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Development of electrochemical sensor for folic acid based on hydroxyapatite nanoparticles

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Abstract

We report the synthesis of hydroxyapatite (HA) nanoparticles (NPs) by a simple microwave irradiation method and its application as sensing element for the precise determination of folic acid (FA) by electrochemical method. The structure and composition of the HA NPs were characterized using XRD, FTIR, Raman and XPS. SEM and EDX studies confirmed the formation elongated spherical shaped HA NPs with an average particle size of about 34 nm. The HA NPs thin film on glassy carbon electrode (GCE) were deposited by drop casting method. Electrocatalytic behaviour of FA in the physiological pH 7.0 was investigated by cyclic voltammetry (CV), linear sweep voltammetry (LSV) and chronoamperometry. The fabricated HA/GCE exhibited a linear calibration plot over a wide FA concentration ranging from 1.0×10^{-7} to 3.5×10^{-4} M with a detection limit of 75 nM. In addition, the HA NPs modified GCE showed good selectivity towards the determination of FA even in the presence of a 100-fold excess of ascorbic acid (AA) and 1000-fold excess of other common interferents. The fabricated biosensor exhibits good sensitivity and stability, and was successfully applied for the determination of FA in pharmaceutical samples.

***Keywords*:** Hydroxyapatite, nanoparticles, Raman spectroscopy, folic acid, biosensor.

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1. Introduction

The development of reliable bio-functional materials is of great importance in medical, pharmaceutical and other relevant industrial fields. Many analysts focus their research on the development of new materials with good biocompatibility for improving the behaviour of biosensors. Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA), a bioceramic analogous to the mineral component of bone with great biocompatibility, bioactivity and particular multi-adsorbing sites, has attracted a lot of attention because of its extensive applications such as bone and tooth implants, absorbents, protein separation, immunosensor and biosensors [1]. Conventionally, HA bioceramic materials have been synthesized by adopting different approaches like dry process, wet process, hydrothermal, sonochemical, microwave irradiation, etc.,. Microwave method is an environmentally friendly, non-polluting, clean and safe approach. The great potential offered by microwave irradiation process is the acceleration of chemical reaction. In the current study, the HA nanoparticles (NPs) were prepared by microwave irradiation method and the product was used as sensing material for the detection of folic acid in the presence of excessive amount of common interferent ascorbic acid (AA).

A water soluble vitamin folic acid (FA, N-[p-{[(2-amino-4-hydroxy-6-pteridinyl) methyl] amino} benzoyl]-l-glutamic acid, Vitamin M) is synthesized in nature by plants (green leaves, algae) and micro-organisms (yeast, bacteria). Determination of FA in the pharmaceutical, clinical and food samples has drawn significant attention due to the fact that lack of FA in diet is firmly related to the presence of neural tube defects in newborns and to an increased risk of cancer, megaloblastic anemia, cardiovascular disease, Alzheimer's disease and some psychiatric disorders [2]. Electroanalytical methods have attracted more attention in recent years for determination of biological compounds due to their sensitivity, accuracy, lower cost, and simplicity [3]. However, direct electrochemical reduction of FA is

kinetically slow and takes place at a relatively high reduction potential on common electrodes. Different inorganic and organic materials such as multi walled carbon nanotubes [4], ZrO_2 nanoparticles [5], nickel-poly(O-anisidine) [6], 5-amino-2-mercapto-1,3,4-thiadiazole [7] and molybdenum (VI) complex carbon nanotubes [8] etc., have been used to fabricate modified electrodes for the detection of FA. Here, we report the precise determination of FA by using nanostructured hydroxyapatite modified GCE for the first time.

The fabricated HA/GC electrode (HA/GCE) shows high electrochemical activity towards the oxidation of folic acid at 0.77 V in 0.1 M PBS (pH 7.0) in comparison with other folic acid sensors. The sensor was successfully applied for the study of pharmaceutical samples.

2. Experimental Procedure

2.1 Reagents

Folic acid (FA, $C_{19}H_{19}N_7O_6$) was obtained from Sisco Research Laboratory, Mumbai. Calcium nitrate ($Ca(NO_3)_2.4H_2O$) and diammonium hydrogen orthophosphate ($(NH_4)_2HPO_4$), were purchased from Merck, Mumbai. All the chemicals were of analytical reagent (AR) grade and used without further purification. 0.1 M phosphate buffer (PB) solution was prepared from Na_2HPO_4 and NaH_2PO_4 (Merck, Mumbai) and the pH was adjusted to 7.0 by adding 0.1 M HCl. The deionized water used in all experiments was produced by ultra purification system.

2.2 Preparation of hydroxyapatite (HA) nanoparticles

Hydroxyapatite nanoparticles were prepared by a simple microwave method. The typical synthesis process is as follows; 1 M $Ca(NO_3)_2.4H_2O$ solution was added drop wise into 0.6 M $(NH_4)_2HPO_4$ solution with vigorous stirring for 1 h at room temperature. During the reaction, the pH of the solution was maintained at 10 using ammonia solution. The obtained clear aqueous suspension was placed in a microwave oven (900 W) for 20 minutes.

Then the product was washed with deionised water and dried at 80 °C in a hot air oven. This process yielded white colour powder which was identified as phase pure HA.

2.3 Electrode preparation and modification

Prior to modification, the glassy carbon (GC) electrode (2 mm in diameter) was polished with 0.05 μm alumina powder on polishing cloth, rinsed thoroughly with doubly distilled water between each polishing step, sonicated in doubly distilled water, then dried at room temperature. The modified electrodes were prepared by a simple drop casting method. The HA NPs suspension was prepared by dispersing 5 mg HA in 1 mL water solvent with sufficient ultrasonication, until a relatively uniform suspension was obtained. The HA NPs suspension (10 μL) was coated on the surface of the pretreated GC electrode and the electrode was dried in air to form a HA nanoparticles modified GC (HA/GC) electrode.

2.4 Apparatus and measurements

The structure and morphology of HA were characterized by powder X-ray diffraction (XRD, Rigaku X-ray diffractometer with Cu Kα of 1.5406 Å), and field emission scanning electron microscopy with energy dispersive X-ray spectroscopy (FESEM-EDX, JSM-6700F, JEOL Ltd). Fourier transform infrared (FT-IR) spectra were recorded at room temperature with an FT-IR spectrometer (Thermo Scientific Systems, Nicolet-6700) using the KBr pellet technique. Raman spectra were recorded at room temperature using a Raman spectrometer (JASCO corporation, NR-1800, Rev. 1.00). X-ray photoelectron spectra were recorded at room temperature by using ESCA 3400 apparatus with Mg Kα (1253.6 eV) X-ray source.

Electrochemical measurements were carried out on CHI 608D electrochemical workstation (CH Instruments, Austin, USA). A three-electrode cell was used with a modified glassy carbon electrode (GCE, 3 mm dia) as the working electrode, Ag/AgCl (3 M KCl) as the reference electrode, and a platinum wire electrode as the counter electrode and all experiments were carried out at room temperature. Cyclic voltammograms (CVs) were

acquired from 0.2 V and 0.9 V at a scan rate of 50 mV/s in 0.1 M phosphate buffer containing 1.0 mM FA. Electrochemical impedance spectroscopy (EIS) measurements were made by applying ac potential of amplitude 5 mV over the dc potential of 250 mV in the frequency range 100 kHz to 1 Hz. The impedance data are presented in the form of Nyquist plots. The value of the charge transfer resistance (R_{ct}) was determined using Zsimpwin software simulations. The amperometric response of biosensor to folic acid was recorded in stirring (400 rpm) PB solution at +0.77 V.

