of stability. The direct oxidation of UA on unmodified electrodes requires a high overpotential and invites interference due to coexisting easily oxidizable biomolecules, mainly ascorbic acid (AA). Both AA and UA undergo electrochemical oxidation on unmodified electrode almost at the same potential and it is a challenging task to measure the concentration of UA in the presence of AA.\textsuperscript{12-15} Moreover, unmodified electrodes are not appropriate for electrochemical sensing applications due to sluggish electron transfer kinetics and fouling of electrode surface by the adsorption of the intermediate species or the products. Rational modification of electrode surface is critically required for the enzyme-free amperometric sensing of UA. Several approaches have been made in the past for the chemical modification of electrode surface to develop amperometric sensors.\textsuperscript{16} Carbon nanomaterials are widely being used for sensing purpose.\textsuperscript{17} For instance, self-assembled monolayer, metal nanoparticles, carbon nanostructure-based electrodes have been well exploited for the sensing of UA.\textsuperscript{18-20} In the recent years the as-synthesized hetero atom doped and undoped carbon nanomaterials and their composite materials, nanoparticles and polymers have been utilized for the selective detection of UA.\textsuperscript{18-25} The electrode materials should be either capable of facilitating the oxidation of only UA and suppressing the oxidation of AA or it should favor
the oxidation of both AA and UA at two different potentials with large potential separation. As the oxidation of both AA and UA involves two electrons and the diffusion coefficient of AA and UA is very similar, achieving large separation between the oxidation peaks or suppressing the oxidation of AA is a challenging task. Herein, the development of a new electrochemical interface for the selective amperometric detection of UA has been demonstrated using Au electrode modified with a hybrid material based on reduced graphene oxide, sol-gel derived silicate network and Au nanoparticles. The approach involves the self-assembling of the hybrid material on the electrode and the chemisorption of Au nanoparticles on the free thiol groups of the hybrid material. The thiol groups of the silicate moiety chemisorb on Au electrode and hold the hybrid material on the electrode surface. rGO is obtained after reduction of GO moiety by NaBH₄ treatment. GO has dual role: (i) it anchors the MPTS network on both the sides and edges by covalent bond and (ii) it enhances the overall conductivity after the reduction of the oxygen containing functionalities. Free thiol groups have been functionalized with Au nanoparticles. The active surface of Au nanoparticles enhances the oxidation of uric acid (UA). The sensing of UA in presence of AA was achieved without any compromise in the sensitivity and selectivity.

Materials and Methods

Reagents and materials

Graphite, H₂O₂ (30%), (3-mercaptopropyl)trimethoxysilane (MPTS), HAuCl₄·3H₂O, UA and AA were obtained from Sigma-Aldrich. All other chemicals used in this work were of analytical grade (99.9%) and used without further purification. Phosphate buffer solutions (PBS, 0.1 M) of required pH were used as supporting electrolyte in the voltammetric and amperometric measurements. All the solutions used in this work were prepared with Millipore water (Milli-Q system).

Covalent functionalization of GO with MPTS

GO was synthesized from pristine graphite according to modified Hummers method (supporting information). Functionalization of GO with MPTS was achieved according to literature procedure. Typically, required amount of GO was dispersed in 10 mL water and the dispersion was deaerated by purging argon gas. An aliquot of 100 µL MPTS was injected into the GO dispersion in
inert atmosphere at room temperature under constant stirring. Then HCl (100 µL of 0.1 M) was injected into the above mixture to initiate the hydrolysis and the stirring was continued for 16 h in inert atmosphere. The product (MPTS-GO) was collected by centrifugation, washed extensively with water and ethanol and dried at 70 °C in vacuum (Scheme S1).

**Preparation of MPTS sol**

An aliquot of MPTS (24 µL) was injected into 2 mL of water. To it 25 µL of 0.1 M HCl was added and the resulting mixture was stirred for 45 min at room temperature to obtain MPTS sol.

