I J C R M

International Journal of Contemporary Research In Multidisciplinary

Research Paper

Preparation And Characterization of Vincristine and Cisplatin Hydrogel Scaffolds for The Treatment of Transmissible Venereal Tumour in Dogs

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DOI: https://doi.org/10.5281/zenodo.12180186

Abstract

The present study was undertaken to evaluate the effects of vincristine sulphate, cisplatin and their scaffolds on canine transmissible venereal tumour in twenty-four sexually mature adult dogs affected with naturally occurring canine transmissible venereal tumour (CTVT). Preparation and characterization of hydrogel scaffolds were consisting of FT-IR measurements, ultrastructure studies and electrochemical analysis. The oncolytic potential of the different chemotherapeutic agent (vincristine sulphate, cisplatin and their scaffolds) was evaluated on the basis of physical and cytological parameters, histopathological studies, haemato-biochemical parameters (Hb, PCV, TLC, DLC and platelets, total protein, glucose, BUN, creatinine, ALT, ASTand GGT) and apoptotic effect on HeLa cell line. The animals were randomly divided into four groups (n=6) and subjected to administration of different oncolytic drugs and drugs scaffolds. The animals of group A were administered vincristine sulphate @ 0.025 mg/kg intravenously once in a week for four consecutive weeks and animals of group B were administered cisplatin @ 2.14 mg/kg intravenously and repeated after 21 days. The animals of group C and D were subjected to the administration of scaffolds of vincristine sulphate @ 0.025 mg/kg intravenously once in a week for four consecutive weeks and scaffolds of cisplatin @ 2.14mg/kg intravenously and repeated after 21 days respectively. Genomic DNA from HeLa cells was isolated and subjected to electrophoresis in agarose gel (1.8%) and 1kb DNA ladder. DNA fragments were visualized under a UV trans-illuminator and compared with a standard marker. Lane C showed no fragmented DNA, however, 20 µg/ml vincristine and 20 µg/ml cisplatin showed fragmented DNA in the form of ladder 1 and 3 after 24 h. Vincristine scaffolds and cisplatin scaffolds showed mild DNA fragment in lane 2 and 4. On the basis of parameter observed in this study, it is concluded that the early and best regression of the CTVT was observed in the animals treated with vincristine scaffolds. Cisplatin regressed the CTVT masses upto some extent; however, cisplatin scaffolds are moderately effective when it is used in appropriate dose. Vincristine alone is effective drug for the treatment of CTVT even in metastatic conditions, however the vincristine scaffolds are more effective as it has early regression of tumour as compare to vincristine alone. This may be due to decreasing the side effects caused in healthy cells. These vincristine scaffolds may be used safely by field veterinarian for the treatment of TVT in canines.

Manuscript Information

- **ISSN No:** 2583-7397
- **Received:** 18-05-2024
- **Accepted:** 17-06-2024
- **Published:** 20-06-2024
- **IJCRM:**3(3); 2024: 117-129

- **Plagiarism Checked:** Yes
- **Peer Review Process:** Yes

How to Cite this Manuscript

Arun Kumar, Narendra Singh Jadon, M. G. H. Zaidi, Manjul Kandpal, Rashmi Saini. Preparation And Characterization of Vincristine and Cisplatin Hydrogel Scaffolds for The Treatment of Transmissible Venereal Tumour in Dogs. International Journal of Contemporary Research in Multidisciplinary.2024; 3(3): 117-129.

KEYWORDS: Hydrogel formulations, freeze-dried gel, CTVT, transmissible venereal tumour.

INTRODUCTION

Hydrogel formulations of polymeric material from natural or synthetic sources combined with therapeutic agents have gained great attention in the recent years for treating various diseases. New forms of treatments to attack cancer cells are required while simultaneously decreasing the side effects caused in healthy cells. There are several advantages from using encapsulated antineoplastic agents, including increased drug solubility, better bioavailability, high stability, controlled drug release, prolonged half-life, selective organs or tissue distribution and reduction of the total dose required. Together, all the benefits outlined above can help minimize adverse side effects to a dramatic degree. Hydrogels are three dimensional, hydrophilic polymeric networks which are capable of absorbing large amounts of water and biological fluids or molecules. Another important property of hydrogels is the ability to swell and dissolve in water. Hydrogels can be divided into chemical and physical gels depending on the nature of crosslinking. These systems possess unique properties to improve the efficacy of the therapeutic agents and minimize undesirable side effects (Haq *et al.*, 2017). It has a significant challenge with regard to their cure, because of drug resistance and considerable side effects. So effective drug delivering into the body is required to reduce their toxicity and side effects in view of the above facts, this study was undertaken with a primary objective to compare the chemotherapeutic effect of vincristine sulphate alone and along with hydrogen scaffolds on transmissible venereal tumour in dogs.

Preparation and characterization of hydrogel scaffolds for control drug delivery of vincristine and cisplatin

N-Isopropylacrylamide (NIPAM, Polysciences), N, N′-Methylenebisacrylamide (Otto), and Azo-bis-isobutryo nitrile (Molychem), were used as supplied. The monomer used were selected acrylamide derivatives and crosslinker were bisacrylamide.

Synthesis of pNIPAAm hydrogel

A 150 ml flat bottom flask with 5.6 gm (0.05 mol) of NIPAM, 0 .75 gm (0.005 mol) of MBBA and 0.70 gm (0.005 mol) of AIBN, was placed over temperature controlled magnetic stirrer. Initially the content was stirred at 70±1 ^{o}C for 6 h in Ethanol (99.9%, 20 ml). Further temperature is increased to 90±1 ^{o}C. After 24 h continuous stirring a viscous, transparent semi solid gel is obtained.

Characterization of porous poly (N-isopropylacrylamide) hydrogel

FT-IR Measurements

The FT-IR scans were obtained at Perkin Elmer FTIR spectrometer in KBr. The hydrogel samples and vincristine sulphate scaffold were analyzed by FT-IR in the region of 4000-500cm^{-1}. Before the measurement, the originally swollen hydrogel samples were kept at room temperature for 48 h and then freeze-dried (-48 °C) for 24 h.

Ultrastructure studies of hydrogel, vincristine sulphate scaffold

Ultrastructural (SEM) study of vincristine sulphate scaffold was undertaken. Degree of ultrastructural differentiation and molecular characteristics were recorded. The surface morphology of the hydrogels was studied using a scanning electron microscope. Specimens of the freeze-dried gels were glued to the brass holders and coated with gold for 40 s using a coating machine prior to the SEM examination.

Scanning electron microscopic studies were carried out at appropriate acceleration voltage and magnification range.

Electrochemical Analysis

Electroanalytical experiments have been conducted at IVIUM Potentiostat Galvanostat with reference to the potential of Ag/AgCl using glassy carbon as working and platinum as counter electrodes repectively. Mechanical cleaning and polishing of the glassy carbon electrode alumina slurries 3 µm followed by rinsing with water. *In situ* cleaning of the electrode between successive voltammetric runs was carried out by applying a negative potential (-1.0V) for 40 s followed by keeping electrode in an open circuit for 20 s with moderate stirring for providing a short pre-concentration period.

Synthesis of pNIPAAm Hydrogel

A 150 ml flat bottom flask with 5.6 gm (0.05 mol) of NIPAM, 0.75 gm (0.005 mol) of MBBA and 0.70 gm (0.005 mol) of AIBN, was placed over temperature controlled magnetic stirrer. Initially the content was stirred at 70±1 ^{o}C for 6 h in Ethanol (99.9%, 20 ml). Further temperature is increased to 90±1 ^{o}C. After 24 h continuous stirring a viscous, transparent semi solid gel is obtained.

