Determination of Phosphate in Human Serum with Zirconium/Reduced Graphene Oxide Modified Electrode

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An electrochemical phosphate sensor based on zirconium and reduced graphene oxide modified pencil graphite electrode (Zr/rGO-PGE) is proposed. The XRD, CV and EIS confirmed that GO was partially reduced on the PGE. Scanning electron microscopy (SEM) exhibited the layered and wrinkled structures for the rGO-PGE and Zr/rGO-PGE, respectively. Cyclic voltammetry showed the immobilized rGO was highly stable and had high activity toward zirconium adsorption. The prepared electrode was used for the electrochemical determination of phosphate. Based on the optimum condition using differential pulse voltammetry, the limit of detection and sensitivity for phosphate was obtained as \[0.011(±0.004) \mu M\] \((S/N=3)\) and \[622.4(±9.6) \mu A \mu M^{-1} cm^{-2}\], respectively. The sensor was successfully evaluated for phosphate determination in human serum samples. In practical terms, the construction of this sensor was exceptionally simple, fast, cost effective and reproducible.

Keywords Graphene oxide, phosphate, zirconium, pencil graphite electrode

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Introduction

Inorganic phosphate is a salt of phosphoric acid, in the form of negative ion in mineral substances or in the form of organophosphates, in biological molecules.\(^1\) Phosphate is an essential component of most living organisms that plays a major part in biological processes such as the synthesis of ATP and DNA and pH control.\(^2\) In clinical trials, serum phosphate levels are commonly used to diagnose diseases such as hypertrophy of the thyroid gland, vitamin D deficiency, and so on.\(^3\) Phosphate has an important role in the environment.\(^4\) Several techniques have been extended for measuring phosphate ion in industrial, environmental and clinical samples, including chromatography,\(^5\) spectrophotometry,\(^6\) and electrochemical\(^7\) methods. Electrochemical methods include potentiometric approaches based on single-electron electrodes,\(^8\) enzyme-modified biosensors\(^9\) and modified electrodes by self-assembled monolayers.\(^10\) The powerful ionic-coordinative interaction between zirconium ions and phosphate groups can be used in many cases, such as the formation and functionalization of thin films on solid substrates, such as metals and non-metallic materials, as well as the creation of self-supporting supermolecular arrays for various purposes.\(^11\) According to early electrochemical studies, based on the strong absorption of phosphate ion on the surface of the Zr(IV) immobilized on gold-mercaptopropionic acid self-assembled monolayer (Au-MPA-Zr(IV) SAM) electrode mentioned in our previous study, a simple, inexpensive and fast analytical method for measuring phosphate ion in a human serum sample is presented.\(^12\)

In spite of its unique features, its simplicity, low price and excellent detection limit, Zr/rGO-PGE has not been used up until now for the electrochemical determination of phosphate. In our recent work, a PGE was modified for the first time with partially reduced graphene oxide.\(^13\) In this report, a reduced graphene oxide modified PGE (rGO-PGE) was modified with Zr for the first time. The step-by-step modification of the PGE was monitored by XRD, SEM, EIS and cyclic voltammetry. The Zr/rGO-PGE was used for the sensitive determination of phosphate using differential pulse voltammetry.

Materials and Methods

**Synthesis of graphene oxide (GO)**

The improved Hummer method was used for the synthesis of graphite oxide.\(^14\) In this method, a 9:1 (v/v) of concentrated \(\text{H}_2\text{SO}_4/\text{H}_3\text{PO}_4\) and a 1:6 (w/w) of \(\text{KMnO}_4\) and graphite powder was mixed slightly. Then the mixture was heated under stirring. After about 10 h the mixture was cooled and then 120 mL ice and 2 mL \(\text{H}_2\text{O}_2\) were added to the reaction. Finally, the reaction was centrifuged and the supernatant was washed successively with water, hydrochloric acid 30%, and ethyl alcohol. The suspension of GO was set by adding 100 mg of GO powder to 100 mL of phosphate buffer solution (PBS) 0.1 M, pH 9, and followed by 15 min of sonication to find a steady dispersion.