2.5 Real sample preparation

Pharmaceutical products were selected for FA real sample analysis. Foligen (Biogen Pharmaceutical Co.) and folvite (Vee Excel Drugs and Pharmaceutical Ltd, Mumbai) were obtained from a local drugstore. One tablet (5.0 mg) was grinded and dissolved in 100 mL of deionized water, sonicated for 30 minutes and then filtered through Whatman 42 (125 mm Dia) filter paper which yielded a clear solution. The solution was diluted with phosphate buffer (pH 7.0) to the required concentration and amperometry was performed with standard addition method.

3. Results and discussion

3.1 Characterization of hydroxyapatite

Fig. 1 shows the XRD pattern of the synthesized HA powder. The observed planes with (h k l) indices (0 0 2), (2 1 0), (2 1 1), (1 1 2), (3 0), (2 2 2) and (2 1 3) exactly match with the powder diffraction file (ICDD PDF Card No. 09-0432 of hexagonal phase, space group $P6_3/m$) confirming the HA phase formation. There are no peaks corresponding to possible impurities, such as calcium hydroxide and other calcium phosphates. The results indicate that the phase pure HA could be prepared under the proposed experimental condition without requiring high temperature heating. The lattice constants and unit cell volume were calculated as a=b=9.420 Å, c=6.868 Å and V=527.77 $Å^3$, respectively. Average crystallite

size for the HA samples for all the crystallographic planes (h k l) were calculated by using Scherrer's equation (i.e. $D = K \lambda / \beta \cos \theta$, where K is the shape factor (0.9), β is the full width at half-maximum (fwhm) of diffraction peaks measured in radians, λ is the wavelength of the X-rays (λ=1.5406Å) and θ is the Bragg's diffraction angle). The average crystallite size of HA was estimated as 29 nm.

3.2 SEM and EDX analysis of HA

Fig. 2 shows SEM image and EDX spectrum of the HA nanoparticles. The HA NPs have elongated spherical morphology with the average particle size of about 34 nm. The EDX results show that the HA is primarily composed of Ca, P and adequate amount of O. There is no indication of any other impurities up to the resolution limit of the instrument. The Ca/P atomic ratio obtained from EDX results is 1.678 which is very close to the stoichiometric value of HA (Ca/P= 1.67).

3.3 FTIR and Raman analyses of HA

Fig. 3 shows the FTIR and Raman spectra of the HA nanoparticles. All the observed peaks correspond to functional groups of HA. Two adsorption bands located at 561 and 606 cm^{-1} are ascribed to the ν_4 bending mode of PO_4^{3-}. The stretching vibration band of OH^- is observed at 637 and 3570 cm^{-1}. The band at 960 cm^{-1} is also related to the ν_1 stretching mode of PO_4^{3-}. The characteristic bands observed at 1040 and 1098 cm^{-1} are assigned to the ν_3 stretching vibration of PO_4^{3-}. The peaks found at 3440 and 1640 cm^{-1} are attributed to the ν_2 bending mode of adsorbed water [9]. The carbonated peak appeared at 1380 is related to CO_3^{2-} group adsorbed from atmosphere during synthesis process which might have partially substituted the PO_4^{3-} groups of the HA [10].

The observed Raman wave numbers are in quite good agreement with the literature [11]. The spectral bands appear at ~433 and ~448 cm^{-1} correspond to the ν_2 bending vibrations of the PO_4^{3-} ion. The bands present at ~580, 592 and 611 cm^{-1} belong to the ν_4

fundamental vibrational mode and arise from the triply degenerate bending vibrations. The mode at 963 cm^{-1} has very high intensity, which is a specific characteristic of the Raman spectrum of HA and is attributed to the ν_1 mode of P-O-P symmetric stretching with free tetrahedral phosphate ion associating HA. The significant increase in the peak intensity shows an improved crystalline nature of hydroxyapatite NPs. The bands at 1055 and 1079 cm^{-1} corresponds to the P-O asymmetric stretching vibrations of the ν_3 vibrational mode.

3.5 XPS studies of hydroxyapatite

X-ray photoelectron spectra of HA nanoparticles is shown in Fig. 4. Absence of any peaks corresponding to traces of metallic and other impurities in the survey spectrum (Fig.4a) in general scan up to 1200 eV confirms the HA NPs to be pure and clean in nature. The major peaks found in the XPS spectra are attributed to Ca, O and P elements in HA (the presence of H cannot be directly detected in XPS). Fig. 4b-c shows the core level spectra of Ca 2p, P 2p and O 1s respectively. The Ca 2p core level presents the doublet bonds at a binding energy of 348.8 and 351.8 eV, which is attributed to $2p_{3/2}$ and $2p_{1/2}$ of Ca 2p orbit with a separation of 3.0 eV. Spectrum of P 2p bond at 134.8 eV results from the PO_4^{3-} group of HA, and the O 1s spectrum at 532.6 eV is assigned to the phosphate oxide.

3.6 Electrochemical impedance spectroscopy **(EIS) studies of HA/GCE**

Fig. 5 shows the impedance data obtained for bare GC and HA modified GC electrodes in $[Fe(CN)_6]^{3-/4-}$ as a redox couple containing 0.1 M KCl at scanning frequencies from 0.1 to 100 000 Hz. The charge transport process of the HA modified GC electrode was studied by monitoring charge transfer resistance (R_{ct}) at the electrode/electrolyte interface. The R_{ct} value was obtained by Randles equivalent circuit [R_S (Q_{CPE} (R_{ct} W)], where R_S is the solution resistance, W is the Warburg impedance and Q_{CPE} is the constant phase element (CPE). The R_{ct} values for bare GCE (curve a) and HA (curve b) modified GCE have been estimated as 188 and 1689 Ω/cm^2 respectively.

3.7 Electrochemical behaviour of the folic acid (FA) at HA/GCE

The electrochemical behaviour of folic acid at HA/GCE was investigated by cyclic voltammetry (CVs). Fig. 6A depicts the cyclic voltammograms for the electrochemical oxidation of 1mM folic acid at bare GCE (curve a) and HA/GCE (curve b) at a scan rate of 50 mVs^{-1} in pH 7.0. As can be seen, the peak potential of FA oxidation is 770 mV at HA/GCE (curve a) and 791 mV at bare GCE respectively. The observed negative shift in the peak potential with increase in peak current indicates that the HA/GCE has improved electrocatalytic activity towards oxidation of FA. Fig. 6B shows the CVs of HA modified GC electrode recorded with different scan rates (100-400 mV/s) in 0.1 M PB solution (pH 7.0) containing 1mM of FA. The inset of this figure shows the plot of peak current (I_p) versus the square root of the scan rate ($V^{1/2}$), in the range of 100-400 mV s^{-1} with correlation coefficients of 0.991. This plot was found to be linear, suggesting that the catalytic electrochemical oxidation of FA at HA/GCE is diffusion controlled. The HA NPs modified GC electrode did not show any oxidation peak in the absence of FA (Fig. 7A). Further, it involves two electron oxidation of FA to dehydrofolic acid at the HA NPs modified GC electrode and is represented as follows [12].