Characterization of porous poly (N-isopropylacrylamide) hydrogel

FT-IR Spectra of hydrogel samples, vincristine sulphate and cisplatin scaffolds: -

Fourier transmission infra-red scans were obtained at Perkin Elmer FTIR spectrometer. The hydrogel samples and vincristine sulphate scaffolds were analyzed by FT-IR in the region of 4000-400cm^{-1}. Before the measurement, the originally swollen hydrogel samples were kept at room temperature for 48 h and then freeze-dried (-48 °C) for 24 h. The FT-IR spectra of the pNIPAM hydrogel and vincristine scaffolds samples, which have been freeze-dried, are shown in **Plate 1, 2 and 3**. The FT-IR spectra of hydrogel samples and vincristine sulphate scaffolds showed the following spectrum, which are depicted in following tables: -

Table 1: FT-IR spectra of hydrogel scaffolds and their typical bands

Band	Spectrum
G	3433.0
I	3301.1
D	3074.2
D	2974.3
D	2936.0
d’	2876.9
G	1655.3
I	1546.4
i’	1460.4
A	1386.8
A	1368.1
B	1326.7
d’	1244.4
C	1172.9
F	1130.31
c’’	1065.0

Table 2: FT-IR spectra of vincristine scaffolds and their typical bands

Band	Spectrum
G	3393.5
I	3290.3
D	2970.7
D	2939.3
d’	2917.1
d’	2702.0
g’	1630.4
E	1630.4
i’	1459.3
i’	1427.5
A	1387.3
A	1352.6
B	1318.3
d’	1280.4
d’	1261.4
C	1197.0
c’’	1083.9
F	1020.3

4.2.2 Ultrastructure studies of hydrogel and vincristine sulphate scaffolds

Ultrastructural (SEM) study of hydrogel vincristine sulphate scaffolds was undertaken. Degree of ultrastructural differentiation and molecular characteristics were recorded. The surface morphology of the hydrogels was studied using a scanning electron microscope. Specimens of the freeze-dried gels were glued to the brass holders and coated with gold for 40 s using a coating machine prior to the SEM examination.

Scanning electron microscopic studies were carried out at appropriate acceleration voltage and magnification range. In order to have further insight into hydrogel nature of scaffolds, there were image through scanning electron micrographs **(Plate 4, fig-a)**. Scaffolds display the characteristic morphology at x220 and 100 µm. Increase in magnification to x1000, 10 µm has clearly presented the porous morphology of scaffolds as a monolith **(Plate 4, fig-b)**. Loading of vincristine sulphate has transformed the monolith morphology of scaffold into crystalline needle-like structure. This represents the well-organized dispersion of vincristine sulphate **(Plate 5, fig-a)**. Further increase in magnification x220, 100 µm to x1000, 10 µm has clearly indicated the dense dispersion of vincristine sulphate in to porous gel-like structure of pNIPAM **(Plate 5, fig-b)**. The pore length and width of scaffold was found to be 237.54 and 138 µm respectively. Microscopic data reveals the hydrogel nature of pNIPAM.

Electrochemical Analysis

Electroanalytical experiments have been conducted at IVIUM Potentiostat Galvanostat with reference to the potential of Ag/AgCl using glassy carbon as working and platinum as counter electrodes repectively. Mechanical cleaning and polishing of the glassy carbon electrode alumina slurries 3 µm followed by rinsing with water. *In situ* cleaning of the electrode between successive voltammetric runs was carried out by applying a negative potential (-1.0V) for 40 s followed by keeping electrode in an open circuit for 20 s with moderate stirring for providing a short pre-concentration period.

Electrochemical standardization of release of oncolytic drugs from scaffolds

1. Stock solution

Stock solutions (1mg/ml) of oncolytic drugs and drug loaded with scaffolds were prepared in deionized water and stored at refrigeration at 4°C.

2. Square Wave Voltammetry

Experiments have been conducted at IVIUM Potentiostat Galvanostat using a triple electrode assembly. The assembly was constructed in a glass cell equipped with Ag/AgCl, Glassy carbon (2 mm dia) and platinum foil (1 cm^2) electrodes. PBS (0.1 M, pH-7.4) was served as electrolyte media. Prior to electrochemical experiments, glassy carbon electrode was well polished with moist alumina slurry (3 µm) followed by successive rinsing with deionised water and finally a thorough cleaning under sonication over 10 min. square wave voltammograms were recorded in presence of varying concentrations of scaffolds dispersed in PBS under gentle stirring. The SWV experiments have been performed in the potential range from -0.1 to 2.0 V using the following optimal parameters: pulse amplitude 100 mV, equilibration time 10s and frequency 50 Hz. Most favourable conditions, in terms of sensitivity and speed, were explored using the SWV **(Plate-7, fig-a)**.

3. Monitoring drug release from scaffolds

One week old stock solutions of oncolytic drug and its scaffolds were employed for square wave voltammetry. Serial dilution of oncolytic drugs and its scaffolds (1 mg/ml) were made and applied to PBS (20 ml) for square wave voltammetry. The voltammograms with increasing concentration of oncolytic drug and its scaffolds show linear increase in the intensity of current signals with concentration of drug **(Plate-8, Plate-9, Plate-10 and Plate-11)**.

Square wave voltammograms of scaffolds derived from oncolytic drug ranging 0.1 to 0.6 mg/ml in PBS (7.4 pH) revealed peak current corresponding to vincristine sulphate at 0.16 (1.45

Volt). From calibration plots **(Plate-9 fig-b and Plate-11 fig-b)**, it was revealed that the release of vincristine from scaffolds was accomplished within 16.0 min. On the basis of the release time of oncolytic drug from their scaffolds, it was revealed that lower the release time of drug from their scaffolds shows higher the toxic effects on body. The electrochemical relation between peak current and voltage revealed electrical nature of the oncolytic drug. Such electrical nature of drug was assessed through presence of polar linkage in their molecular structure. **Plate-1, 2 & 3** reveals the various linkages associated with scaffolds of the drug in terms of their respective FTIR spectra. Vincristine sulphate indicated the characteristic functionalities of carbon-carbon bond of single (h) and multiple nature (e), along with C-N (g'), N-H (a), C=O(g). FTIR spectra further reveals the hydrogel nature of the scaffolds due to appearance of wave numbers corresponding to bending and stretching modes of O-H group associated with NIPAM **(Plate- 2 & 3)**. Such polar functionalities associated with drug allows well electron transport in PBS medium that renders current -voltage characteristics in SQV. In order to have further insight into hydrogel nature of scaffolds, there were image through scanning electron micrographs **(Plate 4, fig-a)**. Scaffolds display characteristic morphology at x220 and 100 µm. Increase in magnification to x1000, 10 µm has clearly presented the porous morphology of scaffolds as a monolith **(Plate 4, fig-b)**. Loading of vincristine sulphate has transformed the monolith morphology of scaffold into crystalline needle-like structure. This represents the well-organized dispersion of vincristine sulphate **(Plate 5, fig-a)**. Further increase in magnification x220, 100 µm to x1000, 10 µm has clearly indicated the dense dispersion of vincristine sulphate in to porous gel-like structure of pNIPAM **(Plate 5, fig-b)**. The pore length and width of scaffold was found to be 237.54 and 138 µm respectivelyMicroscopic data reveals the hydrogel nature of pNIPAM.

