

Biosensor for detection of selective anticancer drug gemcitabine based on polyaniline-gold nanocomposite

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ABSTRACT

Electrochemical biosensor is an effective tool for pharmaceutical analysis due to its simplicity, specificity, sensitivity, fast, cost-effective and repetitive measurements with miniaturized and portable devices. The paper illustrates the detail methodology for development of an amperometric biosensor based on polyaniline-gold nanocomposite film modified horseradish peroxidase for anticancer drug gemcitabine in bulk and in parenteral formulation. Scanning Electron Microscopy, Cyclic Voltammetry, Fourier Transform Infra Red Spectroscopy and Electrochemical Impedance Spectroscopic studies of the electrodes and after immobilizing of HRP shows the successful formation of a selectivity of the electrode. The proposed polyaniline-gold nanocomposite based biosensor allow quantitation over the range 0.10 to 1.10 ngmL⁻¹ with detection limit of 0.031 ngmL⁻¹, biosensor sensitivity of 2.934 μ Ang mL⁻¹ has distinct advantages over other existing methods. Precision and accuracy were also checked and were within the limits. The procedure has been applied to the assay of the drug in dosage form with mean percentage recoveries of 99.00 \pm 0.08%. The suggested biosensor method can be successfully applied to the detection and determination of anticancer drug gemcitabine in different drug formulations. Copyright © 2015 VBRI press.

Keywords: Biosensor; polyaniline-gold nanocomposite; HRP; electrochemistry; anticancer drug gemcitabine.



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Introduction

Gemcitabine [4-amino-1-[(2R, 4R, 5R)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)-oxolan-2-yl] pyrimidin-2-one monohydrochloride] belongs to a class of drugs known as antimetabolites, approved by FDA in 1996 and used to treat a wide variety of solid tumors including pancreatic, breast and non-small lung cancer [1-6], bladder cancer [7]. Keeping in view of the importance of gemcitabine in the treatment of solid tumors several methods have been developed for quantitative analysis of gemcitabine viz. chromatographic methods [8-25], voltammetry [26], electrochemical sensor [27] and spectrophotometry [28].

Biosensor offers a very promising tool with its application in the field of pharmaceutical, biomedical and

environmental. For the last two decades, conducting polymers have emerged as one of the most interesting materials for the fabrication of biosensors and electrochemical sensors [29]. Polyaniline (PANI), one of the promising and unique conducting polymers has various advantages in the field of fabrication of gas sensors, biosensors and field emitters [30-32]. Furthermore, PANI is not only environmentally stable but also dramatically changeable in its electronic structure and physical properties in pro-activate state [33]. Above facts facilitates functionalization of PANI with metal nanoparticles that will provides a new class of advanced hybrid nanocomposite materials.

Gold nanoparticles (AuNPs) enhance polyaniline performance in a neutral pH [34]. AuNPs display unique structural, optical, electronic, magnetic and catalytic properties making this material a very attractive material for biosensor, chemisensor, electrocatalyst, drug delivery and sensing [35-38]. The high surface-to-volume ratio and high surface energy of AuNPs provide stable immobilization of biomolecules retaining their bioactivity. The presence of amine groups and cysteine residues in the enzymes are known to bind strongly with gold colloids [39, 40] making AuNPs a favorable candidates for the immobilization of enzymes [41, 42]. The application of AuNPs as a detection probe for the analysis of pharmaceuticals [43-45] was reported. Menon *et al.* reported a strategy for detection and quantification of gemcitabine by colorimetric method using AuNPs as complexing agent [24]. Gemcitabine has electroactive groups and its electrochemical behaviour has been investigated by Keerti *et al.* [46] using gold electrode.