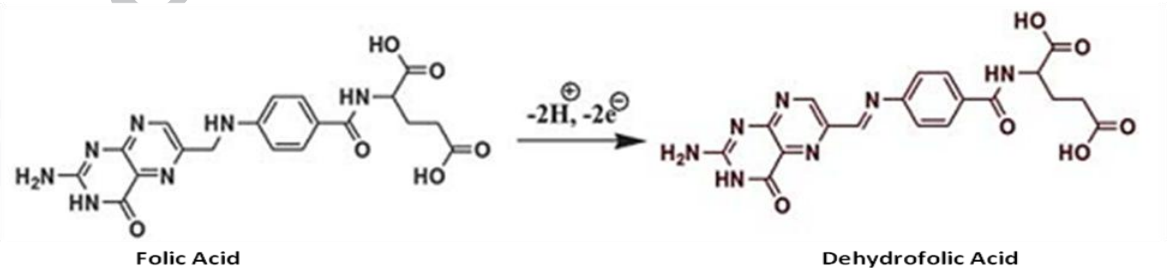

Fig. 7B shows the linear sweep voltammograms (LSVs) obtained for FA in the concentration range of 0-100 µM at the HA/GCE. The oxidation peak current of FA increased with the increase in FA concentration (each step with the increment of 10 µM).

Further it has a linear relationship with concentration of FA with a correlation coefficient of 0.998 (inset of Fig. 7B).

3.3 Amperometric determination of FA

Amperometric method was used to examine the sensitivity of HA/GCE towards the detection of FA. Fig. 8 shows the amperometric i-t curve for FA at HA/GCE electrode in a homogeneously stirred 0.1 M PB solution with the pH of 7.0 by applying a potential of +0.77 V. The modified electrode shows the initial current response due to 200 nM FA. The current response increases and a steady state current response was attained within 5 s for further addition of 200 nM FA in each case with the sample interval of 50 s. The current response of FA biosensor increases along with the increase of FA concentration and a linear response range is obtained from 1.0×10^{-7} to 3.5×10^{-4} M with a correlation coefficient of $R^2 = 0.999$ and the detection limit was found to be 75 nM. These results are compared with the recently reported ones for various chemically modified electrodes and are given in Table 1. The observed high sensitivity of HA NPs could be attributed to its large effective surface area coupled with improved electrocatalytic activity and high absorbability of HA.

3.4. Determination of FA in the presence of interferents

Ascorbic acid (AA) is a well known electroactive molecule that coexist in a biological system and its concentration is much higher than that of FA, and that can also be oxidized in most conventional solid electrodes. Therefore, it is important to investigate the concentration of FA in the presence of higher concentration of AA. Fig. 9A shows the differential pulse voltammetry (DPV) of HA/GCE obtained for the increment of 10 µM FA in the presence of 1000 µM AA in 0.1M PBS of pH 7.0. The concentration of FA was varied from 10 to 60 µM (curve a-f). A very clear signal was observed for 10 µM FA in the presence of 1000 µM AA, which revealed that the detection of very low concentration of FA is possible even in the presence of 100-fold AA. These results demonstrated that the HA/GCE electrode is more

selective towards FA. The influence of other interferents such as glucose, sucrose, Mg^{2+} and Ca^{2+} on FA was also investigated by amperometric method (Fig. 9B). No change in the amperometric current response was observed for 10 μM FA in the presence of 10 mM of glucose, sucrose, Mg^{2+} and Ca^{2+} indicating that the present modified electrode is highly selective towards the determination of FA even in the presence of 1000- fold excess of these interferents.

3.5 Stability and reproducibility of HA/GCE

The stability and reproducibility are two important parameters for the evaluation of the performance of a sensor. In order to investigate the stability of the HA modified GCE, the amperometry for 100 μM FA in 0.1M PB solution were recorded for every 10 min interval. It was found that oxidation peak current remained same with a relative standard deviation of 3.4 % for 10 repetitive measurements indicating that this electrode has a good reproducibility and does not undergo surface fouling. To further ascertain the reproducibility of the experimental results, five different HA modified electrodes were tested towards the oxidation of 100 μM FA. The peak currents obtained from the five independent electrodes showed relative standard deviations of 5.6 % for FA, confirming that the fabricated sensor is reproducible. These results clearly indicate that the fabricated FA sensor is very much stable and reproducible.

3.6 Analytical performance in real samples

The analytical applicability of the proposed electrochemical sensor was tested through the determination of FA in two different pharmaceutical tablets such as foligen and foliviate by using the standard addition method. The results (Table 2) show that the newly fabricated HA NPs based GC electrode is useful to measure folic acid content in pharmaceutical samples with good recovery rate.

4. Conclusion

We have successfully synthesized hydroxyapatite nanoparticles by a simple microwave irradiation method. Powder X- ray diffraction results proved that the HA belongs to hexagonal system. The FTIR and Raman studies confirmed the presence of functional groups in the HA. The phase purity of HA NPs have been confirmed through EDX and XPS studies. SEM observation revealed that the HA nanocrystals grew as well separated elongated spheres. HA NPs modified GCE was prepared by dropcasting method and the modified electrode exhibited improved electrocatalytic activity towards the oxidation of folic acid. The amperometric current response got increased linearly with increasing FA concentration over a wide range of 1.0×10^{-7} to 3.5×10^{-4}M and the lowest detection limit was found to be 75 nM. High sensitivity, selectivity and reproducibility of the amperometric responses, and very low detection limit, together with the ease of preparation, biocompatibility and surface regeneration, makes the proposed HA modified GC electrode to be very useful for accurate determination of FA. Further, this modified electrode is shown to be useful for the determination of FA in pharmaceutical samples.

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Figure captions

Fig. 1 XRD patterns of as synthesized HA nanoparticles.

Fig. 2 (A) SEM images of HA nanoparticles; (B) EDX spectrum of HA.

Fig. 3 (A) FTIR spectrum of HA; (B) Raman spectrum of HA.

Fig. 4 X-ray photoelectron spectra of HA (a) survey spectrum and core level spectra of (b) Ca 2p, (c) P 2p and (d) O 1s. Ca(LMM) and O(KVV) are Auger type peaks.

Fig. 5 EIS recorded in presence of 1 M KCl solution containing 1 mM $[Fe(CN)6]^{3-/4-}$; (a) bare GCE and (b) HA/ GCE.

Fig. 6 (A) CVs recorded at 50 mV/s for (a) bare GCE and (b) HA/GCE in PBS (0.1 M; pH 7.0) containing 1 mM FA; (B) CVs obtained for 1 mM FA at the HA/GCE in PBS (0.1 M; pH 7.0) at different scan rates (100-400 mV/s). Inset: oxidation current vs. square root of scan rate.

Fig. 7 (A) CVs recorded in the (a) absence and (b) presence of 1 mM FA in PBS (0.1 M; pH 7.0); (B) LSVs obtained for FA in the concentration ranging from 0 to 100 µM. FA was added in steps of 10 µM each at the HA/GCE in PBS (0.1 M; pH 7.0) at a scan rate of 50 mV/s. Inset shows the calibration plot.

Fig. 8 Amperometric response of the HA/GCE with successive additions of FA into PBS (0.1 M; pH 7.0) measured at + 0.77 V. Inset shows the calibration plot.