**Synthesis of colloidal Au nanoseeds**

The colloidal Au nanoseeds (sAu) were synthesized by NaBH₄ reduction without using any additive or stabilizer.²⁸ The reducing agent NaBH₄ (20 µL, 0.04%) was slowly added to aqueous HAuCl₄ (2 mL, 0.0001%) solution under constant stirring at room temperature. The resulting wine-red colored Au sol was stored in dark at room temperature.

**Self-assembly of MPTS-GO on polycrystalline Au electrode**

In order to develop the sensing interface, MPTS-GO was self-assembled on electrochemically cleaned polycrystalline Au electrode by soaking the electrode in MPTS-GO dispersion in water (1 mg mL⁻¹) for 10 min. The free thiol groups of MPTS-GO hybrid chemisorb onto the electrode surface to yield the hybrid self-assembly. The electrochemical cleaning of the Au electrodes involves cycling the potential of the electrode between -0.2 and 1.5 V in 0.25 M H₂SO₄ at the sweep rate of 10 V s⁻¹ for 10 min or until the characteristic cyclic voltammogram for an Au electrode was obtained. The real surface area of the polycrystalline Au electrode was obtained from the charge consumed during the reduction of surface oxides; the reported value of 400 µC cm⁻² was used in the calculation.²⁹ The calculated real surface area was 0.1015 cm². The oxygen functionalities of the MPTS-GO hybrid assembly were partially reduced by immersing the MPTS-GO hybrid self-assembly into 1 mM NaBH₄ solution for 10 min. The electrode was repeatedly washed with water and ethanol to remove the physically adsorbed material and stored in PBS. The electrode after partial reduction of surface
oxygen functionalities is referred as MPTS-rGO. MPTS electrode was prepared by dipping a cleaned Au electrode in MPTS sol.

**Self-assembling of Au nanoseeds on MPTS-rGO electrode**

The MPTS-rGO electrode was soaked in colloidal sAu for 18 h for the self-assembling of Au nanoseeds on to the free –SH groups of the hybrid assembly to obtain MPTS-GO-sAu electrode. The self-assembled sAu was further grown by seed-mediated growth approach. Typically, the MPTS-rGO-sAu electrode was soaked in a solution containing 1:1 molar ratio of hydroxylamine and HAuCl₄, and the solution was stirred gently for 10 min (Scheme 1). The electrode was washed with copious amount of water and kept in PBS before subjecting to any electrochemical experiment. The electrode after hydroxylamine enlargement is referred hereafter as MPTS-rGO-Au.

**Results and discussion**

**Synthesis and characterization of silicate-rGO hybrid material**

The hybrid material was synthesized by the covalent grafting of MPTS on GO moiety according to literature procedure.²⁷ It is well known that the alkoxy silanes undergo facile hydrolysis and condensation in presence of acid or alkali. The condensation reaction of the hydrolyzed MPTS with the –OH groups of GO yields the MPTS-GO hybrid material (Scheme S1). GO, MPTS-GO and MPTS-rGO were characterized by FTIR (supporting information, Figure S1, Table S1), Raman (supporting information, Figure S2), XPS (supporting information, Figure S3), powder XRD and cyclic voltammetric measurements. The interpretation of the results of FTIR, Raman and XPS are also shown in the supporting information. The results of powder XRD and cyclic voltammetry measurements are provided on the following pages. In the XRD profile of GO, the diffraction at 2θ = 9.28° corresponds to (002) plane of the carbon (Figure 1). After the functionalization of GO with MPTS, the diffraction of (002) plane shifted from 9.28 ° to 6.65 °, implying that the covalent grafting of MPTS groups on the GO moiety increases the interlayer distance. The interlayer distance of GO increases from 0.952 to 1.328 nm upon functionalization with MPTS. In case of MPTS-rGO, broadening of the peak at lower angle and appearance of broad peak at higher angle were observed. MPTS-rGO-Au displays peaks corresponding to (111), (200), (220), and
planes of Au (JCPDS 04-0784) (Inset of Figure 1). The Raman spectrum for MPTS-rGO self-assembly on Au surface shows a band at 275 cm\(^{-1}\) assigned to Au–S vibration (supporting information, Figure S4).