In this manuscript we propose an electrochemical preparation of inorganic-organic hybrid probe followed by immobilization of horseradish peroxidase (HRP) enzyme for determination of the gemcitabine in pharmaceutical formulations. The electrochemical investigation of gemcitabine at AuNPs/PANI nanocomposite based biosensor has been undertaken. The study will give some insight into its redox process, which is important for our understanding of its property as well as its metabolism in biological system. Furthermore, there appears to be no electrochemical biosensor method based on polyaniline-gold- horseradish peroxidase (PANI-AuNPs-HRP) for the determination of gemcitabine in pharmaceutical formulation and bulk form.

The present communication reports a validated, rapid and selective PANI-AuNPs-HRP based biosensor method for the simple and direct determination of anti cancer drug gemcitabine in bulk form and pharmaceutical formulation without any time-consuming extraction or separation steps prior to drug assay.

Experimental

Reagents and materials

Gemcitabine hydrochloride (99% purity) was procured from Enzo Life Sciences (USA). Parenteral gemcitabine (*Tabicad*) labeled to contain 200 mg gemcitabine per bottle was obtained from commercial sources. HRP, aniline monomer (99.5%) of analytical grade, Gold (III) chloride trihydrate, indium tin oxide (ITO) coated glass plates was

supplied by Sigma Aldrich (Germany). For experimental investigations, stock solution (1.0 mg mL^{-1}) of gemcitabine was prepared by direct dissolution of 10 mg of gemcitabine in 10 mL freshly prepared 0.05M phosphate buffer solution (PBS) of pH 7.0 ± 0.1 . Simultaneously, standard working solutions were prepared by appropriate dilutions of the stock solution. Potassium ferrocyanide containing phosphate buffer solutions of 200 ml capacity with ionic strength 0.05M in the pH range 5.5-9.0 were prepared in de-ionized water (TKA Millipore water system) by adding appropriately measured amounts of mono sodium dihydrogen phosphate, disodium mono hydrogen phosphate and adjusting with 1M HCl and 0.1 M NaOH solution. All the reagents used in the present study were of analytical and molecular biology grade.

Instrumentation

Electrochemical measurements were made using Autolab Potentiostat/galvanostat (Eco Chemie, Netherlands) with NOVA software and potentiostat/galvanostat/ZRA (Gamry Reference 3000, United States of America) with Gamry Echem Analyst Software in which working electrode was replaced with PANI/ITO, PANI/AuNP/ITO and PANI/AuNP/HRP/ITO. Platinum wire and Ag/AgCl (3M KCl) were used as counter and reference electrodes, respectively. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were carried out in a 20 mL Dr Bob's electrochemical cell stand. For chemical characterization of PANI-AuNPs nanocomposite and its HRP interaction, Fourier transform infrared (FT-IR) spectrophotometer (Spectrum 100 with software version CPU32) has been used. The surface topology of respective films was studied by using Scanning Electron Microscopy (JEOL-JSM-6390LV).

Electrochemical polymerization of PANI-Au nanocomposite film

The nanocomposites of PANI-AuNPs were prepared by electrochemical deposition of a mixture of HCl (1.0M), aniline (0.2M) and AuNPs (500 μL of 3 mg mL^{-1} HAuCl₄.3H₂O in de-ionized water) and thoroughly sonicated for 15 minutes and these were introduced in a three-electrode electrochemical cell of Autolab Potentiostat/Galvonostat (EcoChemie, Netherlands, Model 101N). The cell consists of Ag/AgCl (3M KCl) as reference, Pt wire as counter electrode and ITO glass plate (0.25 cm²) as working electrode. The electro-polymerization was demonstrated at scan rate of 20 mVs^{-1} in the potential range from -2.0 to 1.1 V. The PANI/AuNPs/ITO electrode was washed with distilled water to clean the untreated AuNPs-PANI and was kept for drying.