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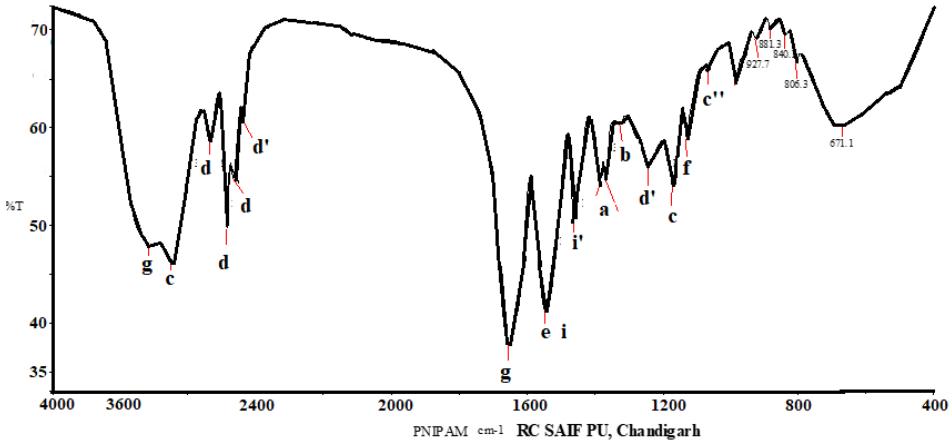
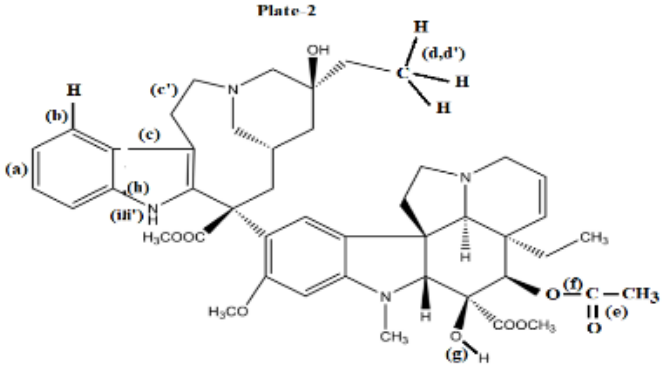
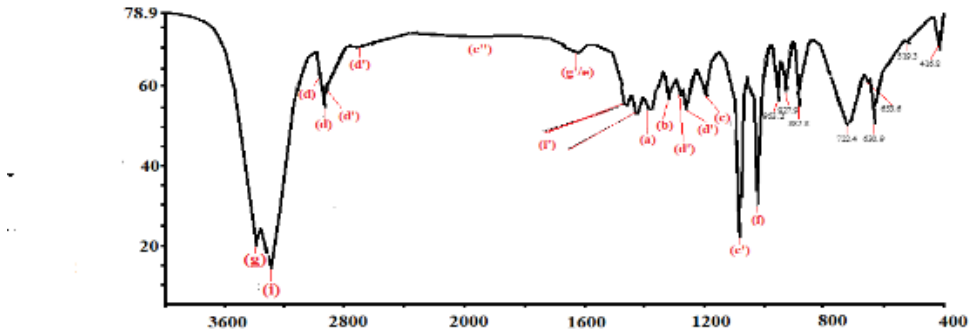
Plate-1



FT-IR pNIPAM

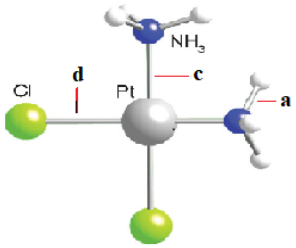
Plate-2

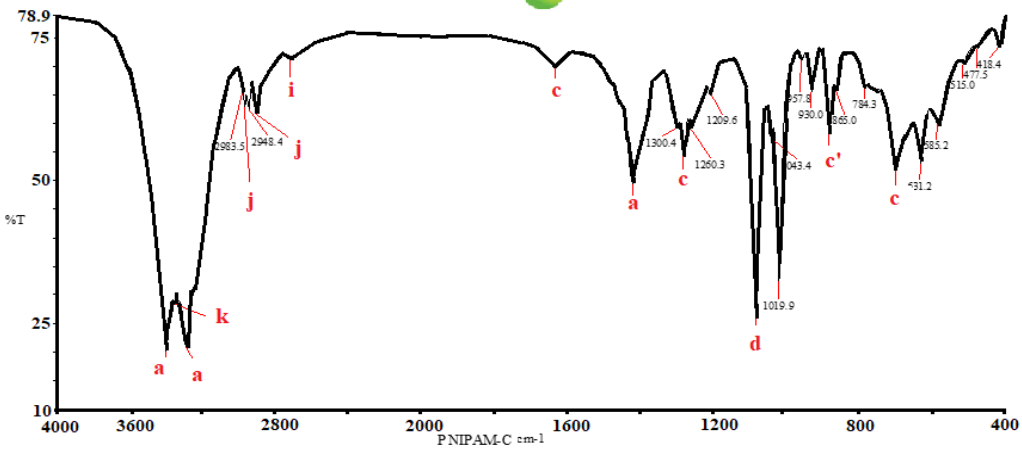




FT-IR Vincristine Scaffolds

Plate-3





FT-IR Cisplatin Scaffolds

Plate No. 4

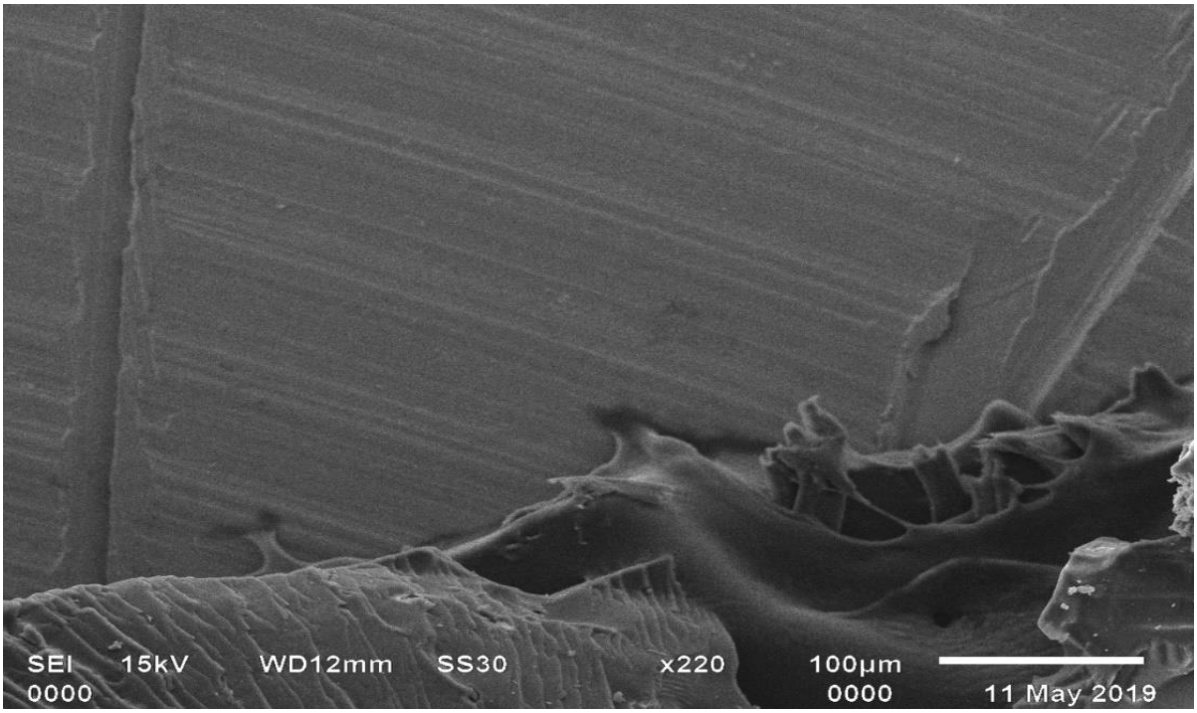


Fig. a: Scanning Electron Microscopic photomicrographs of hydrogel showing characteristic porous morphology at x220.