**Modification of PGE with rGO (rGO-PGE)**

Pencil lead with a diameter of 0.5 mm (Tombow Co., Ltd., Japan) was implanted into a Teflon tube, so only 1 cm of its tip and end were exposed \((A = 0.16 \text{ cm}^2)\). Electrical contact was made by soldering a copper wire to the end exposed side of the PGE. Pretreatment of the PGE was done by immersing for 15 min in \(\text{HNO}_3\) 6 M and then washing with water. The PGE was then introduced into the electrochemical cell containing...
suspension of GO (1 mg mL\(^{-1}\), pH 9) and the potential of the electrode was scanned for 10 cycles at 20 mV s\(^{-1}\) in the range of 0 to \(-1.0\) V. Before potential scanning, the solution was deaerated by purging with argon gas (99.99\%) for about 15 min.

**Modification of rGO-PGE with Zr (Zr/rGO-PGE)**

The rGO-PGE was immersed deep into the stirring solution of 0.1 mM zirconium chloride for about 10 min. Then, the electrode was rinsed and moved to the electrochemical cell containing 0.5 mM [Fe(CN)\(_6\)]\(^{3–}\) in acetate buffer solution (ABS) 0.1 M, pH 5, and the potential of the electrode was scanned at 20 mV s\(^{-1}\) in the range of 0.6 to \(-0.3\) V.

**Measurement of phosphate on Zr/rGO-PGE**

The Zr/rGO-PGE was immersed into the stirring phosphate solution with determined concentration for 5 min. The electrode was rinsed and moved to the electrochemical cell containing 0.5 mM [Fe(CN)\(_6\)]\(^{3–}\) in acetate buffer solution (ABS) 0.1 M, pH 5, and the potential of the electrode was scanned at 20 mV s\(^{-1}\) in the range of 0.6 to \(-0.3\) V.

**Apparatuses**

A Metrohm/Eco Chemie Autolab Potentiostat/Galvanostat30 was used for electrochemical measurements on a conventional three-electrode glass cell containing a Pt electrode as auxiliary, Ag/AgCl (3 M KCl) as a reference and pencil graphite as working electrodes. A D8-ADVANCE diffractometer (Bruker AXS, Germany) was used for X-ray diffraction (XRD) analysis using Cu K\(_\alpha\) radiation with a secondary graphite monochromator. A HITACHI 54160 was used for scanning electron microscopy (SEM) of the electrode.

**Results and Discussion**

**Characterization of Zr/rGO-PGE**

Modification of the PGE by graphene oxide was performed via scanning the potential of the bare PGE at 20 mV s\(^{-1}\) in 10 cycles, applied potential at 20 mV s\(^{-1}\) from 0 to \(-1.0\) V into GO suspension (1 mg mL\(^{-1}\), pH 9) for 10 cycles. The related cyclic voltammograms are shown in Fig. 1.

A distinct reduction peak at a potential about \(-0.7\) V was observed. Since the solution was first deaerated by purging argon gas (99.99\%) for about 15 min this peak can not be due to the oxygen reduction on the electrode surface. Besides, in the same blank experiment in which the GO did not exist in the electrochemical cell, no similar peak was observed in the studied potential range. Therefore, the observed peak may be ascribed to the reduction of the oxygen groups such as hydroxides, carboxylates, and epoxides that exist on the edges and surfaces of graphene oxide and deposition of reduced graphene oxide (rGO) on the electrode surface. It was noticeable when the broader potential range +1 V to \(-1.0\) V was applied, the peaks current increased and an oxidation peak around +0.65 V appeared (Fig. S1, Supporting Information). But our experimental results showed at this situation that rGO was not tightly deposited on the PGE. It was probably due to the reoxidation and resolution of the deposited rGO from the electrode surface at such positive potential. In order to study the structure of the deposited rGO on the PGE, the XRD technique was also applied (Fig. 2). At 2\(\theta = 10.4°\) the characteristic XRD peak of GO was observed. Using Bragg’s Law equation: \(n\lambda = 2d \sin \theta\), an interlayer space of 0.85 nm was obtained. This value is significantly larger than the reported value (0.335 nm) for graphite, due to the presence of oxygen containing functional groups on the edges and sheets of GO (Fig. 2A).

On the rGO-PGE, at 2\(\theta\) around 10° the peak height decreased significantly and at 2\(\theta\) around 25° a new broad peak appeared (Fig. 2B). According to the literature, the observed new peak corresponding to the hexagonal structure of...
resulted due to the electrochemical reduction of GO to graphene on the PGE. Again, it can be concluded that because of the partial reduction of GO on the PGE, the characteristic XRD peak of GO at 2θ around 10° did not disappear completely. This result was in good accordance with obtaining electrochemical results.