Immobilization of HRP on PANI-Au nanocomposite film

The bio-electrode for detection of gemcitabine was prepared by immobilizing HRP enzyme (1 mgmL^{-1} solution in phosphate buffer, pH 7.0 ± 0.1) onto the PANI-Au nanocomposites by adsorption technique (overnight dipping in a special assembled cell). It is further followed by incubation at 4°C for overnight using glutaraldehyde which

acts as a cross linker. The conditions for the immobilization of the enzyme were selected based on our prior studies [47, 48]. To remove loosely-bound material, the biosensors were rinsed with a buffer solution and preserved at 4 °C at pH 7.0±0.1 phosphate buffer solution for further use.

Results and discussion

Cyclic voltammetric (CV) studies

Cyclic voltammograms obtained for (A) PANI, (B) AuNPs/PANI electrode and (C) AuNPs/PANI/HRP bioelectrode in PBS (50mM, pH 7.01±0.1, 0.9% NaCl) containing 5mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ was recorded at different scan rates (5-50 mVs^{-1}) (Fig. 1). The difference between cathodic (E_{pc}) and anodic peak (E_{pa}) shift ($\Delta E_p = E_{pa} - E_{pc}$) was calculated. The linear increased in ΔE_p value with increasing square root of scan rate indicates the slow kinetics of electron transfer on the electrode surface (Fig. 1D). Fig. 1B shows the enhancement of polyaniline performance in a neutral pH with the addition of AuNPs into PANI. After addition of AuNPs to PANI, the slope is enhanced by a factor of about three with the appearance of one more redox peak at higher scan rate [Fig. 1(b)], which may be due to the formation of the AuNPs-PANI thereby absorption spectra and electronic transmission are drastically changed when several particles of PANI bind with AuNPs [49].

The effect of pH on the peak current was investigated in the pH ranges from 5.5 to 9.0 in presence of 0.10 ng mL^{-1} gemcitabine. The plot of current versus pH shows that maximum peak current and good peak shape was obtained at pH 7.01±0.1 of PBS (data not shown). Therefore, in order to maximize the signals of the biosensors current, a phosphate buffer solution (pH 7.01±0.1) was selected as the optimum solution for all further experiments.

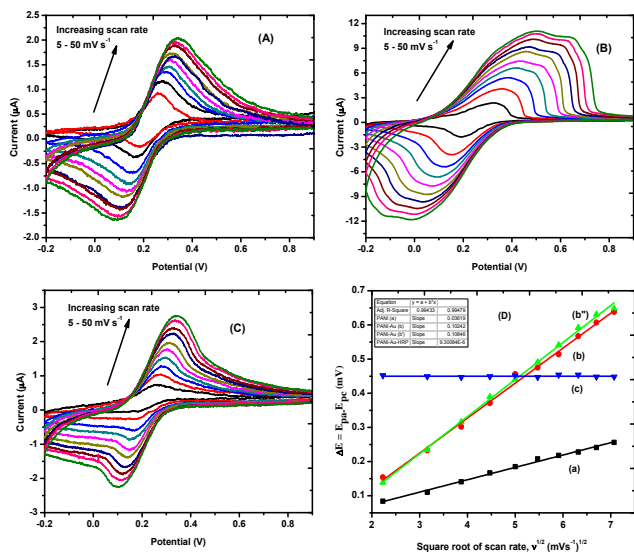


Fig. 1. Cyclic voltammograms of (A) PANI/ITO, (B) AuNPs/PANI/ITO, and (C) AuNPs/PANI/HRP/ITO electrodes and (D) plot of ΔE_p value with square root of scan rate in PBS (50mM, pH 7.0±0.1, 0.9% NaCl) containing (5mM) $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at scan rate from 5 mV s^{-1} to 50 mV s^{-1} .

