Polyaniline as a Sacrificing Template for the Synthesis of Ultra-small Co3O4 Nanoparticles for the Sensitive and Selective Detection of Methotrexate (MTX)

Irum Naz Qureshi
University of Sindh

Aneela Tahira
University of Sindh

Khoulwod Aljadoa
King Saud University

Ali M. Alsalme
King Saud University

Asma A. Al-Othman
King Saud University

Ayman Nafady
King Saud University

Amal Kasry
The British University in Egypt

Zafar Hussain Ibupoto (✉ zaffar.ibhupoto@usindh.edu.pk)
University of Sindh  https://orcid.org/0000-0002-6756-9862

Research Article

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Abstract

The successful monitoring of the anticancer drugs using nanostructured materials is very important but very challenging task. Beside this, uniform and ultra-small size of metal oxide nanoparticles is highly needed in order to enhance the catalytic activity which could result into the development of sensitive and selective electrochemical sensors for methotrexate (MTX). For this purpose, we have used a simple approach involving the polyaniline (PANI) as a sacrificing template for the growth of uniform and ultra-small Co$_3$O$_4$ nanoparticles by hydrothermal method. The structure, shape, composition and phase purity were studied by scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), X-ray diffraction (XRD) and Fourier transform Infrared (FTIR) techniques. The average size of Co$_3$O$_4$ nanoparticles was below 50 nm. The cubic crystallography is confirmed for the Co$_3$O$_4$ nanoparticles. The electrochemical properties of PANI assisted Co$_3$O$_4$ nanoparticles for MTX drug was evaluated by cyclic voltammetry (CV) and linear sweep voltammetry (LSV) in Britton–Robinson buffer (BRB) of pH 3.5. The PANI assisted Co$_3$O$_4$ nanoparticles were found highly sensitive for the MTX drug and exhibited a linear range from 5-75µM of MTX and limit of detection for the modified electrode was estimated 1.98µM. The proposed electrochemical sensor is low cost, simple, highly sensitive and selective towards MTX detection. The synthetic methodology using the conducting polymer as a sacrificing template for the growth of controlled and ultra-small Co$_3$O$_4$ nanoparticles can be utilized for the wide range of electrochemical applications.

1. Introduction

Cancer disease has been remained chronic issue since decades and it causes many deaths annually. The critical handling of this problem resulted the synthesis of several expensive drugs and diagnosis devices. Methotrexate (MTX) is derived from the folic acid antagonist and has been used widely in chemotherapy as a cure for various cancer diseases including leukemia, breast cancer, lung cancer, osteosarcoma, and lymphoma. MTX is also used as cure for the autoimmune diseases like rheumatoid arthritis, psoriasis and Crohn,s illness [1–3]. MTX is also effective for the head and neck cancer. MTX has potential to inhibit the cell development via bonding to dihydrofolate reductase to eliminate the reduction of dihydrofolate to tetrahydrofolate [4]. On other side, MTX has severe side effects like other chemo drugs [5, 6]. It has been found that the MTX value above 10 µM in plasma is highly dangerous for the time of 10 h [7]. The high level of MTX in the blood decreases the development of noncancerous cells and causes heart stroke, liver and poisoning effect to lungs and ulcer of stomach [8]. Hence it is very vital to control on the use of MTX during the chemotherapy and should be monitored by low cost and quick analytical methods with high accuracy in order to improve effectiveness of drug and its safety. In the recent past, the designing a facile, fast, and very sensitive analytical method for the determination of MTX has received considerable attention. Currently, the analytical techniques such as capillary electrophoresis [9, 0], high performance liquid chromatography [10] and fluorescence spectroscopy [11–13] have been found highly accurate for the determination of MTX from biological samples. These standard analytical methods are costly and require multistep for the sample preparation which restricted their clinical applicability. The
electroanalytical methods have more preference due to their excellent sensitivity, selectivity, facile features, inexpensive nature, quick in response and very suitable for on spot detection. For this purpose, various modified electrodes have been fabricated for the electroanalysis of MTX [14–26]. At the present, the scientists and researchers are using variety of compounds in electroanalytical methods and investigating their activity in different conditions such as clinical samples. The use of multiple semiconducting nanostructures for the modification of conducting electrodes has enabled them to quantify biomolecules and drugs sensitively compared to the conventional electrodes [27]. Among the metal oxide nanostructured materials, cobalt oxide (Co$_3$O$_4$) with different shapes have been investigated in different electrochemical applications such as batteries, supercapacitors, and electrocatalysis [28]. Due to unique and attractive properties of Co$_3$O$_4$ such as high theoretical capacity, catalytic activity, excellent thermal and chemical stability [28]. However, Co$_3$O$_4$ nanostructures are experiencing poor electrical conductivity which adversely brings the unfavorable charge transfer kinetics and poor specific capacitance than the expected value. To uplift the electrical conductivity, different effective strategies have been used in producing the Co$_3$O$_4$ composites with conducting polymers, metal oxides and carbon [29–32]. The Co$_3$O$_4$ composite material offers unique architecture and enhanced electrical conducting for the determination of pharmaceutical products. The polyaniline (PANI) is low cost, conducting polymer and it can be produced into nano size, thus it is among the appealing conducting polymers [33]. The use of nanostructured PANI is increasing in the recent time in the field of electrochemical sensors due to its attractive physical and chemical characteristics compared to its bulk phase. The properties like flexible nature, high porosity, large surface to volume ratio and swift charge transfer kinetics are obvious for the PANI [34, 35]. The significant redox catalytic activity of Co$_3$O$_4$ has been exploited for the oxidation of pharmaceutical products [36, 37]. The Co$_3$O$_4$ has been used with PANI and its electron transfer properties were enhanced [38]. The nanostructured PANI has been prepared by the different methods [39–42]. The use of PANI as sacrificing agent for the synthesis of controlled, homogenous, and ultra-small Co$_3$O$_4$ nanoparticles is rarely investigated. The PANI as a sacrificing template provides the excellent dispersion of Co$_3$O$_4$ due to excess of carbon in the prepared sample in water and it also gives a large number of oxygen defects in the composition. Therefore, it fosters the development of sensitive and selective electrochemical sensors for the detection of biomolecules and pharmaceutical products.