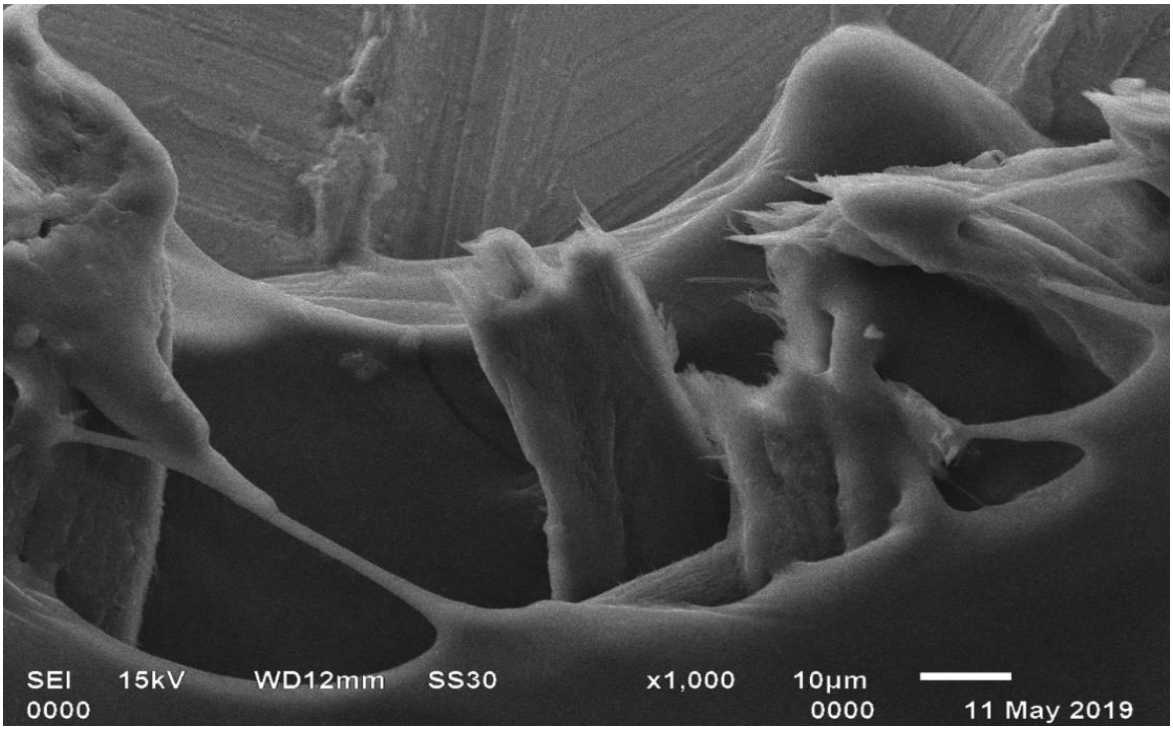


Fig. b: Scanning Electron Microscopic photomicrographs of hydrogel showing characteristic porousmorphology at x1000

Plate No. 5

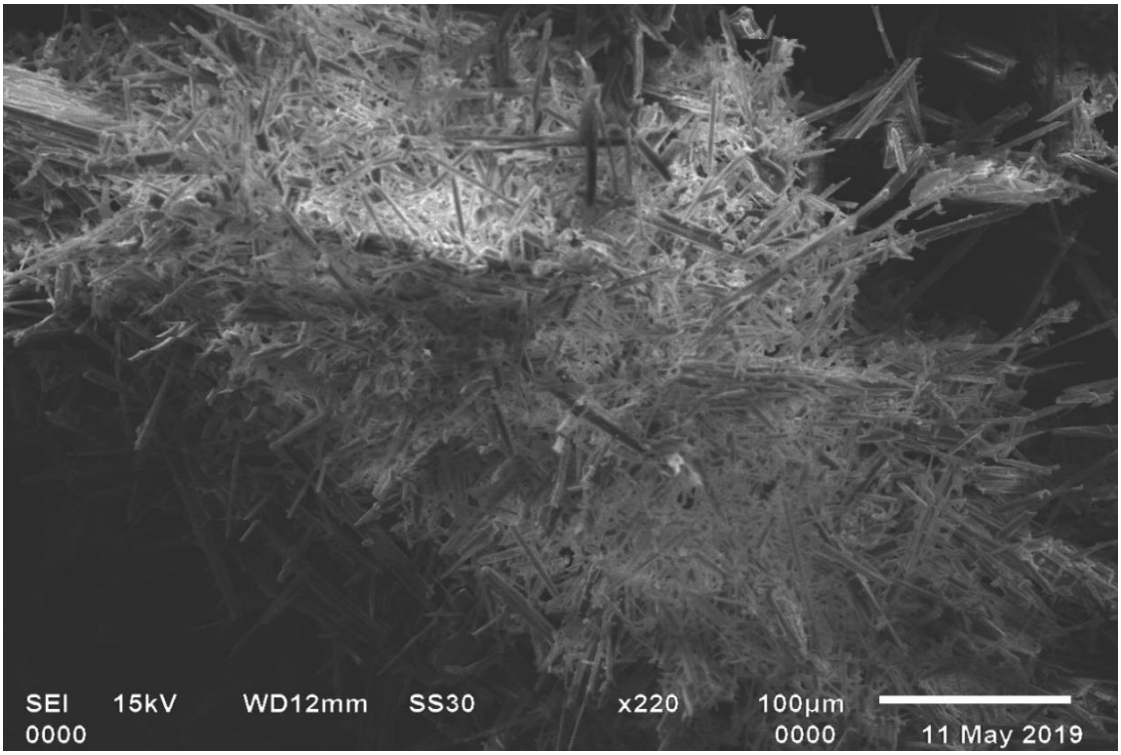


Fig. a: Scanning Electron Microscopic photomicrographs of vincristine scaffolds showing characteristic monolith morphology of scaffold into crystalline needle-like structure at x220.

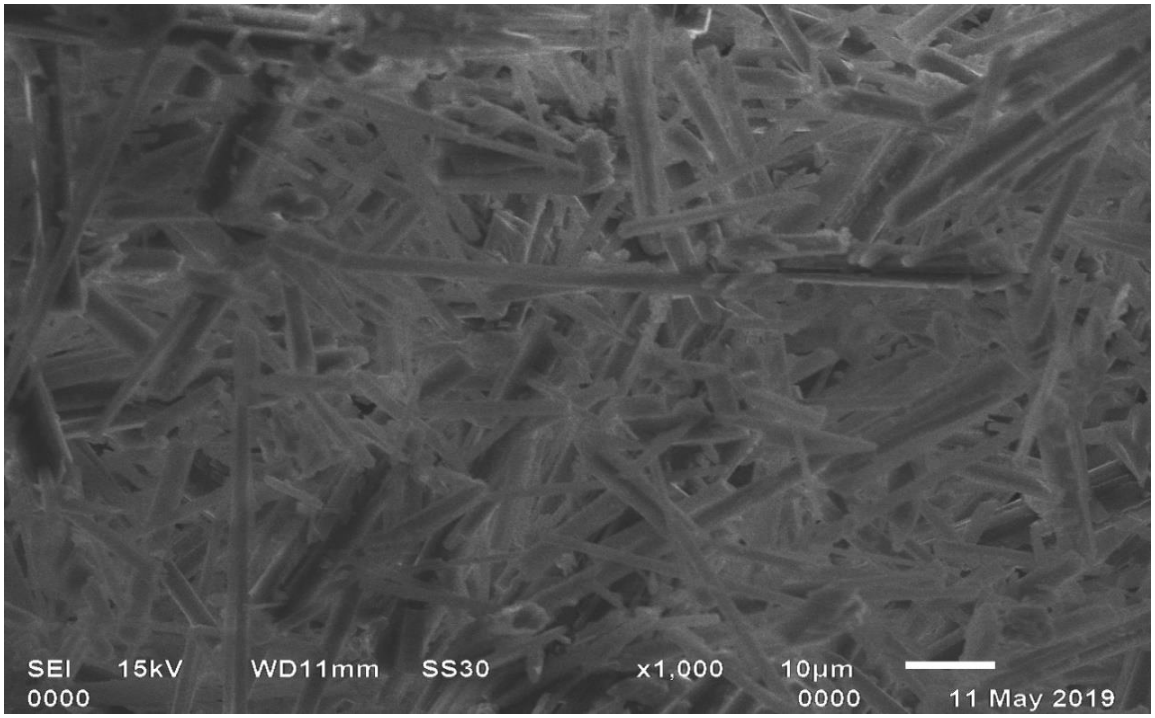


Fig. b: Scanning Electron Microscopic photomicrographs of vincristine scaffolds showing characteristic monolith morphology of scaffold into crystalline needle-like structure at x1000.