In the case of the Zr/rGO-PGE, the XRD pattern was changed significantly; the peak at 2θ around 10° was disappearing and the height of peak at 2θ around 25° was decreased significantly. This observation was probably due to alteration of the crystalline structure of rGO upon immobilization of zirconium ions. In fact, it can be concluded that rGO functional groups were tightly interacted with surface immobilized Zr ions, which was in accordance with those reported in the literature.

Scanning electron microscopy (SEM) was also applied to study the morphology of the rGO-PGE and Zr/rGO-PGE. The resulting SEM images are presented in Fig. 3. As expected, the rGO-PGE had layered and relatively smooth surface with clear boundaries of rGO layers (Fig. 3A). In the SEM image of Zr/rGO-PGE (Fig. 3B), the flat layers of rGO were strongly wrinkled, so that the surface immobilized rGO layered structure was demolished, significantly. This result was in good accordance with obtained XRD results.

The interfacial events induced by successive modification of the PGE were monitored by electrochemical impedance spectroscopy (EIS) in the presence of [Fe(CN)6]3– 0.5 mM as a redox probe. By comparing complex plane plots recorded on the PGE before (plot a) and after (plot b) immobilization of the rGO, the significant increase of charge transfer resistance on the rGO-PGE was made quite clear. The EIS data were approximated with several different equivalent circuit models to investigate the observed behavior. As a result, two constant phase element (CPE) model could explain the electrode behavior. A CPE is an equivalent electrical circuit component which models the behavior of a double layer, which is an imperfect capacitor. The observed two CPE model can be explained by assuming two different charge transfer pathways for the [Fe(CN)6]3– probe on the electrode surface. The smaller time constant (first CPE) originated due to the charge transfer through the reduced graphene oxide-electrode interface and the larger time constant (second CPE) due to the charge transfer through the graphene oxide-electrode interface. Again, this observation can be related to the partial reduction of GO on the PGE, as confirmed by CV and XRD techniques. The charge transfer resistance was decreased significantly after adsorption of Zr on the rGO-PGE. Adsorption of phosphate on the Zr/rGO-PGE was monitored after a 5-min incubation of the Zr/rGO-PGE in the solution containing 100 μM of phosphate. EIS complex plane plot showed that the charge transfer resistance increased significantly on the electrode exposed to the phosphate. Adsorption of phosphate on the Zr/rGO-PGE (recognition system) may prevent [Fe(CN)6]3– from approaching the surface and transferring the charge. The observed behavior can be used to design a new sensor for the electrochemical determination of phosphate.

Electrochemical determination of phosphate on Zr/rGO-PGE

In order to electrochemically determine phosphate, immersion solutions containing different concentrations of phosphate in the range of 1 – 250 μM were prepared. The Zr/rGO-PGE was immersed for 5 min in one solution, and after thorough rinsing, transferred to the electrochemical cell. The obtained cyclic voltammograms on the P/Zr/rGO-PGE in the presence of 0.5 mM [Fe(CN)6]3– redox probe are represented as Fig. 5. The results showed that by increasing the concentration of phosphate in the immersion solution, the peak currents decreased accordingly. A good linear calibration curve (R² = 0.99) was obtained between the currents of oxidation peaks and the logarithm of concentration of phosphate in immersion solutions in the range of 1 to 250 μM (Fig. 5, inset). The limit of detection of phosphate was estimated as: [1.12(± 0.09) μM] (S/N = 3).
For the quantitative determination of phosphate, differential pulse voltammetry under the optimized experimental conditions in ABS, pH 5, containing 0.1 M KCl and 0.5 mM \([\text{Fe(CN)}_6]^{3–}\) was employed. Figure 6A shows differential pulse voltammograms of the P/Zr/rGO-PGE. A good linear calibration curve (\(R^2 = 0.99\)) was obtained between the peaks current and the concentration of phosphate in the range of 0.01 to 1 μM as the following equation (Fig. 6B):

\[
I_p/\mu A = -70.5(\pm 0.1)\text{[phosphate]}/\mu M + 93.4(\pm 0.2)/\mu A,
\]

\(R^2 = 0.99\) (1)

Error bars indicated standard deviations obtained for three independent analyses at each concentration of phosphate. Limit of detection and the concentration sensitivity of phosphate were estimated from Eq. (1) as: \([0.011(\pm 0.004) \mu M] (S/N = 3) \text{ and } [622.4(\pm 9.6) \mu A \mu M^{-1} \text{ cm}^{-2}]\) by assuming \(A = 0.15 \text{ cm}^{-2}\), respectively.