In this study, three steps methodology was used to prepare the ultra-small nanoparticles of Co$_3$O$_4$ using PANI as sacrificing template. The structure and composition of prepared Co$_3$O$_4$ material was studied by SEM, EDS, XRD and FTIR techniques. The Co$_3$O$_4$ nanoparticles were used to modify the glassy carbon electrode (GCE) for the electrochemical determination of MTX in BRB buffer of pH 3.5. The modified electrode has sensitively and selectively quantified the MTX drug with a linear range of 5–75µM and limit of detection of 1.98µM. The successful application of modified electrode for the detection of MTX from blood plasma is also reported.

2. Experimental Section
Cobalt chloride hexahydrate (99%), aniline, urea (99%), glucose (99%), sodium chloride (99%), sucrose, phosphoric acid, acetic acid, potassium chloride (99%) and 5% Naon (E.W. 1100) were of analytical grade and received from Sigma Aldrich Karachi Pakistan. All the solutions were prepared in the deionized water. A 0.01MBRB buffer solution of pH 3.5 was used as supporting electrolyte. The stock solution of MTX was prepared in BRB buffer solution of pH 3.5.

2.2. Growth of ultra-small Co$_3$O$_4$ nanoparticles using PANI as sacrificial template

Hydrothermal method was followed for the growth of ultra-small Co$_3$O$_4$ nanoparticles using PANI as sacrificial template. First, PANI was prepared by oxidative polymerization of aniline using ammonium sulfate as an oxidizing agent. The purpose of using PANI as sacrificial agent was to control on the morphology and uniformity of Co$_3$O$_4$ nanoparticles. Also, the carbon could be added into the ultra-small Co$_3$O$_4$ nanoparticles which can improve the dispersion of ultra-small Co$_3$O$_4$ nanoparticles in water during the preparation of catalyst slurry. Then 1 g of PANI was used as support for the deposition of cobalt hydroxide nanostructures using (0.1M) equi-molar solution of cobalt chloride hexahydrate and urea. The growth solution was covered with aluminum sheet very tightly. The hydrothermal process was performed at 95 °C for 5 h. Then nanostructured material was collected on the ordinary filter paper and left to dry for overnight. Afterwards, the calcination was carried out at 500 °C in air for 4 h in order to complete the transformation of hydroxide phase into oxide and to remove the PANI from the surface of ultra-small Co$_3$O$_4$ nanoparticles. The pristine Co$_3$O$_4$ nanostructures were synthesized by the same method without the use of PANI as a sacrificial agent.