The capacitive performance of the hybrid self-assemblies was examined in neutral pH (Figure S5). The electrochemical response of carbon-based materials is highly dependent on the electronic nature of the carbon network. The capacitance of self-assembly of MPTS-rGO is \(\approx 3.5\) times higher than that of the self-assembly of MPTS-GO. The partial removal of oxygen functionalities and restoration of \(\pi\)-conjugation actually increase the electronic conductivity and hence an enhancement in the capacitive performance. The chemisorption of Au nanoseeds does not significantly alter the capacitive performance of the self-assembly. However, Au nanoseeds after hydroxylamine enlargement show broad voltammetric signature in the potential range between 0.2 and 0.35 V, presumably due to the surface oxides on the nanoparticle enriched with (111) planes (Figure S5).\(^{30}\) The MPTS-GO and MPTS-rGO electrodes do not show such voltammetric response, implying that the peak is associated with the self-assembled Au nanoparticles.

The TEM image of the self-assembly of Au nanoparticles on MPTS-rGO shows the presence of quasi-spherical and polyhedral nanoparticles with a size ranging from 40 to 60 nm (Figure 2A). The high resolution TEM image shows the fringe spacing of 0.2307 nm corresponding to the (111) plane of Au (Figure 2B). Selected area electron diffraction shows spotty pattern corresponding to the (111), (200), (220) and (311) planes (Inset of Figure 2B) of face centred cubic Au. The voltammetric responses of the hybrid self-assemblies towards redox marker K\(_3\)[Fe(CN)\(_6\)] are shown in Figure 3. The self-assembly of MPTS silicate network does not permeate the redox marker and ill-defined redox response was noticed. On the other hand, the MPTS-GO self-assembly shows a sluggish redox response with a peak-to-peak separation (\(\Delta E_p\)) of \(>350\) mV due to the insulating nature of both GO and silicate and it is significantly higher than unmodified electrode (Table S2). Enhancement in the electron transfer kinetics was observed while moving from MPTS-GO to MPTS-rGO due to the partial restoration of \(\pi\)-conjugation. The chemisorption of the Au nanoseeds on the –SH terminal groups followed by the hydroxylamine enlargement of the seeds has significant impact on the
voltammetric response. Enhancement in the peak current due to the increase in the electrochemically accessible surface area and easy accessibility of the redox species and decrease in the $\Delta E_p$ value due to the facile electron transfer were noticed.