Fig. 9 (A) Differential pulse voltammograms (DPVs) for various concentrations of FA (from *a - f:* 10, 20, 30, 40, 50 and 60 µM respectively) in presence of 1000 µM AA recorded using HA/GCE in 0.1M PBS (pH 7.0) under amplitude = 0.05 V and pulse period 0.05 s; (B) Amperometric response of the HA/GCE for the addition of (a) 10 µM FA in the presence of various interferents such as 10 mM each (b) glucose (c) sucrose (d) Mg^{2+} and (e) Ca^{2+} in 0.1 M PBS (pH 7.0).

Table captions

Table 1 Comparison of different chemically modified electrodes for the determination of FA with HA/GCE

Table 2 Determination of FA in pharmaceutical sample using nano-HA/GCE

Fig. 1

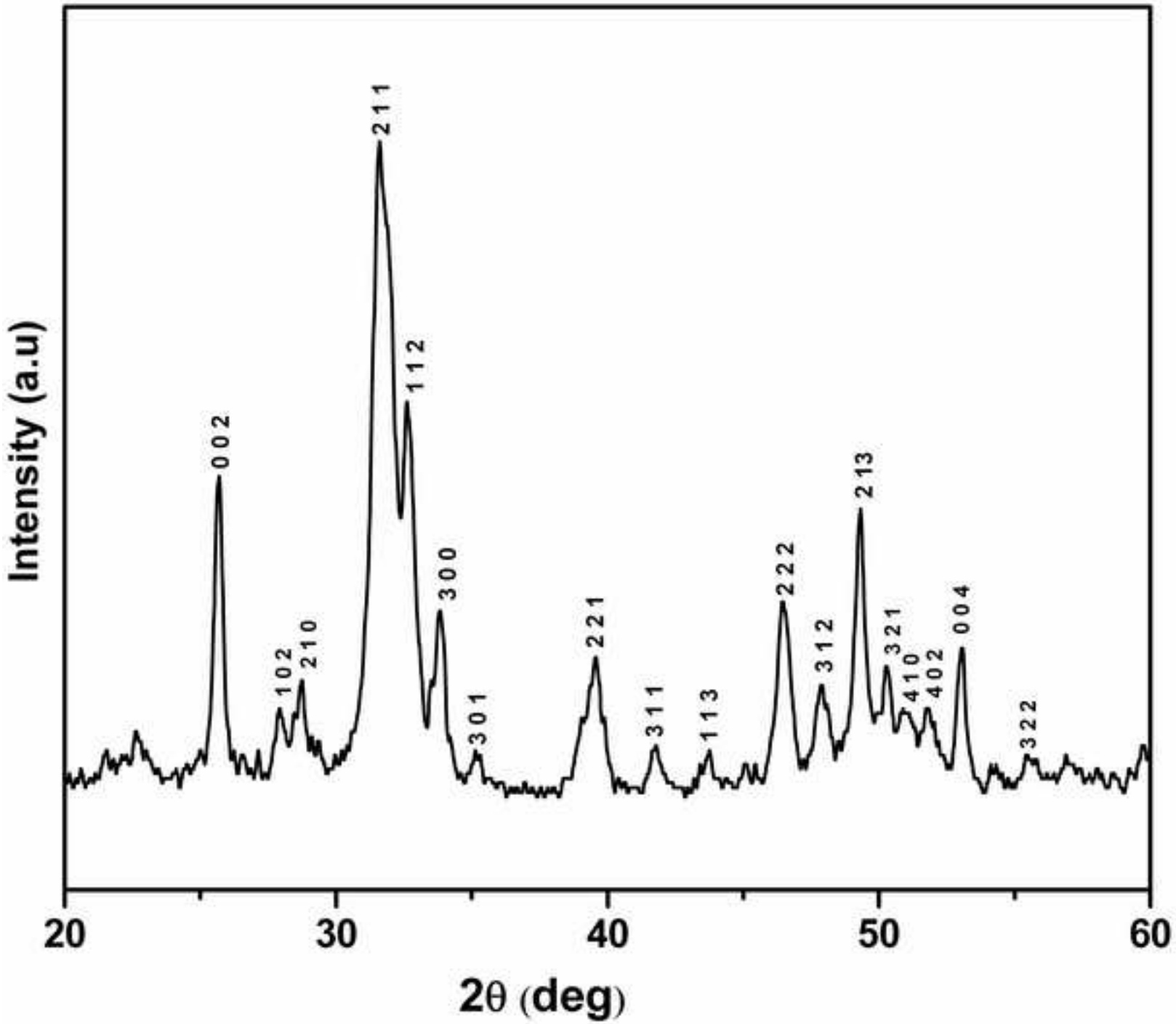
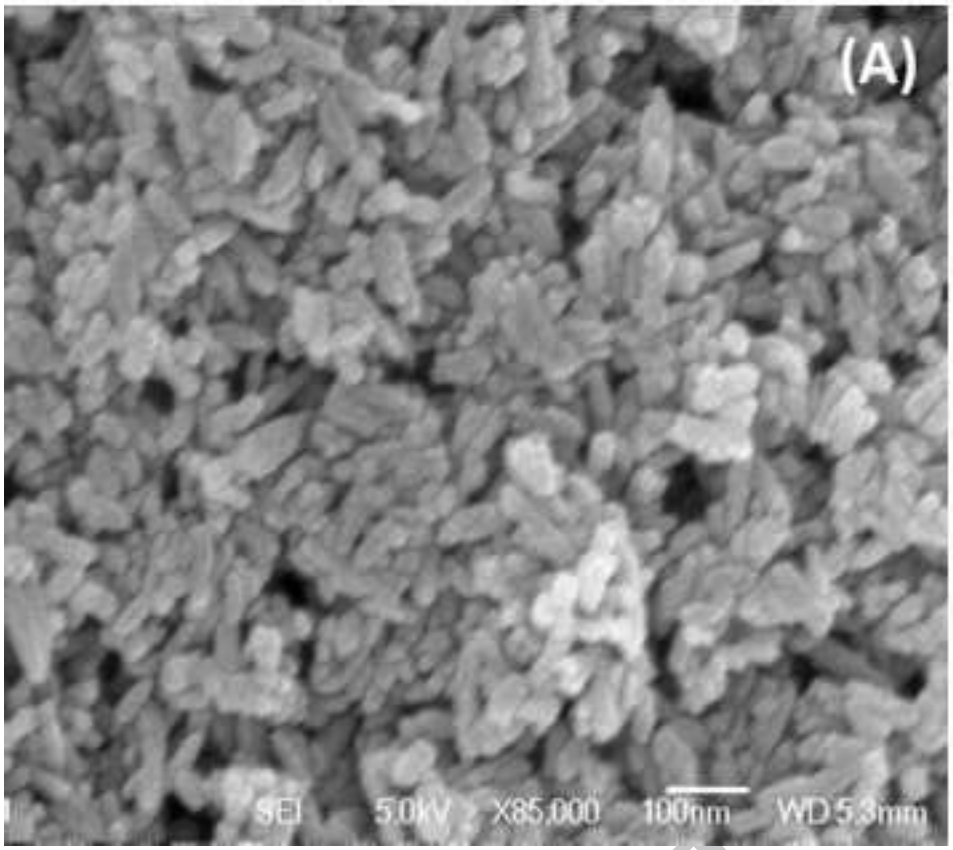