The morphology, composition, and phase purity of synthesized Co$_3$O$_4$ were investigated via SEM attached with EDS at 20 kV. The XRD was applied to measure the crystalline features and phase of prepared nanomaterials using measurement conditions of X-ray source from CuKα radiation (λ = 1.5418 Å), 45 kV and 45 mA. The chemical interaction of Co$_3$O$_4$ with PANI was also studied by FTIR analysis.

2.3. Electroanalysis of methotrexate (MTX) using ultra-small Co$_3$O$_4$ nanoparticles

The suspension of Co$_3$O$_4$ nanoparticles was prepared by dispersing 2 mg in 2 mL deionized water and 50µL of 5% Nafion and homogenous suspension was obtained in ultrasonic bath for 1h. Before the modification of GCE, the polishing was used to clean the surface of GCE using silicon paper and alumina paste, followed by washing with deionized water and drying in air at room temperature. Then, 5 µL of the suspension of Co$_3$O$_4$ nanoparticles was deposited on the surface of GCE and dried at 50 °C in electric oven for 5 minutes. The electrochemical cell system was consisting Co$_3$O$_4$ nanoparticles modified GCE as working electrode, silver-silver chloride (Ag/AgCl) as reference electrode and platinum as counter electrode. The BRB buffer of pH 3.5 was used as a supporting electrolyte. The stock solution of desired
concentration of MTX was prepared in BRB buffer solution. The CV curves were measured at 60 mV/s except scan rate study. The LSV was performed at a scan rate of 10 mV/s.

3. Results And Discussion

3.1. Morphology, crystalline structure and composition characterization of Co₃O₄ nanoparticles

The morphology of Co₃O₄ without and with PANI was studied by SEM as shown in Fig. 1. It could be seen that the pristine Co₃O₄ exhibits a heterogeneous nanostructured like elongated nanoparticles and long rods in the structure. The dimension of elongated nanoparticles could be more than 100 nm as shown in Fig. 1a. However, the use of PANI as sacrificing template has controlled the morphology Co₃O₄ with homogeneous distribution and ultra-small nanoparticles of Co₃O₄ were obtained. The average size of ultra-small Co₃O₄ nanoparticles was measured below 50 nm as shown in Fig. 1c. It is clear from this study that the small particle size of Co₃O₄ shows the large surface area and consequently superior electrocatalytic performance towards the oxidation of MTX was observed. The chemical composition of Co₃O₄ without and with PANI was explored by the EDS analysis as shown in Fig. 1b. The Co₃O₄ sample obtained in the presence of PANI as sacrificing template possess less oxygen concentration compared to the pristine Co₃O₄, indicating oxygen vacancies as shown in Fig. 1d. They have shown a dynamic role in the generation of strong electrical signal during the oxidation of MTX in BRB buffer solution of pH 3.5.

Figure 2a shows the diffraction patterns of pristine Co₃O₄ including 111,220,311, 222,400,422,511, 531,533, and 622 at 2theta degree 18.93°, 31.16°, 36.72°, 38.42°, 44.65°, 55.46°, 59.14°, 68.37°, 77.04°, and 78.10° respectively. Similarly, the XRD diffraction patterns were measured for the Co₃O₄ sample obtained with PANI as sacrificing template and they were noticed for the crystal planes such as 111, 220, 311, 222, 400, 422, 511, 531, 533, and 622 at 2theta degree 18.91°, 31.13°, 36.68°, 38.38°, 44.61°, 55.40°, 59.08°, 68.30°, 76.97°, and 78.01° respectively. All the observed crystallographic patterns are well matched with the reference (card no: 01-080-1542). This indicates that the complete combustion of PANI at 500 °C in air and only leaving behind the diffraction patterns of Co₃O₄. It was also found that the addition of PANI suppressed the crystal planes towards lower angle which might be assigned to steric effect offered by the long chains of PANI on the growth process of Co₃O₄. From XRD analysis, there was not any other phase or impurity present in the PANI assisted Co₃O₄ sample. From SEM and XRD analysis, it is found that the PANI as sacrificing template has suppressed the crystallite size which is further verified by SEM analysis.