Fig. 2 shows the relative variation in CV at constant scan rate (10 mVs^{-1}) in PBS (50mM, pH 7.01, 0.9% NaCl) containing 5mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ for three electrodes. During

the bio-electrochemical reaction, decrease in peak current in Fig. 2(c) is due to slow redox process after immobilization of HRP on AuNPs/PANI electrode. All the three electrodes showed relative variation in the oxidation (I_{pa}) and reduction (I_{pc}) peak current against $v^{1/2}$ (v is the scan rate). The peak current (I) is proportional to $n^{3/2}D^{1/2}v^{1/2}$ for constant surface areas and concentrations; whereas n and D are the number of electrons and diffusion coefficient, respectively. Intuitively, the current intensity (i.e. the flow of electrons) is expected to depend on the surface area of the working electrode and the concentration of the electro-active species. Diffusion coefficient depends on the slope of peak current with square root of scan rate [$d(I)/d(v^{1/2})$] is proportional to $D^{1/2}$, where linear response with the scan rate shows a diffusion controlled process. The drastic decrease in peak current in Fig. 2(c) was observed and is possibly due to strong binding of HRP to AuNPs-PANI matrix and control in transport of ions of the supporting electrolyte.

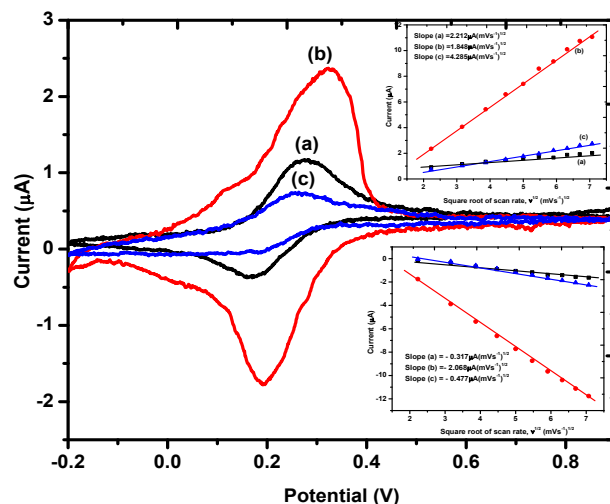


Fig. 2. Cyclic voltammograms curve of (a) PANI/ITO, (b) AuNPs/PANI/ITO, and (c) AuNPs/PANI/HRP/ITO electrodes in PBS (50mM, pH 7.0±0.1, 0.9% NaCl) containing (5mM) $[\text{Fe}(\text{CN})_6]^{3-/4-}$ scan rate at 5 mV s^{-1} . Fig. in inset shows reduction and oxidation peak current with square root of scan rate.

FT-IR studies

FT-IR spectra of PANI (Figure 3a), AuNPs (Fig. 3(b)), AuNPs-PANI (Fig. 3(c)) and AuNPs-PANI-HRP (Fig. 3(d)) films were obtained to characterize the chemical interactions between PANI, AuNPs and HRP. FT-IR spectra of PANI have a strong absorption band between 656.9 cm^{-1} and 752.8 cm^{-1} due to presence of an aromatic ring and deformation of C-H vibrations (Fig. 1(a)). In the spectra, bands between 1192.5 cm^{-1} and 1384.8 cm^{-1} are associated with C-N stretching in aromatic amines. The peak at 1631.5 cm^{-1} is attributed to C=C stretching of benzenoid rings and quinoid rings. The FTIR spectrum of AuNPs-PANI-HRP (Fig. 3(d)) film has shown the characteristics of amide bands at (1632 cm^{-1}), due to the stretching vibration of peptide C=O groups, and amide II band at (1594.6 cm^{-1}), which results from a combination of N-H in plane bending and C-N stretching of peptide groups. The appearance of peaks at 1632 cm^{-1} and 1594 cm^{-1}

confirms of the immobilization of HRP on AuNPs-PANI matrix.

