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DNA Biosensor for Determination of 5-Fluorouracil based on Gold Electrode Modified with Au and Polyaniline Nanoparticles and FFT Square Wave Voltammetry

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Abstract

In the present study, a new biosensor for 5-Fluorouracil was described using modified gold electrode and Fast Fourier transform square wave voltammetry (FFT SWV). Calf thymus DNA immobilization was on a gold electrode decorated with polyaniline and gold nanoparticles. The electrochemical characteristics of the electrodes were investigated by cyclic voltammetry, and electrochemical impedance spectroscopy. After interaction DNA with the analyte, a dramatic decline in $[Fe(CN)_6]/[Fe(CN)_6]]^{3-/4-}$ signal was observed which could be used as the signal. The important parameters of the detection system, such as, pH were optimized. The linear dynamic range of the biosensor was1×10^{-9} to 1×10^{-6} M (r^2=0.996) limits of detection of 2.5×10^{-10} M. In addition to the wide linear range response, the proposed method demonstrated acceptable sensitivity, repeatability and long-term stability. Future experiments could be performed to examine several other effective factors so as to decrease the influence of the environment and investigate its application to FFTSWV detection.

Keywords: Electrochemical DNA biosensor, 5-Fluorouracil, Gold electrode modification, Fast Fourier transform voltammetry.

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Introduction

In recent years, 5-fluorouracil as an antitumor active compounds is widely used in the treatment of cancer (solid tumors), which metabolizes rapidly in the body. However, measuring safe and effective dosage of drugs for chemotherapy is still a considerabletask in the treatment. Therefore, measurement of the concentration of 5-fluorouracil in biological samples is extremely demanded to maintain an optimal concentration of the analyte.In fact,an overdose of 5-fluorouracil may cause very high toxicity by accumulation in cancer patients. Controlling the amount of the analyte in a given formulation and the dose to the sufferers is important in pharmaceutical quality control. Several analytical procedures have been aimed for the detection of 5-fluorouracil based on spectroscopic [1,2], chromatographic capillary [3], electrophoresis [4] and electrochemical methods [5,6]. Nevertheless, the main problem of the detection of 5-fluorouracilin the electrochemical measurements is the poor oxidation of the analyte at all solid electrodes. Therefore, highly sensitive and selective biosensor systems are still in need for the determination of5-fluorouracil.

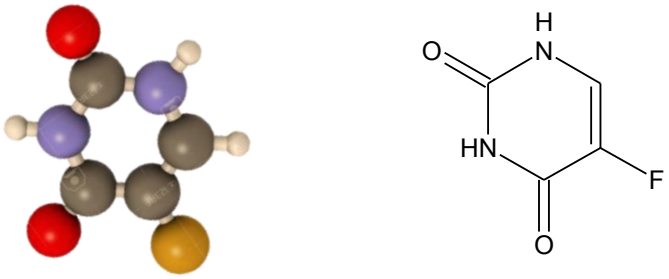

Figure 1. Chemical structure of 5-Fluorouracil.

In this direction, more attention has been devoted to the development of DNA biosensors for5-fluorouracil.Such DNA biosensors are very attractive because of their rapid, simple, and selective detection. The use of conducting polymers, such as polyaniline (PANI), as electrode materials has increased widely in the field of biosensor and can be used to enhance stability, speed and sensitivity. In fact, PANI can be simply prepared from aqueous and organic solvents by either chemical or electrochemical oxidative polymerization. Among them, electrochemical polymerization of PANI is a simple, relatively inexpensive and convenient way to prepare film structures. The synthesis conditions are affected by the properties of the polymeric film such as conductivity. These electrochemical techniques are convenient for being simple and for not requiring a labeling step. The electrostatic interaction between the negatively charged DNA and the positively charged PANI backbone results in DNA immobilization. Also, Au nanoparticles play an importantrole in the electrode transduction enhancement of the affinity reaction as well as in the efficiency of DNA immobilization in a stable form.

Using fast Fourier transform (FFT) method was found very sensitive system in combination by electrochemical method for trace detection of compounds [7-10]. The detection method, fast Fourier transform square wave voltammetry (FFTSWV), which is introduced here, is very sensitive, inexpensive and fast. The square wave voltammetry (SWV) has recently been shown to be advantageous for environmental detection of several compounds [10-14]. In particular, when the magnitude of current is in the range of nano and pico ampere, electrochemical response suffers from existence of environmental noises. In fact, the analyte signal is calculated based on admittance changes related to the changes in electrical double layer.