Fig. 2



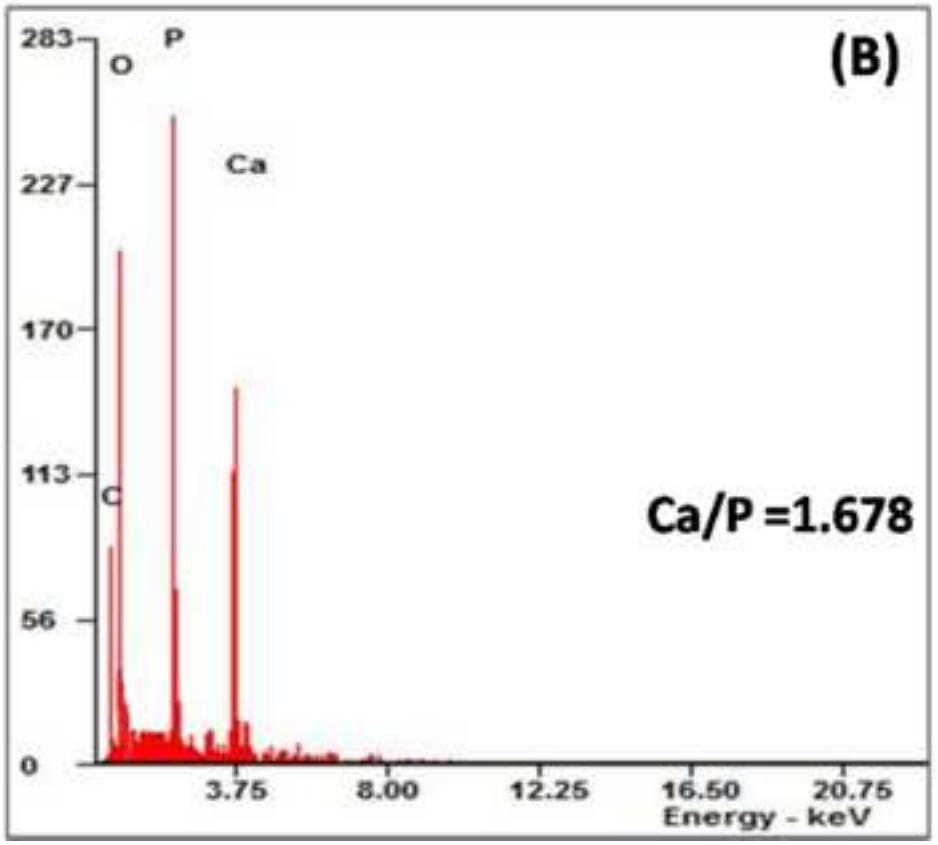


Fig. 3

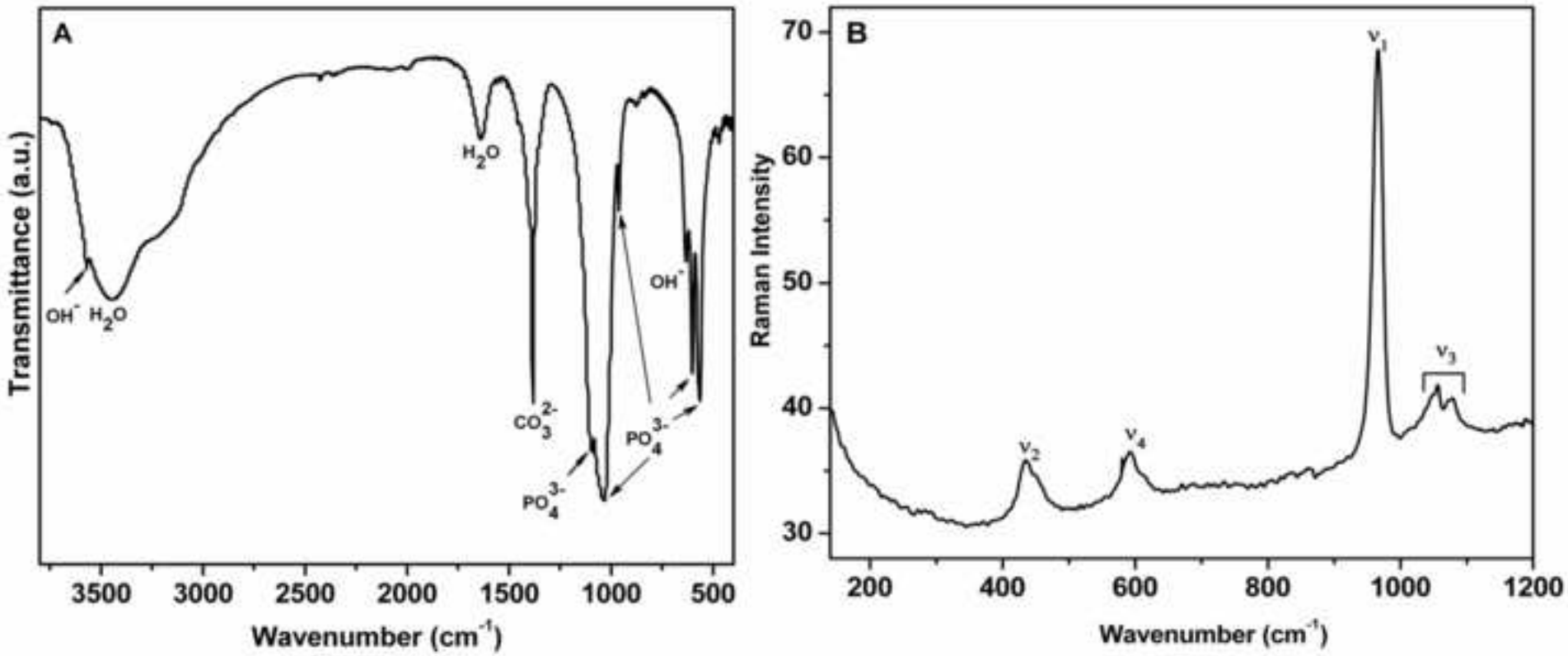


Fig. 4

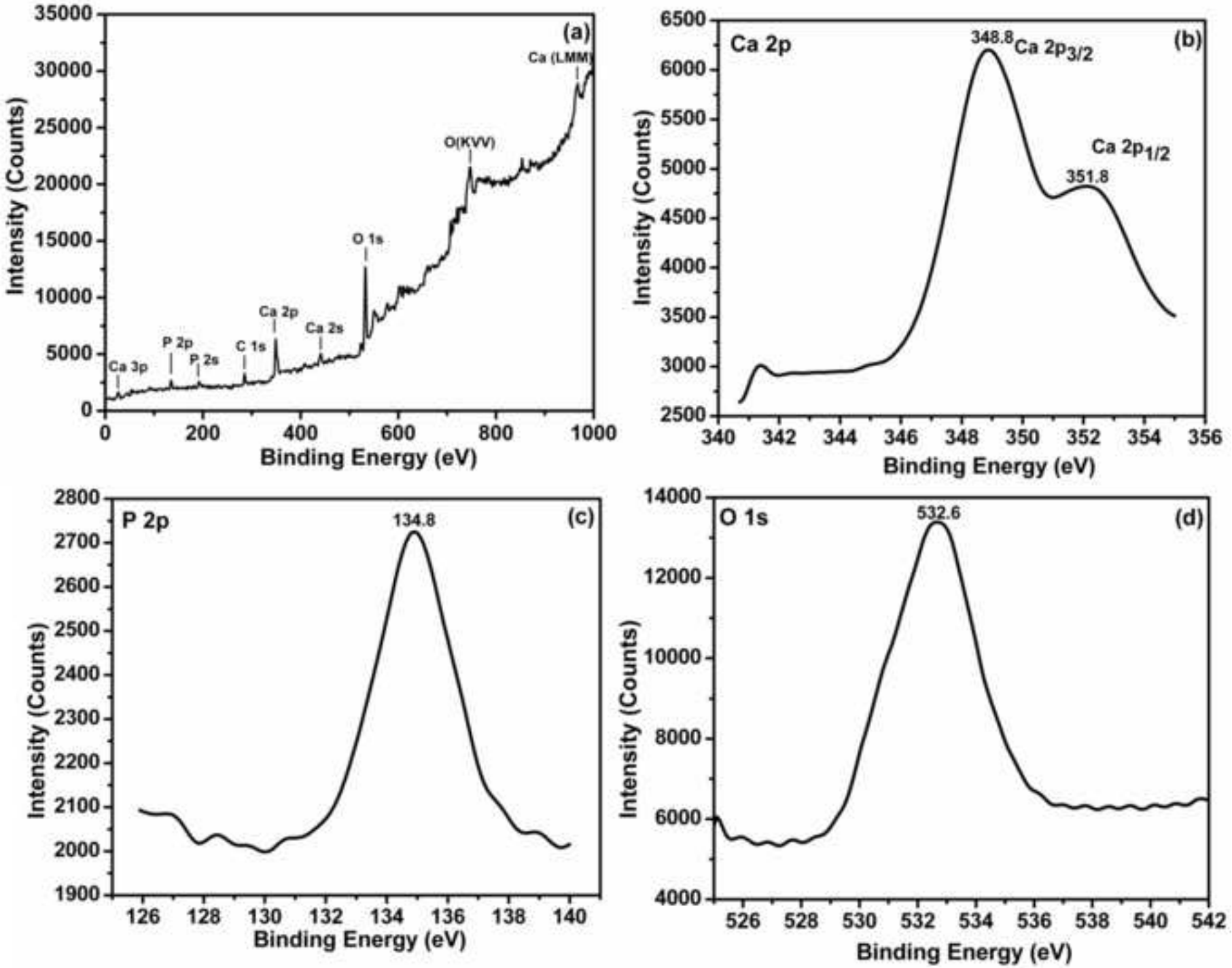


Fig. 5

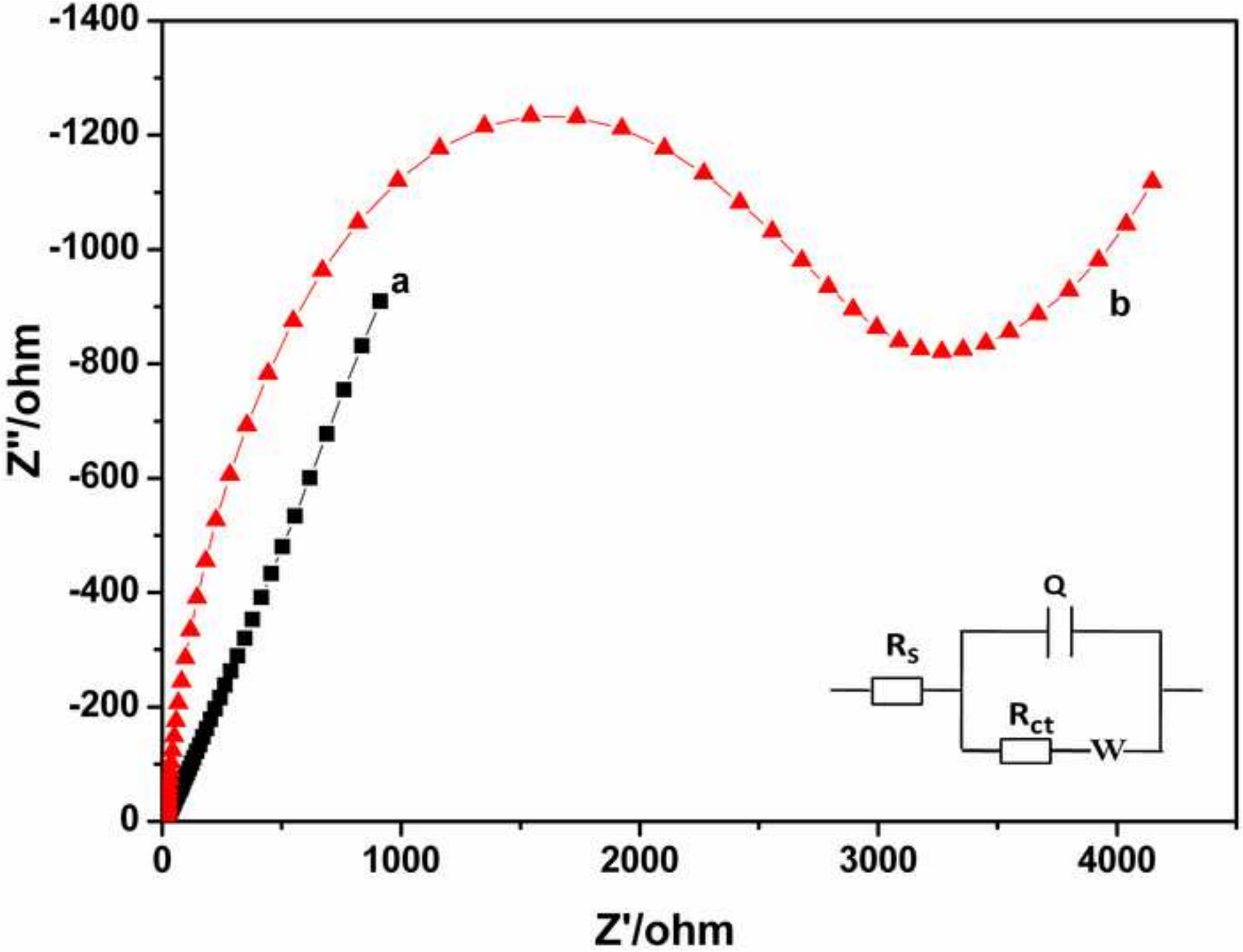


Fig. 6

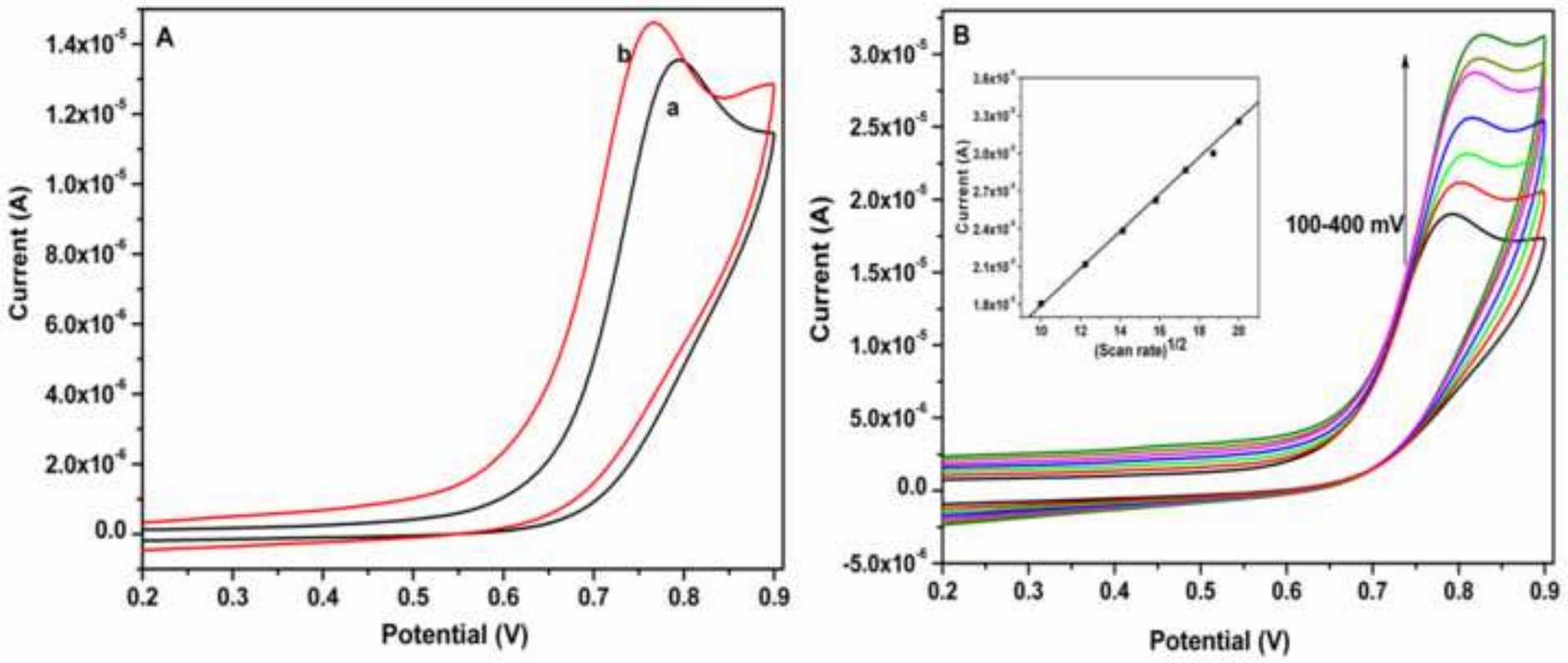


Fig. 7

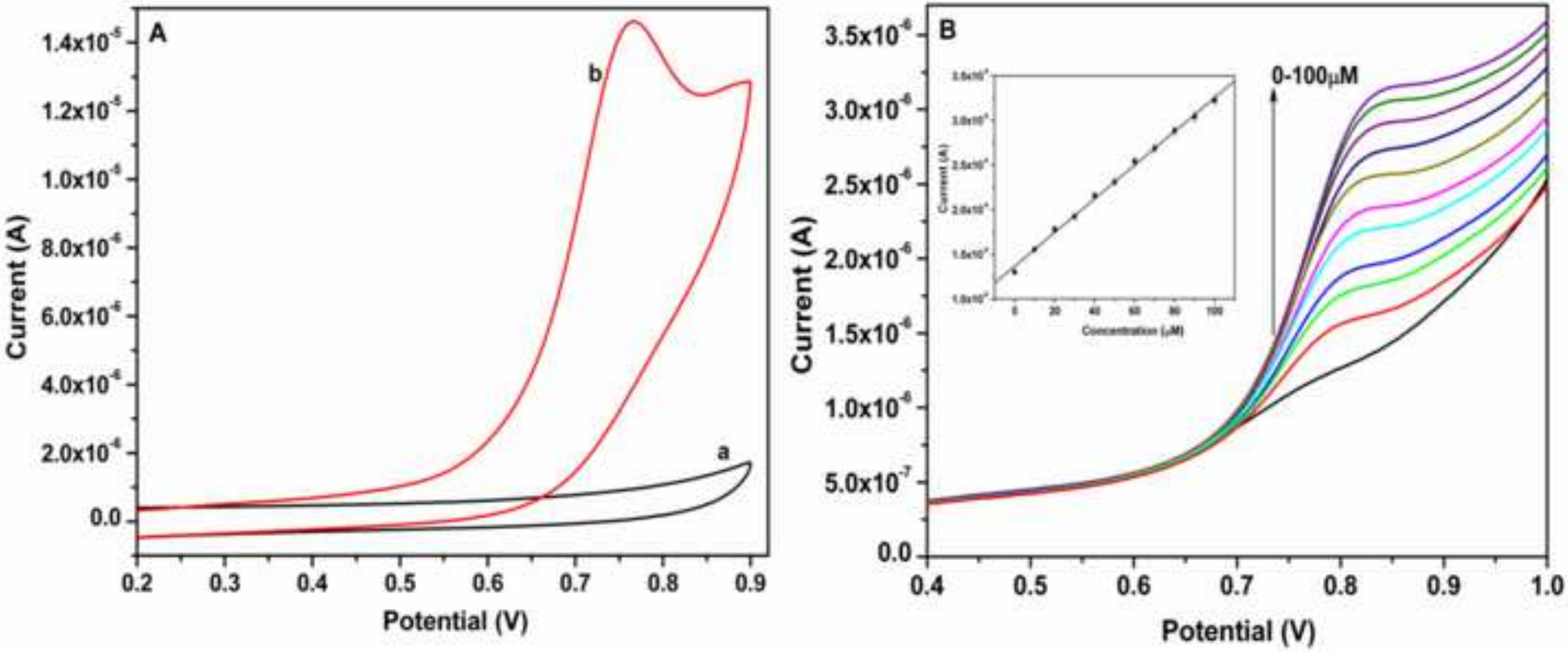


Fig. 8

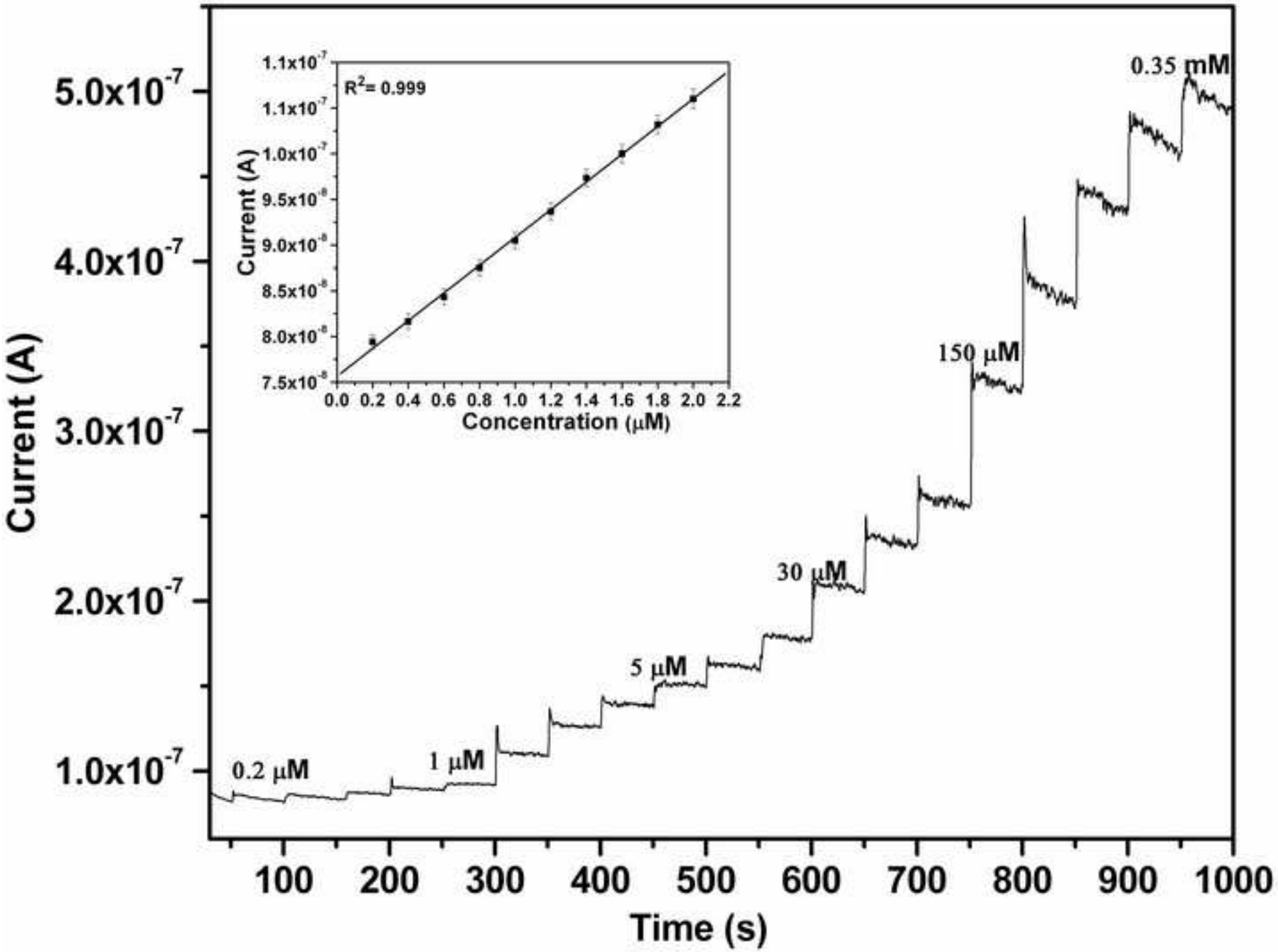


Fig. 9

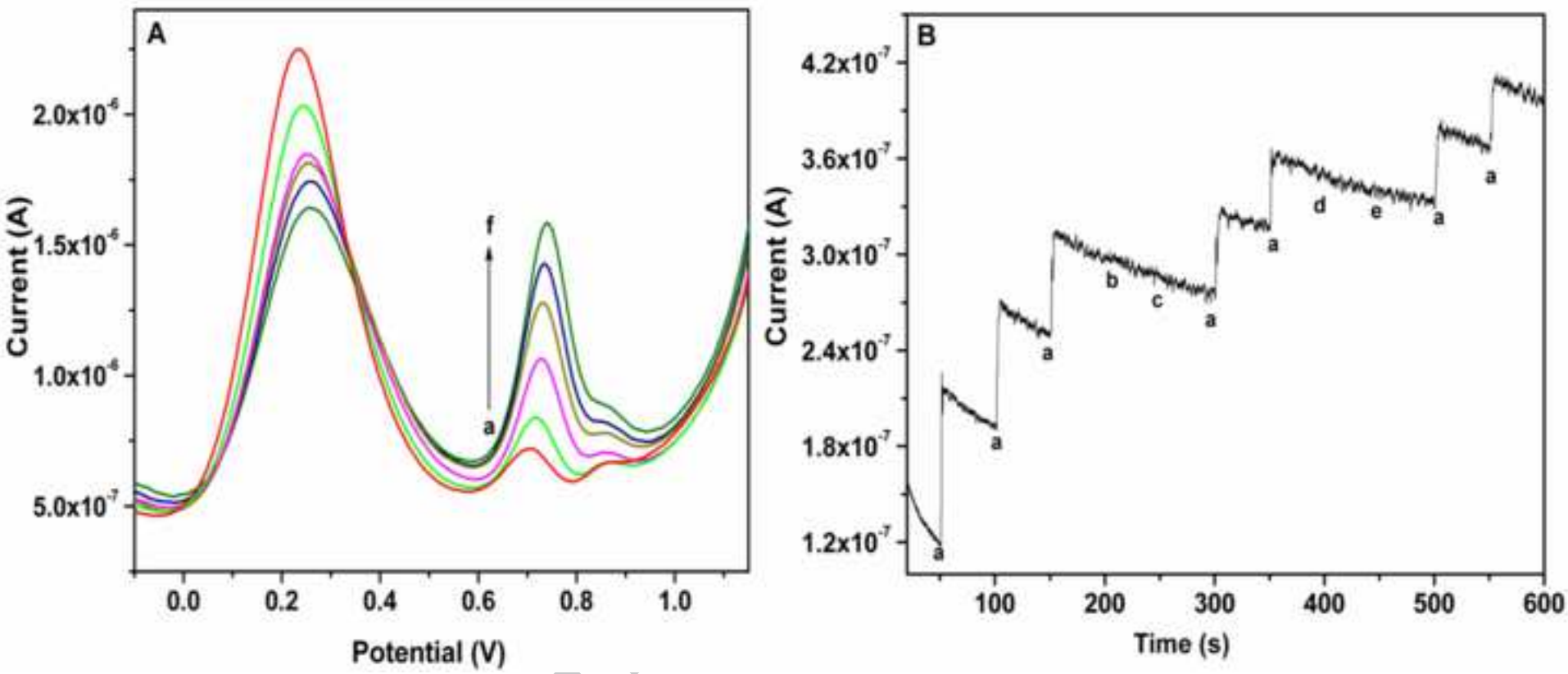


Table 1 Comparison of different chemically modified electrodes for the determination of FA with HA/GCE