Plate No. 6

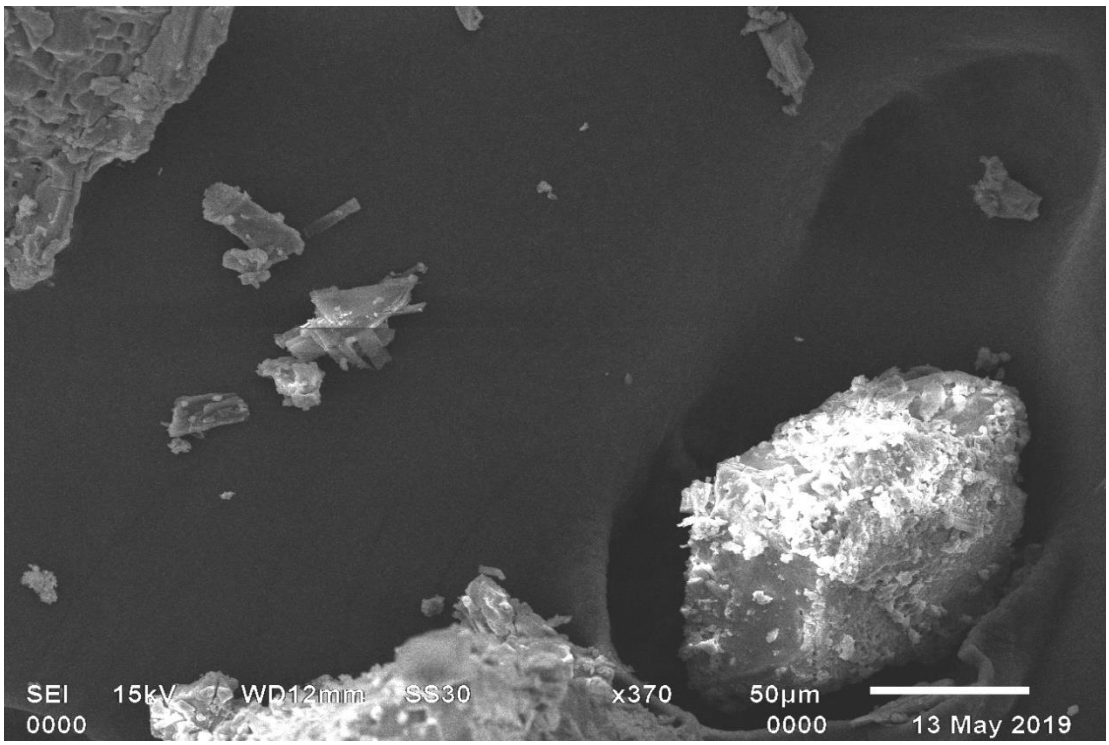


Fig. a: Scanning Electron Microscopic photomicrographs ofcisplatin scaffold showing the porous cavity of scaffold and a well crystalline lump of drug at x370.

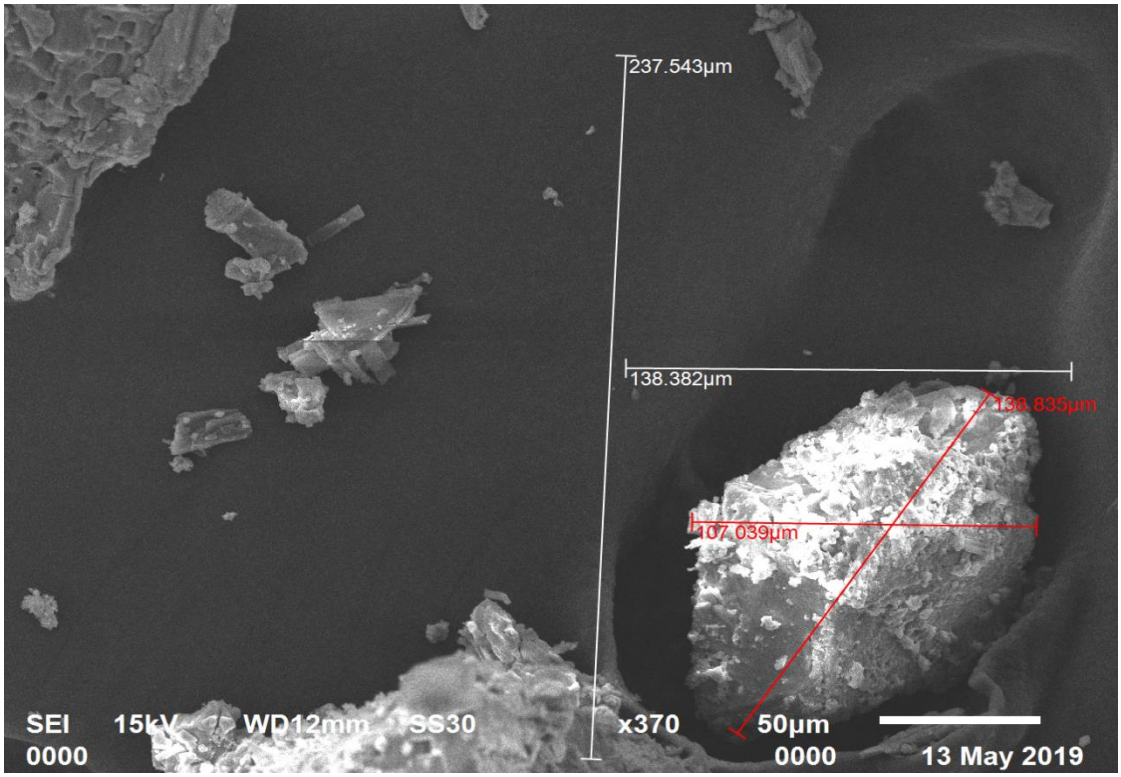


Fig. b: Scanning Electron Microscopic photomicrographs ofcisplatin scaffold showing the measurements of porous cavity of scaffold and a well crystalline lump of drug at x370.