This paper describes a fundamentally different approach to SWV measurement, in which the detection limits are improved, while preserving the information content of the SW voltammogram. A new biosensor sensor exhibited an improved electrochemical response to the presence of 5-Fluorouracil. Where, the electrode response was obtained by a special square wave electrochemical method called coulometric FFTSWV. The electrochemical behavior of5-Fluorouracilwas investigated on the sensor carefully with the electrochemical parameters optimized.

Experimental

Reagents

5-Fluorouracil was purchased from Sigma-Aldrich and was stored in the frozen state. The prepared solutions were kept at 4 °C before use. Double distilled water was used throughout the experiments. All oligonucleotides were purchased from Generay Biotech. Calf thymus DNA (CT DNA obtained from Sino-American Biotechnical) was used as receive solutions of DNA ($\approx 10^{-4}$ M in nucleotide phosphate NP) in were purified 5mM and the $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture containing 0.1M KCl was usedas a redox probe in the electrochemical measurements 5-Fluorouracilinthe buffer solution.

Polyaniline (PANI) solution was synthesized sonochemically. In brief, 1.59 mL of the purified aniline was dissolved in 50 mL of 1 M HCl aqueous solution with maintaining vigorous stirring at room temperature, at the same time, 40 mL of 0.3 M ammonium peroxydisulfate solution was quickly poured into the solution, which followed by 1 h mixing in ultrasonic bath in 4ºCuntil, a green salt obtained, then it was washed with distilled water and HCl until the filtrate was colorless. At the end, the precipitate was dried in vacuum oven at 60ºC for 24 h.

Instruments and electrodes

Voltammetric FFTSWV measurements were performed by using a homemade potentiostat connectedwith a three-electrode system, consists of the biosensor, an Ag/AgCl reference electrode

and a platinum wire as the auxiliary electrode. For controlling, it was connected to a PC equipped by an analog to digital data acquisition board (PCL-818H, Advantech Co.). During the measurements, the memory requirements of the computer were ordered or controlled by the electrochemical software that was developed in our lab. For the experiments, the current data was acquired, and stored by the software. The electrochemical program was employed to generate an analog waveform and acquire admittance readings. It also, processed and plotted the data in real time [15-18].

Fabrication of AuNP/PANI /Au andProbe DNA immobilization and hybridization

The Au disk electrode (3mm in diameter) was polished with 0.3μm and 0.05 μm alumina powders respectively, and then cleaned ultrasonically in 0.01 M NaOH solution and dried with blowing N_2. 10 μLof the PANI suspension (0.04 g/L) was dropped on the electrode surface and then it was dried using by IR lamp, to from PANI/Au electrode. At that point, to complete the modification process the electrochemical deposition of Au NPs was performed in a solution of 0.3 M Na_2SO_4 and $HAuCl_4$ (1.0 mM). The deposition time was about 10-150 s and the potential was -0.2 V. After that, the surface of the Au NPs/PANI/Au electrode was carefully washed with distilled water and dried at room temperature. The mean size of the prepared Au NPs was about 30-90 nm, estimated by transmission electron microscopy. Finally, the modified electrode was activated by several successive potential scans from 0.4 to 1.0 V with a scan rate of 50 mV/s in phosphate buffer solution (pH 6.0) until a steady voltammogram was obtained. 10 μL of 8 μM DNA solution was dropped on modified electrode surface and kept for 30 min in room temperature. The DNA/Au NPs/PANI/Au electrode was rinsed with buffer for 10 s to prevent nonspecific adsorption of DNA.
The DNA analyte reaction was performed by dropping 7μL of appropriate concentration of the analyte solution on DNA/AuNPs/PANI/Au recognition interface and the reaction was kept for 30 min. Then the electrode was washed with buffer for 10 seconds to remove the unbound analyte. Figure 2 represents the electrode modification and the immuno sensor fabrication steps.

Real sample solutions

5-Fluorouracil citrate tablet commercial pharmaceutical formulation samples of were selected for the analysis. To prepare stock solution of the drug sample, a group of 10 tablets was individually weighed, finely powdered and mixed. A portion of each of the powder 40–60 mg was accurately weighed and transferred into a 50 mL volumetric flask containing 25 mL of the buffer solution. The solution was next filtered through a 0.45 μmmilli-pore filter. The desired concentration was obtained by accurate dilution with water. Then an aliquot of 1.0 mL of the sample solution was

diluted with the buffer solution. The 5-Fluorouracil content was measured according to the recommended procedure.