**Amperometric sensing of UA**

Considering the importance of the development of enzyme-free sensing of serum UA, the hybrid self-assembly has been exploited for the sensing and quantification of UA in human serum. The electrochemical oxidation of UA involves $2e^{-}$ and $1H^{+}$ in neutral pH and it is rather sluggish on the conventional unmodified electrodes. The oxidation of UA on unmodified Au electrode requires large overpotential owing to the sluggish electron transfer kinetics and fouling of the electrode surface by adsorption. The MPTS-rGO electrode favors the oxidation of UA at less positive potential than MPTS-GO and unmodified Au electrodes (Figure 4A). The unmodified Au electrode shows broad voltammetric peak for UA at more positive potential and the voltammetric response is highly unstable. On the other hand, MPTS-GO electrode shows a stable broad peak for UA oxidation at around 0.56 V. In case of MPTS-rGO electrode, a sharp peak for UA oxidation was obtained due to the restoration of $\pi$-conjugation. Further enhancement in the voltammetric peak current and negative shift in the oxidation potential (112 mV) were noticed on MPTS-rGO-Au electrode. The facilitated electron transfer for the oxidation of UA can be rationalized by considering the catalytic effects of surface oxides (AuOH or Au(OH)$_3$) on Au nanoparticles. The negative shift in the peak potential associated with enhancement in the peak current implies a strong electrocatalytic effect of the nanoparticle. Figure 4B shows cyclic voltammograms of the electrode in presence of different concentrations of UA. The oxidation current for UA linearly changes with the square root of scan rate (Figure S6), suggesting the diffusion controlled redox process. Figure 5A shows amperometric response obtained for the UA oxidation at the MPTS-rGO-Au electrode. Stable amperometric response was obtained and the sensitivity was calculated to be $64.43 \pm 1.23$ nA $\mu$M$^{-1}$ cm$^{-2}$. The electrode shows linear response up to 130 $\mu$M with a response time of 23 s. This electrode could detect as low as 40 nM of UA ($S/N = 7$) at the potential of 0.4 V at pH 7.2. The excellent analytical response is highly stable and the amperometric response does not change significantly with time. The operational stability of the
electrode was investigated polarizing the electrode at 0.4 V and injecting 50 µM UA (Figure S7). No significant change in the current was observed for long time. The long-term storage stability test of the electrode was examined using the same electrode for seven days and only 10% loss in the initial amperometric response after seven days was noticed (Figure 5B). The interference due to coexisting other small molecules in the real sample is a serious concern in the enzyme-free sensing of UA. For instance, presence of AA in real sample seriously interferes the amperometric measurement of UA. In order to examine the selectivity of this electrode towards UA, voltammetric measurements have been performed in presence of AA. Interestingly, two well-separated voltammetric peaks for AA and UA were observed at the potential of 0.2 and 0.4 V, respectively and the amperometric current due to oxidation of UA does not change significantly (slightly decreased) with addition of AA (Figure 6). This implies that AA does not interfere UA sensing. To further examine the effect of AA in UA sensing flow injection analysis with a bipotentiostat has been used. The dual electrode was modified with MPTS-rGO-Au and the potentials of the two electrode terminals were controlled at +0.2 and +0.4 V using a bipotentiostat. The sample containing mixture of AA and UA was injected at a regular interval. The amperometric currents for oxidation of AA and UA at the electrode terminals were measured simultaneously (Figure 7A). The electrode terminal polarized at +0.2 V gives the current due to the oxidation AA and the electrode terminal polarized at 0.4 V gives the current due to the oxidation of UA. In another experiment only UA was injected and only one electrode terminal was polarized at 0.4 V. The electrode terminal polarized at 0.4 V (Figure 7B) gives almost the same current obtained for the mixture of UA and AA. This further suggests that AA does not interfere UA sensing. The selectivity of UA with respect to AA can be attributed to the fast electron transfer kinetics and anti-fouling property of the electrode, and favourable interaction of AA with the electrode compared to that of UA. Practical application of the sensor is demonstrated by measuring UA in human serum samples. This electrode is capable of quantifying the concentration of UA without any interference from the coexisting other analytes. Table S3 compares the analytical performances of the developed UA sensor with the previous UA sensor in the literature. The validity of the results obtained with this electrode is authenticated with the conventional clinical laboratory procedure. As shown in Table 1, the concentration of UA obtained with this electrode (method for
human serum sample analysis has been described in the supporting information with Figure S8) is very close to that of the clinical method. The recoveries of the samples were in the range of 97–104 %.

Conclusions

The selective amperometric sensing of UA has been demonstrated using the Au nanoparticle self-assembled hybrid assembly. The Au nanoparticle-based hybrid assembly favors the oxidation of UA at less positive potential. The carbon network of the hybrid assembly and the surface oxides on Au nanoparticle facilitate the electron transfer. The carbon network enhances the conductivity and the Au nanoparticles favor the oxidation of UA. The hybrid assembly prevents the unwanted adsorption of the oxidation products on the electrode surface. The electrode can amperometrically quantify UA in presence of AA without any compromise in the sensitivity. The practical application of this UA sensor is demonstrated with human serum samples and the results are in close agreement with those obtained by clinical method, authenticating the validity of the measurement.