Furthermore, the FTIR study was carried out for Co₃O₄ with and without PANI as a sacrificing template as shown in Fig. 2b. The observed bands at 574 and 676 cm⁻¹ in the FTIR spectrum are suggesting the typical characteristic features of spinal Co₃O₄ [43, 44]. The FTIR band at 1033 cm⁻¹ are assigned to the
stretching mode of C-O and CO$_3^{2-}$ negatively charge species which could be in the sample during the combustion and growth process of Co$_3$O$_4$ [45]. The measured band at 1424 cm$^{-1}$ is corresponded to the stretching mode of nitrogen [45]. In the case of PANI with Co$_3$O$_4$, the peak at 1662 cm$^{-1}$ is attributed from a minor impurity introduced by the quinoid ring and N-H stretching band of PANI [46, 47, 48, 49]. FTIR study reveals that the PANI was completely burnt thus we could not observe any typical FTIR band for PANI. However, a small variation in the band position of Co$_3$O$_3$ sample with PANI suggests its role towards the control on the morphology during the growth process of Co$_3$O$_4$ [50].

3.2. The electroanalysis of Co$_3$O$_4$ nanoparticles towards MTX drug

Figure 3 shows the CV curves of bare GCE, modified GCE with pristine Co$_3$O$_4$ and PANI assisted Co$_3$O$_4$ at a scan rate of 60 mV/s in the absence and presence of 20µM MTX concentration in BRB buffer solution of pH 3.5. It can be visualized that the bare GCE showed no activity in buffer solution and pristine Co$_3$O$_4$ modified GCE has also shown only the change in the current intensity but no obvious redox peaks even in the presence of 20µM MTX. It indicates that neither bare GCE nor pristine Co$_3$O$_4$ are the good electrode materials for the oxidation of MTX. However, the PANI assisted Co$_3$O$_4$ samples has shown a very slight oxidation peak in the BRB buffer solution and a prominent oxidation peak in 20µM MTX concentration was measured as shown in Fig. 3. The oxidation peak for the MTX was found at 1.02 V versus Ag/AgCl. From this electroanalysis, we see a dynamic role of PANI as a sacrificing template towards the growth process of Co$_3$O$_4$ which has not only controlled on the morphology and composition but also has demonstrated well deserving electrochemical properties for the sensing of MTX drug. These findings suggest that the capability of PANI assisted Co$_3$O$_4$ nanostructures to determine the MTX in acidic conditions and the surface of metal oxide nanoparticles is associated with the adsorbed hydroxyl groups. These adsorbed hydroxyl groups on the PANI assisted Co$_3$O$_4$ nanostructures caused an electrostatic interaction between the PANI assisted Co$_3$O$_4$ nanoparticles and carboxylic groups carried by the MTX itself [51, 52]. Therefore, the PANI assisted Co$_3$O$_4$ nanostructures have been used for the modification of GCE for all other measurements.

The influence of scan rate on the oxidation peak current was investigated by CV using PANI assisted Co$_3$O$_4$ nanostructures in 20µM MTX concentration as shown in Fig. 4a. The oxidation peak current was irreversibly increased with increasing scan rate and revealing an excellent electrochemical properties of the modified GCE. A linear fitting of oxidation peak current versus square root of scan rate is shown in Fig. 4b, which characterized the diffusion controlled kinetics of PANI assisted Co$_3$O$_4$ nanostructures towards MTX. To observe the applicability of PANI assisted Co$_3$O$_4$ nanostructures for the sensitive detection of MTX, a concentration window was built ranging from 5–75µM and was tested in BRB buffer solution of pH 3.5 using CV curves as shown in Fig. 5a. The CV curves have demonstrated a successive increase in the oxidation peak current with increase in the MTX concentration. A linear plotting was made between the oxidation peak current and MTX concentration for PANI assisted Co$_3$O$_4$ nanostructures as
shown in Fig. 5b. This analysis illustrated an acceptable linear range within 5–75µM MTX concentrations. The limit of detection of the PANI assisted Co$_3$O$_4$ nanostructures was found 1.98 µM and limit of quantification was estimated 6.6µM which were calculated according to the published work [53].