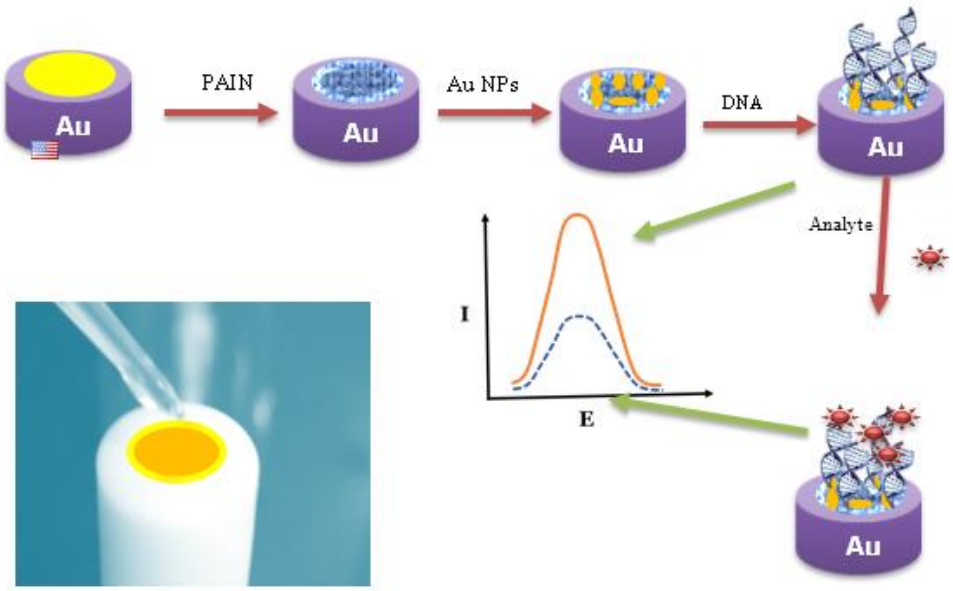

Figure 2. Schematic presentation of thebiosensor fabrication.

Results and discussion

Surface characterization of fabricated biosensor

In order to characterize the modified surface of the electrode, the surface morphologies of Au NPs/PANI/Au was examined by SEM method. Figure 3(A) shows the SEM image of composited film PANI/Au, which clearly illustrates the film is a homogeneously distributed modified electrode film without aggregation and highly entangled network structure. Figure 3(B) indicates that the deposited Au NPs consist of homogeneous spherical-like nanoparticles, in which the amount of deposited NPs on the surface of the substrate following its specific morphology. However, application of a longer deposition time is used the electrochemical deposition may capable to develop its specific composite structure.

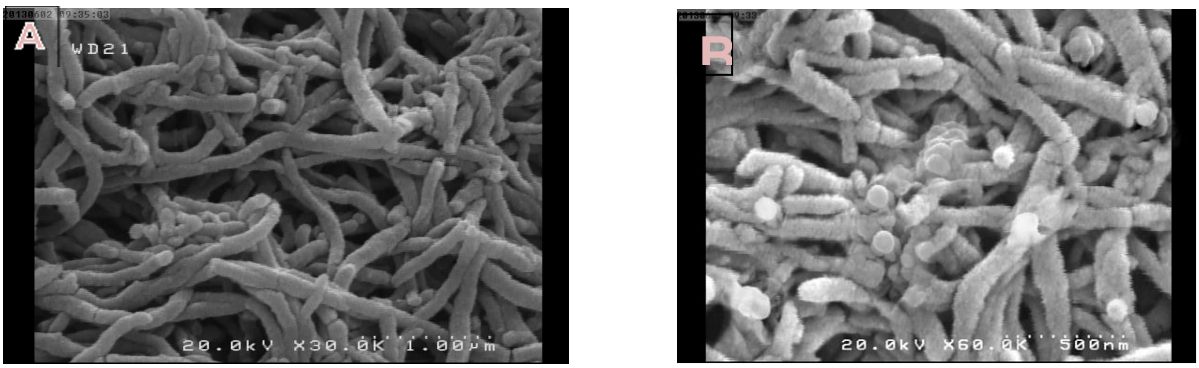

Figure 3.SEM images of A)PANI/Au, B)/Au NPs/PANI/Au.