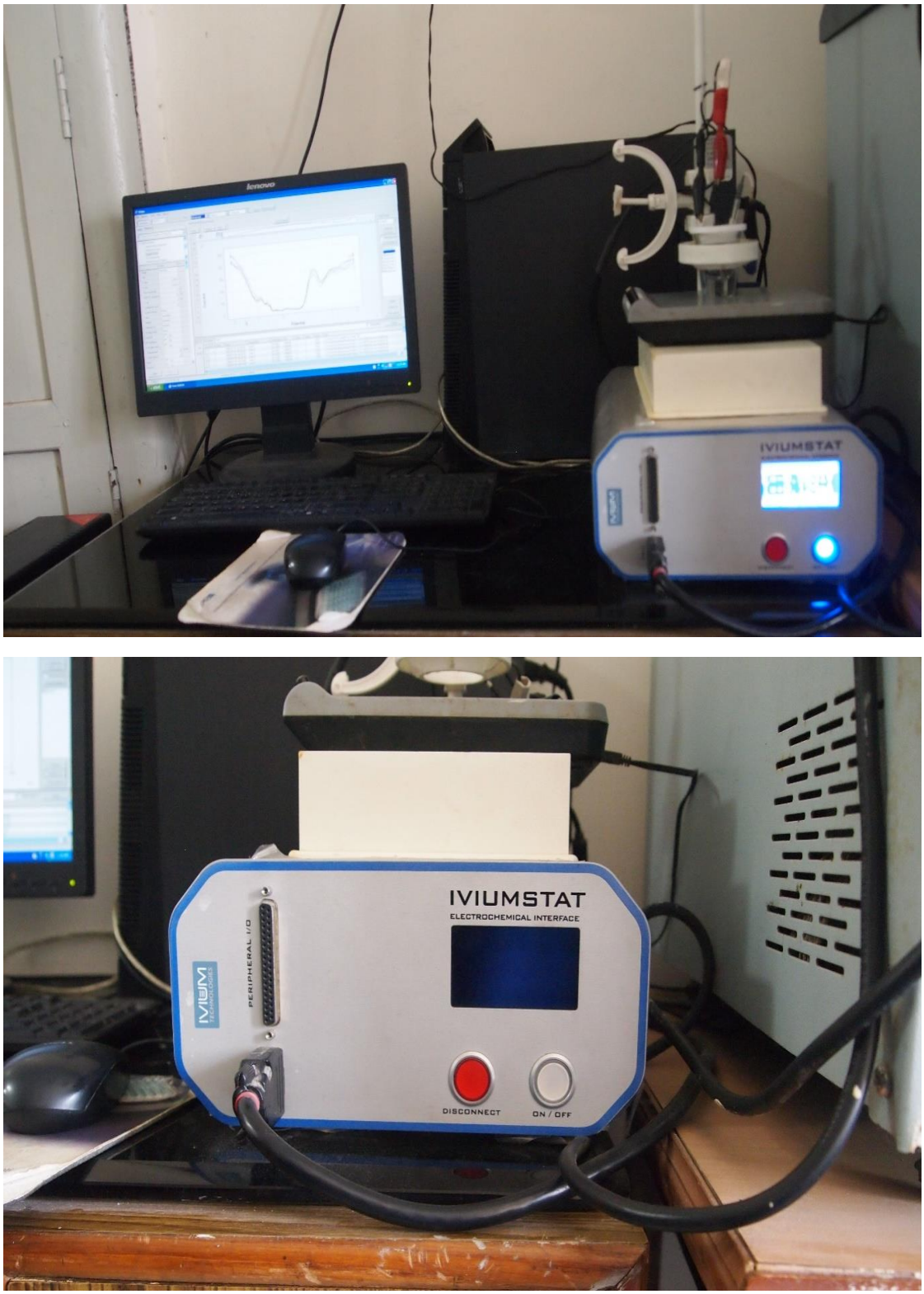


Plate 7: IVIUM Potentiostat Galvanostat using a triple electrode assembly

Plate No. 8

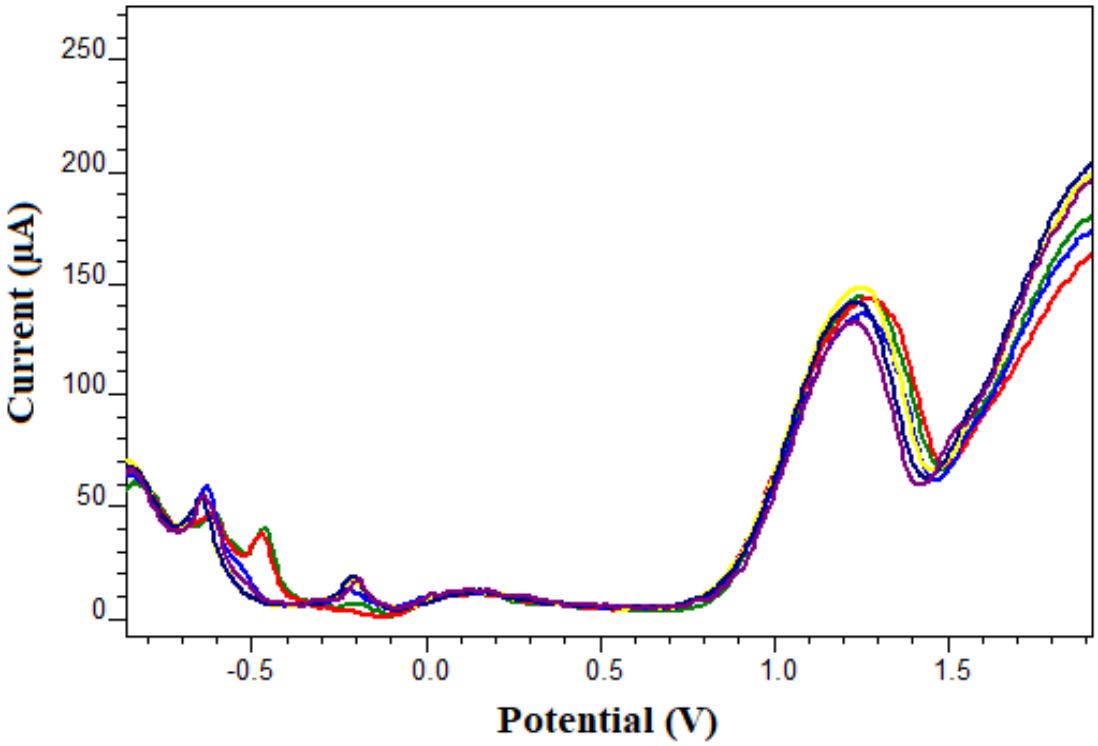


Fig. a: SWV scan of vincristine sulphate at various concentrations

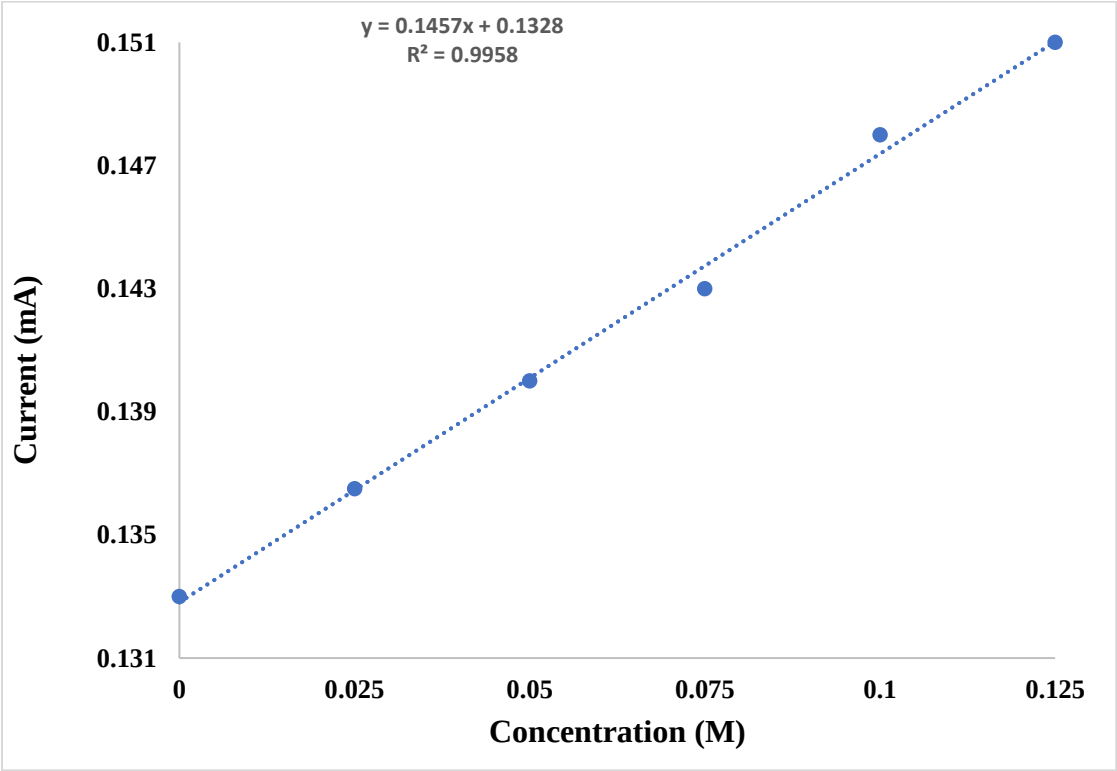


Fig. b: Calibration curve for vincristine sulphate.