The LSV was also used to develop a calibration plot of MTX drug using PANI assisted Co$_3$O$_4$ nanostructures as shown in Fig. 6a. It can be seen that the oxidation peak current was linearly increased with addition of higher concentration of MTX. A linear plot was constructed between the peak current and various MTX concentrations as shown in Fig. 6b. It indicates a well-established linear range for the PANI assisted Co$_3$O$_4$ nanostructures from 20–70µM of MTX. The limit of detection was obtained 1.18µM and limit of quantification was measured 4µM. To evaluate the selectivity of PANI assisted Co$_3$O$_4$ nanostructures towards MTX, interference study was carried out in 20µM MTX concentration and the same concentration of potential interfering species was used. The CV curves were measured and the change in the peak current was noticed for each interfering substance and correlated with the peak current of MTX drug. The measured change in the electrical signal is listed in the Table 1 which suggests that the tested interfering substances such as paclitaxel, mitoxantrone, glucose, sucrose, urea and chloride ions has shown negligible effect on the peak current. This demonstrates the significant selectivity of PANI assisted Co$_3$O$_4$ nanostructures towards the sensing of MTX drug.

The practicality of developed PANI assisted Co$_3$O$_4$ nanostructures was investigated for two tablets of MTX drug by standard addition and recovery methods and the obtained recovery results are enclosed in Table 2. This study suggests that PANI assisted Co$_3$O$_4$ nanostructures have high potential to measure MTX drug precisely. The PANI assisted Co$_3$O$_4$ nanostructures were successfully applied to the real blood sample. The blood sample was collected from the Liaquat University of Medical and Health Sciences Jamshoro Sindh Pakistan. The sample preparation was performed by centrifugation for 10 minutes at 12000 rpm. Then plasma was separated out and frozen at -10 °C. Afterwards, 100 µL of plasma was added to the 100 µL of methanol, for 30 s and centrifuged for the 10 minutes. After a careful centrifugation supernatant was obtained and used for the electroanalysis and the measured results are given in Table 3. The measured MTX concentration was 0.01µM without the addition of MTX and recovery mhid was also used for the detection of MTX from blood plasma and observed results are satisfactory. The biological matrixes in the blood plasma did not interfere with electrical signal of PANI assisted Co$_3$O$_4$ nanostructures, thus the presented protocol has high ability to monitor the MTX drug from real samples.

4. Conclusions

In summary, PANI was used as sacrificial template for the uniform growth of ultra-small Co$_3$O$_4$ nanoparticles by hydrothermal method. The ultra-small Co$_3$O$_4$ nanoparticles were physically characterized by SEM, EDS, XRD and FTIR techniques. The Co$_3$O$_4$ nanoparticles exhibit acceptable oxygen vacancies which played a dynamic role in lifting the electrochemical properties of Co$_3$O$_4$ for the oxidation of MTX in BRB buffer of pH 3.5. The PANI assisted Co$_3$O$_4$ nanostructures have shown a
significant linear range from 5–75µM for MTX drug with limit of detection of 1.98µM. The PANI assisted Co₃O₄ nanostructures were used practically for the analysis of MTX from real blood plasma sample. The modified electrode is low cost, and very sensitive for the determination of MTX. The proposed strategy involved the PANI as a sacrificing template for the growth of ultra-small Co₃O₄ nanoparticles which can be capitalized into different electrochemical applications.

**Declarations**

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# Tables

## Table I. Interference Studies

| Interfering reagent | Concentration (µM) | Signal Increase |
|---------------------|--------------------|-----------------|
| Paclitaxel          | 60                 | 2.54            |
| Mitoxantrone        | 60                 | -3.32           |
| Glucose             | 60                 | 0.2             |
| Sucrose             | 60                 | -2.9            |
| Urea                | 60                 | 1.3             |
| Chloride            | 60                 | 2.65            |

## Table II: Recovery results

| Tablet Number | Added µM | Found µM | % Recovery | RSD (%) |
|---------------|----------|----------|------------|---------|
| 1             | 30       | 31.01    | 103.36     | 3.23    |
|               | 40       | 39.62    | 99.05      | 0.98    |
|               | 50       | 49.99    | 99.98      | 1.34    |
| 2             | 30       | 30.09    | 100.3      | 1.45    |
|               | 40       | 40.07    | 100.7      | 0.59    |
|               | 50       | 50.07    | 100.7      | 1.59    |

## Table III: Real sample analysis

| Blood Plasma | Added µM | Found µM | % Recovery | RSD (%) |
|--------------|----------|----------|------------|---------|
| 1            | 00       | 0.01     | 103.36     | 1.23    |
|               | 30       | 29.02    | 96.73      | 3.98    |
|               | 35       | 37.06    | 105.88     | 4.34    |