Electrochemical characterization of the biosensor

Cyclic voltammetry (CV) can provide useful information on the barrier changes of the electrode surface during the fabrication process Therefore, the CV of The redox signal of $[Fe(CN)_6]^{3-/4-}$ couple 5mM $[Fe(CN)_6]^{3-/4-}$ containing 0.1 M KCl was extensively used as a marker ion to investigate the changes of the electrode behavior before and after each assembly step. Figure 4 displayed the CVs of differently modified electrodes: Au, (b) PANI/Au, (c) /Au NPs/PANI/Au and (d) DNA/Au NPs/PANI/Au, electrodes atscan rate of 50 mV/s. A couple of redox peaks could be observed in all CV plots. As expected the current responses of PANI/Au decreased and then Au NPs/PANI/Au increased noticeably compared to those of \/PANI/Au, indicating that the electron transfer rate at the electrode surface was accelerated thanks to large surface area and excellent electrical conductivity of nanocomposite film. After immobilization ofDNA probe on the modified electrode, the peak current decreased significantly, which could be attributed to the electrostatic repulsion between the negatively charged DNA and $[Fe(CN)_6]^{3-/4}$ anions. The electroactive surface area of the electrodes was calculated according to Randles–Sevcik equation $i_{pa}=2.69\times10^5 AD^{1/2}n^{3/2}\nu^{1/2}$ C,where i_{pa} is the anodic peak current, n the electron-transfer number,A the surface area of the electrode, D the diffusion coefficient, C the concentration of $[Fe(CN)_6]^{3-/4-}$ and v the scan rate. It is clear that the AuNPs /PANI/Au surface area is higher than that of the corresponding bare electrode, indicating the synergistic effect of Au NPs and PANI on enhancing the electroactive surface area of modified electrode.

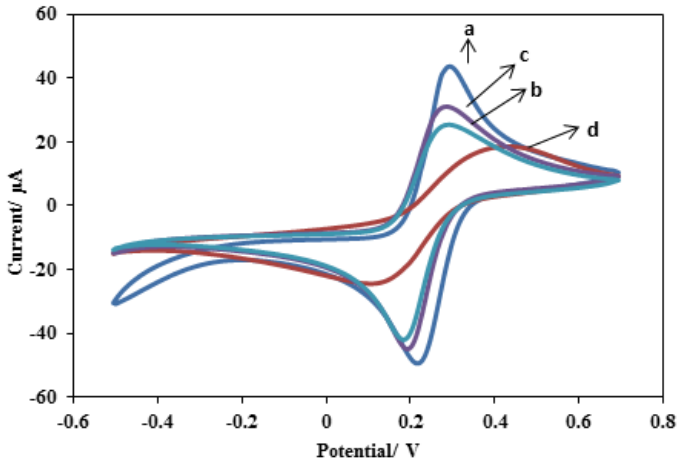

Figure 4. Cyclic voltammograms of (a) bare Au, (b) PANI/Au, (c) /Au NPs/PANI/Au, (d) DNA/Au NPs/PANI/Au, electrodes in PBS solution, pH 7.4, containing 50 mMKCl and 5mM $K_3[Fe(CN)_6]$ and 5mM $K_4[Fe(CN)_6$, Scan rate 50 mVs^{-1}

Using EIS method with $[Fe(CN)_6]^{3-/4-}$ as a probe has been extensively used as an effective tool for electrochemical study of electrode fabrication. Where, the impedance spectrum, Nyquist diagram, includes a semicircle part at high frequencies and a linear part at lower frequencies. The semicircle

diameter corresponds to the electron-transfer resistance (R_{ct}), which used as the successfulness of the electrode fabrication process. Therefore, for this electrode EIS measurement was measured in frequency range from 0.1Hz to 1 MHz, with a DC potential of 0.2 V and AC amplitude of 0.01 V,in PBS solution, pH 7.4, containing 250 mMKCl and 5mM$K_3[Fe(CN)_6]$ and 5mM $K_4[Fe(CN)_6$,Figure 5, curves a to d display the EIS for the electrode fabrication steps. Curve a displays the Nyquist diagram of the bare Au electrode, which is almost straight line that points to existence of diffusion controlled electrochemical process at the electrode surface. In case of PANI/Au electrode, the value of R_{ct} significantly increased, due to the high blocking effect of the formed polymer layer on diffusion of $[Fe(CN)_6]^{3-/4}$ions (curve b).