Plate No. 9

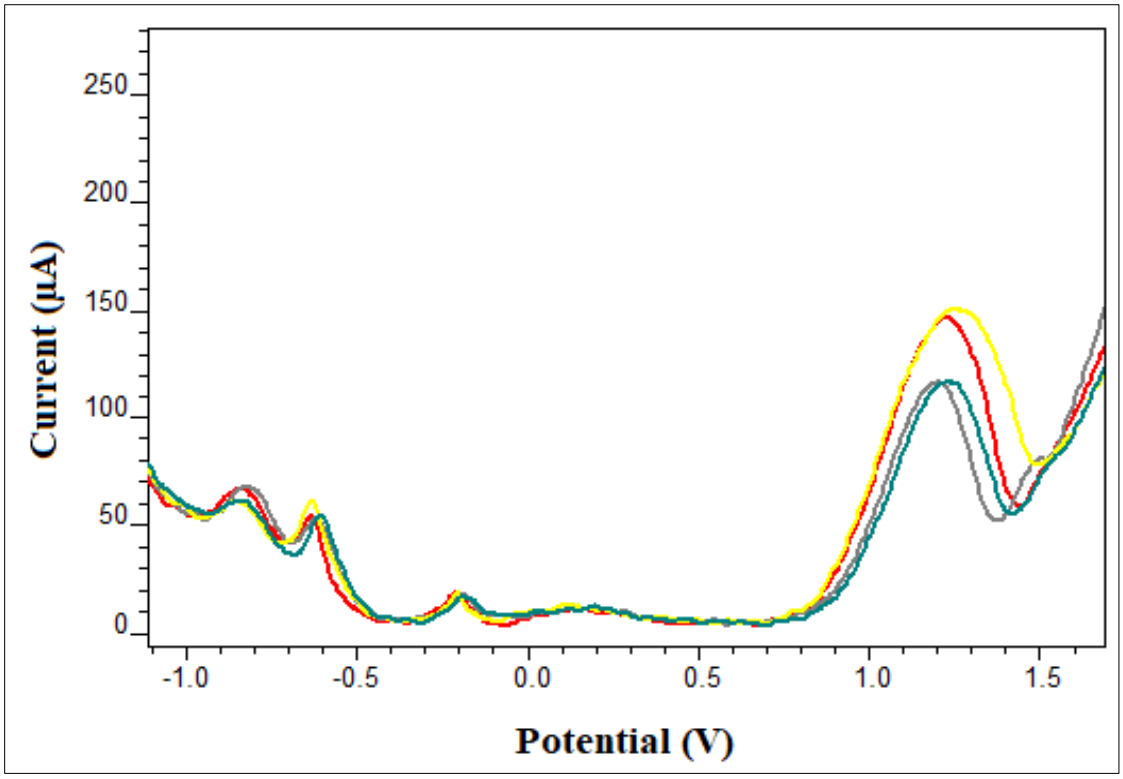


Fig. a: SWV scan of vincristine scaffolds at various concentrations

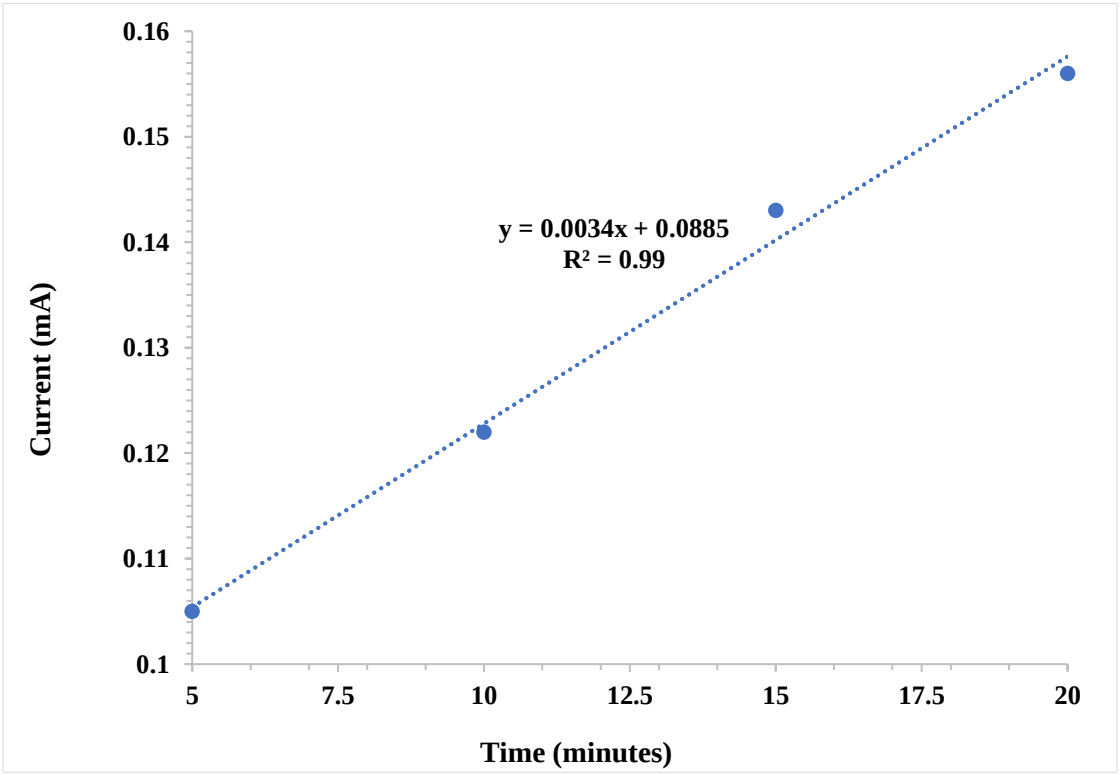


Fig. b: Calibration curve for vincristine scaffolds

Plate No. 10

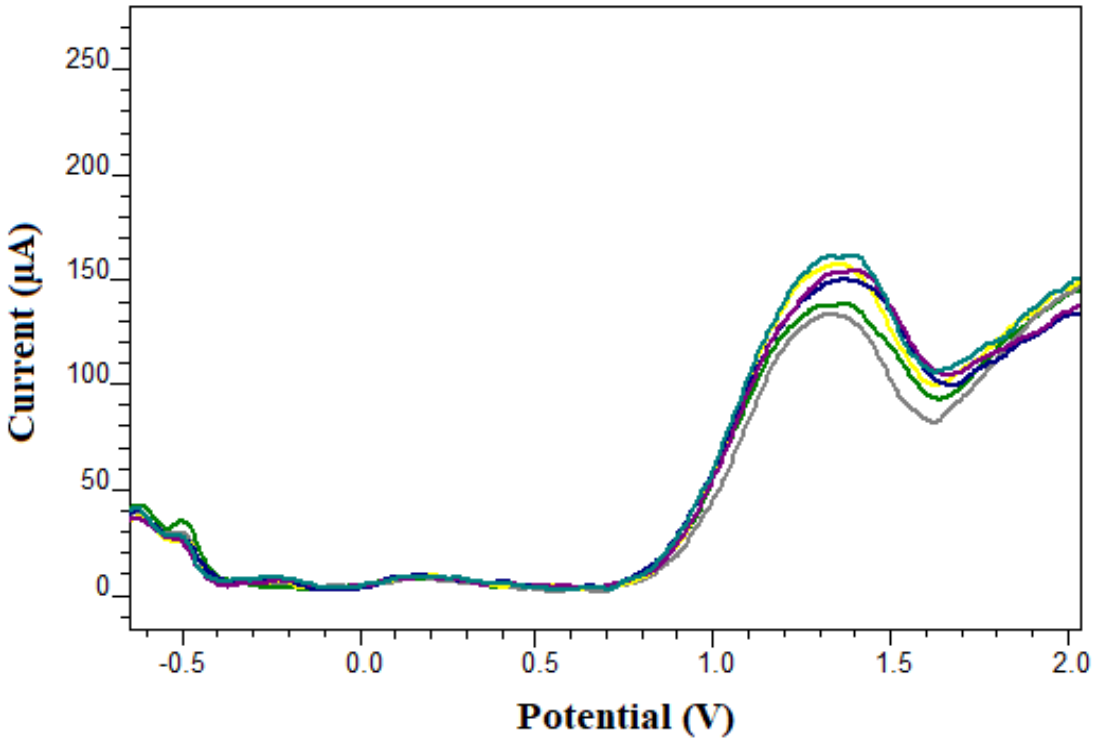


Fig. a: SWV scan of cisplatin at various concentrations