Although in our previous report phosphate was determined on the Au-MPA-Zr(IV)SAM electrode with almost the same mechanism, the present work had several advantages.

(i) The pencil graphite electrode was extraordinarily cheap and accessible and its modification by Zr/rGO was very simple and fast. While the gold electrode was very expensive, its pretreatment was time consuming, and the formation of the SAMs required precision and time.

(ii) Since during removal of the coordinated phosphate ions by the KOH solution some Zr ions were also removed from the electrode surface, regeneration of the Au-MPA-Zr(IV)SAM electrode was a two-step procedure (recovery/repairing). The Zr/rGO-PGE was reproducible, very simple, fast, and inexpensive, no further attempt was carried out to verify reusability of the electrode. In our previous work, it was seen that during removal of the coordinated phosphate ions by the KOH solution some Zr ions were also removed from the electrode surface. So, the regeneration of the electrode was a two-step procedure (recovery/repairing). The Zr/rGO-PGE was meant to be used once and then discarded as a disposable electrode.

The effect of some co-existing compounds on the response of the Zr/rGO-PGE for phosphate was investigated by DPV at the optimized condition. The results showed that the peak current for 1.0 μM phosphate was not affected significantly by the presence of 10-fold of dopamine, ascorbic acid, uric acid, Cl–, NO3–, and ClO4–. Citrate, carbonate and sulfate had some interference effects (Fig. S2, Supporting Information).

Real sample analysis
Real samples were the serum of volunteer blood donations, which were obtained from the local blood transfusion organization. There are a lot of compounds in the serum sample with a tendency for adsorption on the surface immobilized Zr ions. Our preliminary results showed that without dilution of the serum samples, no confident and reproducible results could be obtained. Moreover, the detection limit of the sensor was about 5 orders of magnitude greater than the phosphate concentration range in the human serum. Therefore, in order to minimize the effects of interference with other coexisting species, the serum sample was diluted 1000 times by the addition of 0.1 M ABS solution. In typical procedure, five 50-mL volumetric flasks were each filled with 10 mL of the

Fig. 5 Cyclic voltammograms obtained in ABS, pH 5, containing 0.1 M KCl and 0.5 mM \([\text{Fe(CN)}_6]^{3–}\) on P/Zr/rGO-PGE after immersion of Zr/rGO-PGE in immersion solutions containing phosphate in the range of 1 – 250 μM. Inset shows linear calibration curve on a logarithmic scale.

Fig. 6 Differential pulse voltammograms obtained in ABS, pH 5, containing 0.1 M KCl and 0.5 mM \([\text{Fe(CN)}_6]^{3–}\) on P/Zr/rGO-PGE after immersion of Zr/rGO-PGE in immersion solutions containing phosphate in the range of 0.01 – 1.0 μM. Inset shows linear calibration curve.
diluted serum samples. Then the standard (5 μM phosphate) was added in differing amounts, such as 0, 5, 10, 15, and 20 mL. The flasks were filled to the mark and mixed well. The differential pulse voltammograms of the P/Zr/rGO-PGE were recorded for each sample three times. The average values of three peaks current were used for standard curve drawing. An average value of 1.4 μM phosphate in the 1000-times diluted serum samples was obtained. The obtained result, 1.4 mM phosphate in the undiluted serum samples, was in the range of 0.8 to 1.45 mM phosphate for the same samples reported by spectroscopic methods.12

Conclusions

In the present work an electrochemical phosphate sensor based on a pencil graphite electrode (PGE) modified with zirconium and reduced graphene oxide (Zr/rGO-PGE) was developed. The XRD, CV and EIS confirmed that GO was partially reduced on the PGE. The rGO-PGE showed high activity toward zirconium adsorption (Zr/rGO-PGE). The prepared electrode was used for the electrochemical determination of phosphate. Using differential pulse voltammetry the limit of detection of [0.011 (± 0.004) μM] (S/N = 3) and concentration sensitivity of [622.4 (± 9.6) μA μM⁻¹ cm⁻²] for phosphate were obtained in the optimized conditions. Phosphate concentration in human blood serum samples was determined as 1.4 mM which was comparable with those measured by traditional spectrophotometric methods. All in all, it should be said that the preparation of Zr/rGO-PGE was exceptionally fast, simple, inexpensive and reproducible, with a low limit of detection and high sensitivity for phosphate, which made it a good candidate for practical usage.

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Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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