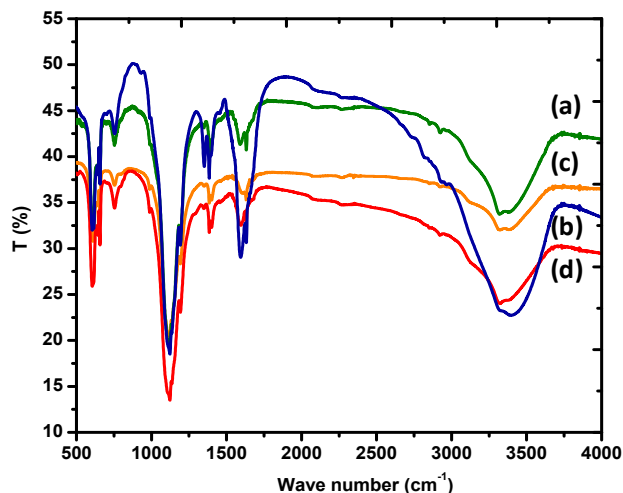


Fig. 3. FT-IR spectra of (a) PANI/ITO (b) AuNPs/ITO (c) AuNPs/PANI/ITO (d) AuNPs/PANI/HRP/ITO.

Scanning electron micrograph (SEM) study

In order to get insight into the surface morphology of the working electrodes SEM micrograph was used to investigate. **Fig. 4 (a)** shows SEM study at lower magnification that reveals electrodes surface consisting of porous matrix of PANI, but at higher magnification, porous morphology along with fiber like was observed in PANI (**a'**). **Fig. 4 (b)** shows agglomeration particles of AuNPs around 53.33 nm (**b'**) at higher magnification which were found embedded in PANI. Successful adsorption of the HRP on the surface of AuNPs-PANI nanocomposite has been observed in **Fig. 4 (c)**, it is also clearly observed in the higher magnification morphology of AuNPs-PANI-HRP (**c'**).

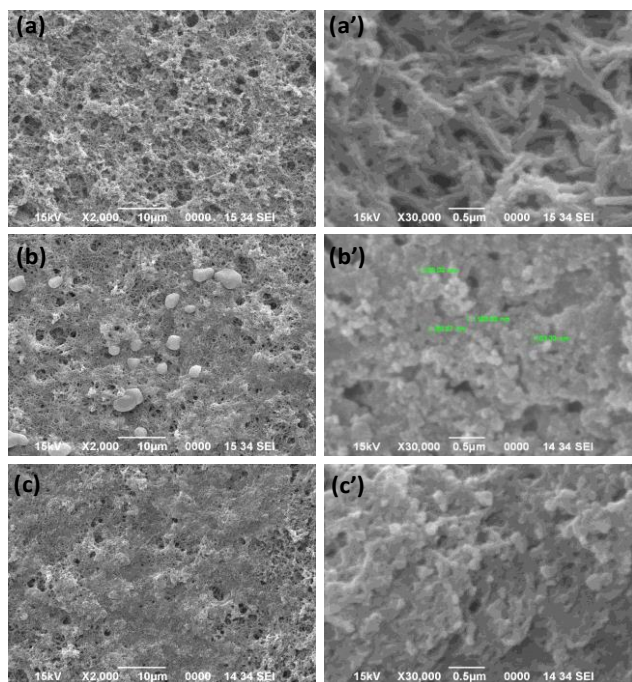


Fig. 4. SEM micrograph of the surface (a & a') PANI, (b & b') AuNPs/PANI, (c & c') AuNPs/PANI/HRP electrodes.

Electrochemical impedance spectroscopic (EIS) studies

Fig. 5 shows the Nyquist plots of (a) PANI/ITO, (b) AuNPs/PANI/ITO, and (c) AuNPs/PANI/HRP/ITO bio electrodes carried out in 0.05 M phosphate buffer solution at pH 7.01±0.1 containing 0.9 % NaCl. The R_{CT} value of AuNPs-PANI-HRP-ITO bioelectrode increases as compared to the AuNPs/PANI/ITO electrode. This is in agreement with the facts that, immobilization of HRP on the electrode surface has offered electron transfer resistance in redox probe resulting in threefold increase of R_{CT} value. The above fact is further supported by the CV studies (**Fig. 2**) in which observation relating to decrease in peak current at AuNPs_PANI_HRP/ITO electrode (c) compared to AuNPs/PANI/ITO electrode (b) suggests successful immobilization on the electrode.