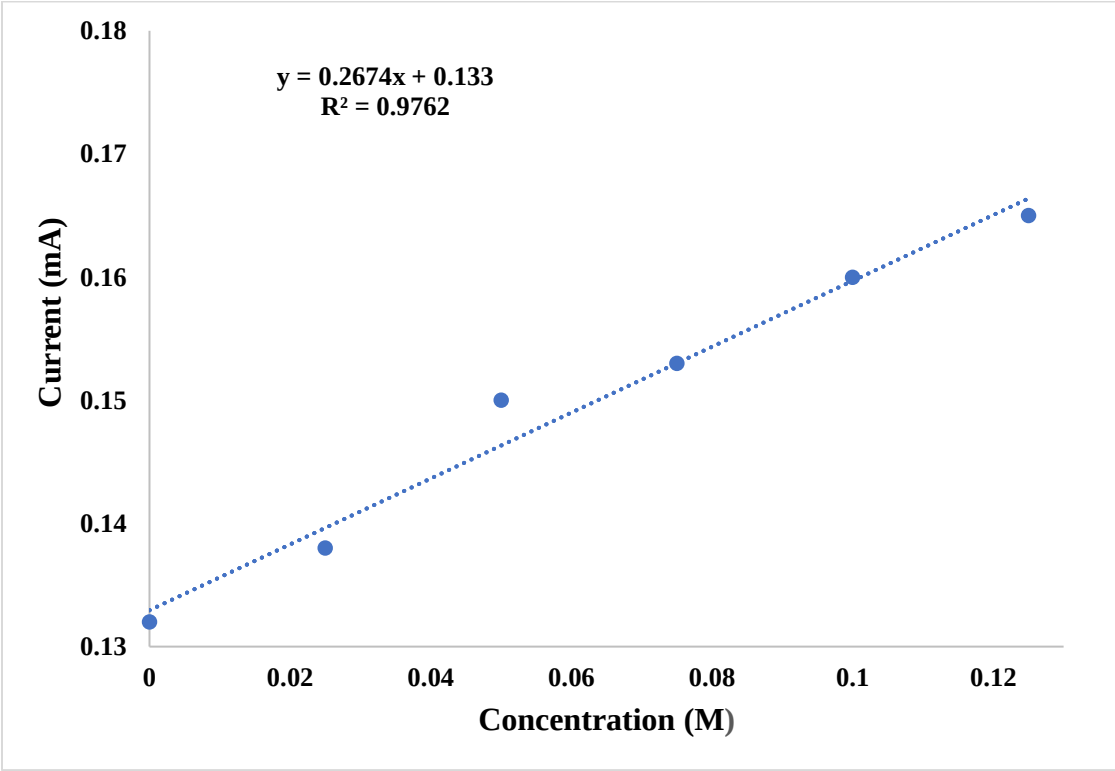


Fig. b: Calibration curve for cisplatin

Plate No. 11

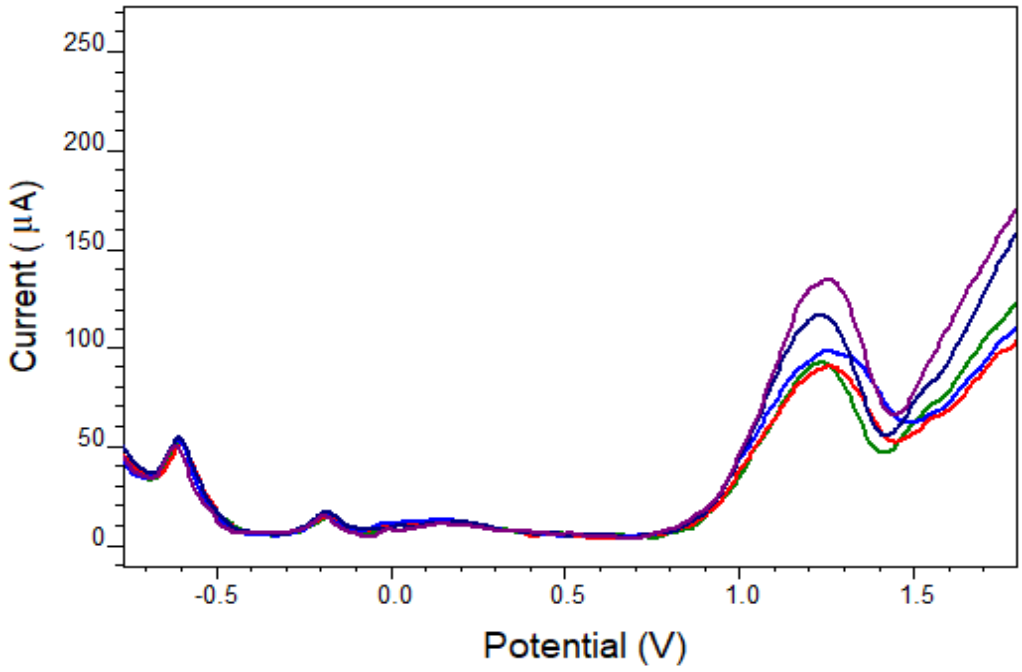


Fig. a: SWV scan of cisplatin scaffolds at various concentrations

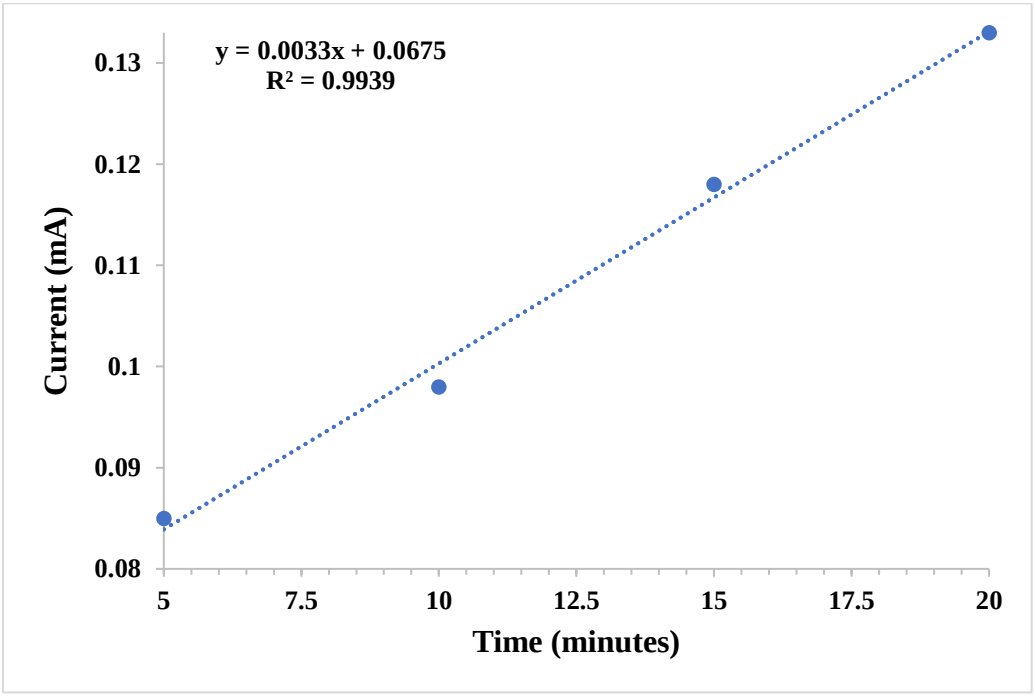


Fig. b: Calibration curve for cisplatin scaffolds