Electrode	Method	pH	Linear range (μM)	Detection limit (μM)	Reference
α-Fe_2O_3 nanofibers/GCE	i-t curve	7.0	0.06 - 60	0.00011	Maiyalagan et al., 2012 [13]
DNA/PGE	DPV	4.8	0.1 - 10	0.0106	Mirmoghtadaie et al.,2013 [14]
ZrO_2 nanoparticles/CPE	DPV	7.0	10 - 2000	0.089	Ardakani et al., 2010 [5]
P-AMT/GCE	i-t curve	7.2	0.1 - 800	0.00023	Palraj et al., 2009 [7]
Ni-POA/CPE	CV	13	100 - 5000	91	Ojani et al., 2009 [6]
MWCNT/GCE	Strippping voltammety	6.4	0.3 - 80	0.13	Xiao-li et al., 2009 [4]
Hydroxyapatite/GCE	i-t currve	7.0	0.1 - 350	0.075	This work

GCE: glassy carbon electrode; CPE: carbon paste electrode;
PGE: pencil graphite electrode; Ni-POA: nickel-poly(O-anisidine);
P-AMT: 5-amino-2-mercapto-1,3,4-thiadiazole; MWCNT: multiwall carbon nanotubes.

Table 2 Determination of FA in pharmaceutical sample using nano-HA/GCE

Sample type	Number	Added (μM)	Found (μM)	Recovery (%)
Foligen	1	20.0	20.56	102.8
	2	30.0	29.63	98.76
	3	40.0	41.30	103.25
Folvite	1	10.0	9.98	99.80
	2	15.0	14.86	99.06
	3	25.0	24.62	98.48

Highlights

- Hydroxyapatite (HA) nanoparticles have been synthesized by Microwave irradiation method.
- A novel amperometric folic acid biosensor has been fabricated using HA/GCE.
- The fabricated sensor exhibits a wide linear range, good stability and high reproducibility.
- The sensor was applied for the detection of folic acid in pharmaceutical samples.