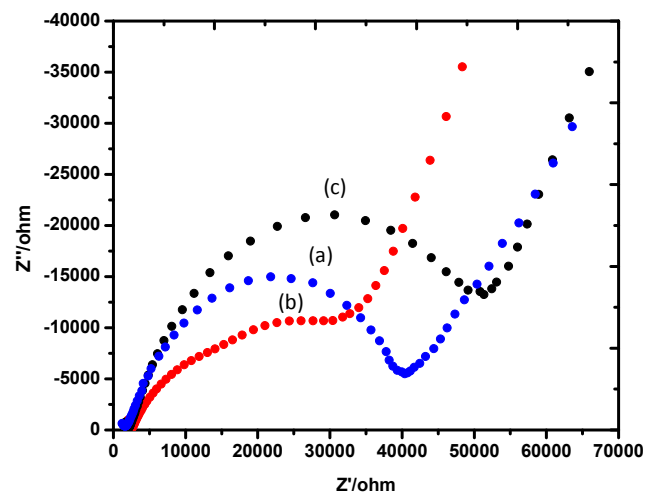


Fig. 5. EIS Nyquist plots of (a) PANI/ITO (b) AuNPs/PANI/ITO (c) AuNPs/PANI/HRP/ITO electrodes.

Reproducibility and storage stability of the biosensor

In order to check the performance of the HRP based nanocomposite biosensor for determination of gemcitabine, reproducibility and storage stability of the biosensor were carried out by keeping the biosensor electrode in the dark at 4 °C for 30 days and were analyzed every day. It has been seen that repeatable peak currents of gemcitabine (0.1 ng mL⁻¹) occurred up to 16 days and after that the peak current decreased significantly. Reproducibility and storage stability were calculated in terms of residual standard deviation yielding a value of 1.23% (n=5). So, the present approach on AuNPs/PANI/HRP/ITO electrode as biosensor has demonstrated better reproducibility for a comparatively longer period and storage stability at 4 °C.

Application of method to the pharmaceutical dosage forms

The developed amperometric biosensor was successfully applied for determination of gemcitabine in parenteral form (*Tabicad*) **Fig. 6**. Parenteral gemcitabine labeled to contain 200 mg of gemcitabine was transferred to 100 mL volumetric flask and completed to volume with freshly prepared 0.05M phosphate buffer solution (PBS) with pH 7.01±0.1 to make a stock solution of 2.0 mg mL⁻¹. There was no need for filtration of injection extracts from

undissolved excipients, just dilution of an aliquot with the supporting electrolyte (PBS, pH 7.01±0.1) is required before measurement. Voltammograms of gemcitabine in 0.5 M phosphate buffer solution of pH 7.0±0.1 containing 0.9% NaCl exhibit well defined cathodic peak at the potential range 0.10±0.05V. The current is mainly diffusion controlled and proportional to the concentration over a convenient range. **Fig. 6** (*in inset*) shows that under the optimized condition the linear relationship between the gemcitabine concentration and peak current is due to the generation of peak product hydrogen peroxide. The amount of compound in the dosage form was calculated by standard addition method. The effect of excipients on the voltammetric response of gemcitabine was studied and it was shown in the proposed method, co-administered drugs did not interfere. The analytical performance data of the proposed method are compiled in **Table 1**.