Notes

The author declares no competing financial interest.

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Supporting Information

Supporting information includes instrumentation; synthesis of GO; characterization of the materials; scheme for MPTS-GO synthesis: FTIR, Raman spectra of the GO, MPTS-GO and MPTS-rGO; XPS spectra of GO and MPTS-GO; Raman spectra of self-assembled MPTS-GO and MPTS-rGO on Au; cyclic voltametric responses of MPTS-GO, MPTS-rGO and MPTS-rGO-Au electrodes; cyclic voltametric responses of MPTS-rGO-Au electrodes toward UA oxidation at different scan rates; amperometric response for operational stability test; method of human serum sample analysis and
resulting figure; tables for peaks in FTIR spectra for different functionalities, peak-to-peak separation for the voltametric responses of different electrodes towards $K_3[Fe(CN)_6]$ and comparison of analytical responses with several other sensors. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.
References


Scheme 1

Scheme representing the chemisorption of sAu on the free thiol groups of the MPTS-rGO hybrid self-assembly and enlargement of sAu by hydroxylamine seeding.
Figure captions

Figure 1

XRD profiles of GO (a), MPTS-GO (b) and MPTS-rGO (c). Inset shows XRD profiles of MPTS-rGO (c, magnified) and MPTS-rGO-Au (d).

Figure 2

TEM (A) and HRTEM (B) images of the Au on the hybrid assembly. Gold grid was used as a substrate for the TEM measurement. Inset in B is the SAED pattern.

Figure 3

Voltammetric responses of unmodified Au (a), MPTS (b), MPTS-GO (c), MPTS-rGO (d) and MPTS-rGO-Au (e) electrodes towards K₃[Fe(CN)₆] (5 mM) in 0.1 M PBS (pH 7.2) at 100 mV s⁻¹.

Figure 4

(A) Voltammetric response of unmodified Au (a), MPTS-GO (b) and MPTS-rGO (c) and MPTS-rGO-Au (d) electrodes towards oxidation of UA (200 µM) in 0.1 M PBS (pH 7.2) at 100 mV s⁻¹.

(B) Voltammetric response of the MPTS-rGO-Au electrode towards UA at different concentrations in 0.1 M PBS (pH 7.2). Each addition increased the concentration by 25 µM. Scan rate: 100 mV s⁻¹.
(A) Amperometric i-t curve for the oxidation of UA at MPTS-rGO-Au electrode. The electrode was polarized at 0.4 V in a stirred solution of 0.1 M PBS (pH 7.2). Each addition increased the concentration of UA by 10 µM. Inset shows the corresponding calibration plot.

(B) The plot illustrates the long-term stability of the MPTS-rGO-Au electrode.

Figure 6

Square wave voltamograms illustrating the interference-free sensing of UA in presence of AA. [UA]: 200 µM. Each addition of AA increased the concentration by 25 µM. Electrolyte: 0.1 M PBS (pH 7.2), frequency: 15 Hz, potential step: 4 mV, amplitude: 25 mV.

Figure 7

Flow injection amperometric response illustrating the detection of UA. (A) Response towards the mixture of AA and UA. The electrode terminals of the dual electrode were polarized at 0.4 and 0.2 V and an aliquot of 20 µL of an aqueous mixture containing 10 µM each of UA and AA was injected at a regular interval. (B) 20 µL of 10 µM UA was injected and the electrode was polarized at 0.4 V. Mobile phase: 0.1 M PBS of pH 7.2, flow rate: 0.5 mL min⁻¹.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Table 1

Sensing of UA in human serum samples.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Measured with this sensor&lt;sup&gt;a&lt;/sup&gt; (mg dL&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Measured by clinical method (mg dL&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>RSD&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Recovery&lt;sup&gt;a&lt;/sup&gt; (%)</th>
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<sup>a</sup> Average of three independent measurements. <sup>b</sup> RSD obtained from three independent measurements of each sample.