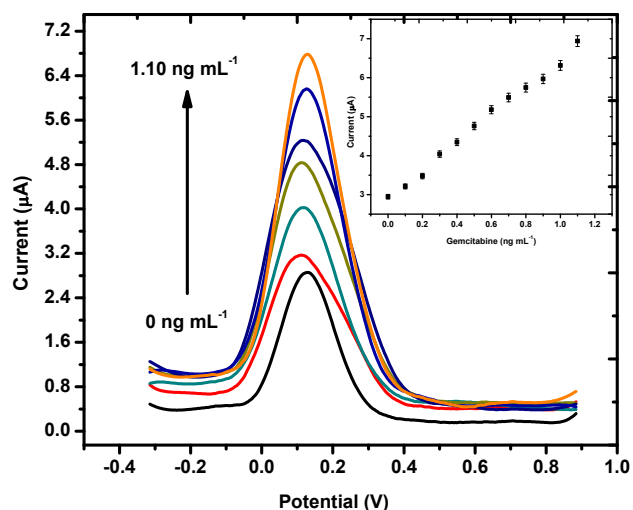


Fig. 6. Biosensors response with increases in concentration of gemcitabine and inset calibration curve shows linearity for gemcitabine as 0.1 to 1.10 ng mL⁻¹.

Table 1. Regression data of the calibration lines for quantitative determination of Gemcitabine by hybrid nanocomposite AuNPs/PANI/HRP based biosensor in bulk form, dosage form using DPV waveform.

Parameters	Bulk form	Injection
Linearity range (ng mL ⁻¹)	0.10 – 1.10	0.10 – 1.10
Slope(A/µg mL ⁻¹)	4.01416 × 10 ⁻⁶	3.555 × 10 ⁻⁶
Intercept (A)	2.80613 × 10 ⁻⁶	2.5576 × 10 ⁻⁶
Correlation coefficient (r ²)	0.994	0.992
RSD of the intercept	4.675 × 10 ⁻⁸	3.690 × 10 ⁻⁸
RSD of the slope	8.740 × 10 ⁻⁸	9.10 × 10 ⁻⁸
Reproducibility (%RSD)	1.05	1.09
Repeatability (%RSD)	1.03	1.11
LOD (ng mL ⁻¹)	0.034	0.031
LOQ(ng mL ⁻¹)	0.106	0.103

The percentage recovery of gemcitabine based on the average of five replicate measurements was found as 99.00 ±0.08% (**Table 2**). The accuracy of the proposed procedure was also judged by applying the standard addition method and excellent percentage recovery of added gemcitabine was achieved. Comparison with previously developed

methods for analysis of gemcitabine with newly developed biosensor based on hybrid nanocomposite AuNPs/PANI/HRP is presented in **Table 2**.

Table 2. Application of the proposed AuNPs/PANI/HRP biosensor for the analysis of gemcitabine in dosage form.

Parameter	AuNPs/PANI/HRP ^a biosensor
Labeled amount (mg)	200
Amount founded (mg)	198±0.08
Standard error	0.080
Added (ng mL ⁻¹)	0.100
Founded (ng mL ⁻¹)	0.099
Recovery (%)	99.00±0.08
%RSD of recovery	1.4±0.08

^aEach value is the mean of five experiments.

Conclusion

Organic-inorganic AuNPs/PANI/HRP/ITO biosensor based nanocomposite probe for direct determination of gemcitabine in bulk and dosage form is developed and studied for the first time. The electrochemical behavior of gemcitabine under the conditions described in this work is an irreversible process controlled by diffusion. The proposed method with detection limit of 0.031 ng mL⁻¹ has distinct advantages regarding sensitivity, time saving and minimum detectability over other existing methods HPLC [22-24] with LOD in the range 50.0 ng mL⁻¹ to 187.0 ng mL⁻¹, electrochemical sensor [26] with LOD 2.61 ng mL⁻¹, voltammetry [25] with LOD 317.0 ng mL⁻¹ and Spectrophotometry [27] with LOD 440.0 ng mL⁻¹ electro-oxidation at gold electrode [46] with LOD 17.979 ng mL⁻¹. In addition no sophisticated instrumentation is required. Consequently, the proposed methods have the potential of a good analytical alternative for the determination of anticancer drug gemcitabine in pharmaceutical formulation. Further, it is proposed that the biosensor method could also be successfully applied for determination and detection of gemcitabine in biological fluids after few studies.

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