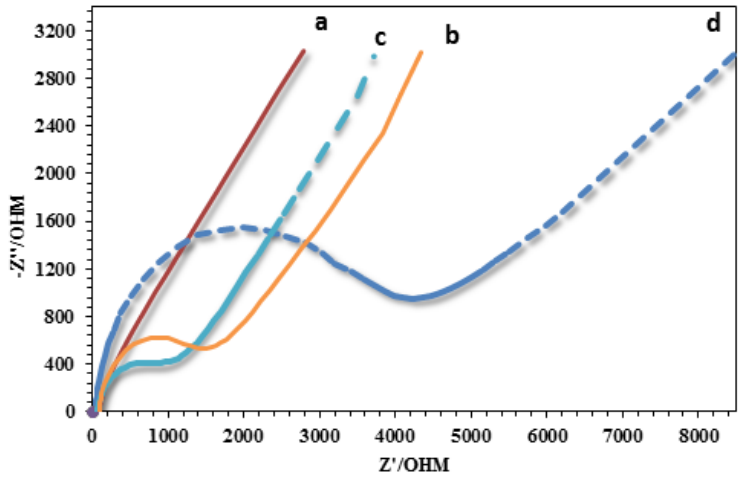

Figure 5. EIS plots of(a) bare Au, (b) PANI/Au, (c) /Au NPs/PANI/Au, (d) DNA/Au NPs/PANI/Au, electrodes in PBS solution, pH 7.4, containing 250 mMKCl and 5mM $K_3[Fe(CN)_6]$ and 5mM $K_4[Fe(CN)_6$, Frequency range 0.1Hz to 1 MHz, DC potential of 0.2 V and AC amplitude of 0.01 V.

However, when Au nano-particles deposited the value of R_{ct} is dramatically decreased (curve c). As mentioned in the CV section, the enhancement of the conductivity of the deposited NPs. As shown in curve d, in case of DNA/Au NPs/PANI/Au electrode showed higher R_{ct} value. Fig.5 shows FFTSW the changes in voltammetric of the biosensor in the potential range of 500 to1200 mV. The time axis represents the time passing of the experiment and the sweep number [20-28].The figure shows that at the begging of the experiment there is not a significant signal in the voltammograms, but after addition of 1.0×10^{-6} M 5-Fluorouracil in the buffer solution at pH 7.4.

Dependence of the electrochemical detection method on the experimental conditions needs to be investigated to obtain the best performance of the sensor, which the most important are, pH and the parameters for square-wave voltammetry (frequency, pulse amplitude and scan increment).

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Optimization of the parameters

In FFTSWV technique the admittance voltammetric response of the biosensor depends on the applied conditions of excitation waveform, where the square wave frequency and amplitude on the analyte response. In order to optimize the sensor response to 5-Fluorouracil the SW parameters, frequency, SW amplitude in CFFTAV method were studied and amplitude 5 to 50 mV. The effect of frequency on the sensor response was studied in the range of 100–1000 Hz. Furthermore, the background noise and peak shape of 5-Fluorouracil are depends on the factors in the square wave form. In Figure 6 the results of change in the response at various value of the square wave frequency and amplitude is demonstrated for solution of 1 µM of 5-Fluorouracil.

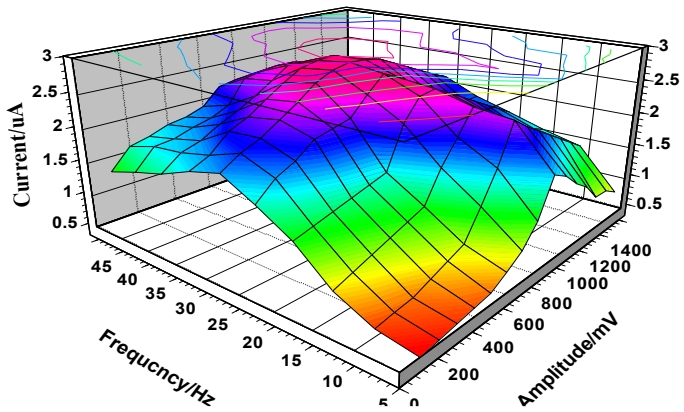

Figure 6. The effect of frequency and amplitude on the response of the biosensor to addition of 1.0×10^{-6} M 5-Fluorouracil in the buffer solution at pH 7.4.

The outcome of varying the SW amplitude of the applied potential was studied from 5 to 50 mV. The value of 30 mV was chosen for the biosensors. The best analytical signal obtained was at 500 Hz. These experimental conditions were selected for subsequent experiments. The enhancement of the signal may due to improvement is similar to the enlargement of current in cyclic voltammetry with potential scan rate. However, after that value the signal decline. This may due to kinetic limitation in electron transfer rate. Therefore, it is expected that the value of the sensor response is limited at high values of the square wave amplitudes.

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Optimization of pH and the biosensor composition

Figure 7displays the change in response of the sensor for addition of 1.0×10^{-6} $molL^{-1}$ 5-Fluorouracil in the buffer solution at pHs4 to 10. The following measurements were recorded at frequency 700 Hz and amplitude 20 mV. The results showed that when pH of the buffer solution was increased up to 7.4, oxidation current peak improved and oxidation potential shift to less positive potential. This is an induction of facilitated electron transfer at the senor surface. Conversely, at pHs higher than

the magnitude of the biosensor response decreased. Therefore, pH 7.4 was selected as the optimal pH for detection in the following experiments.

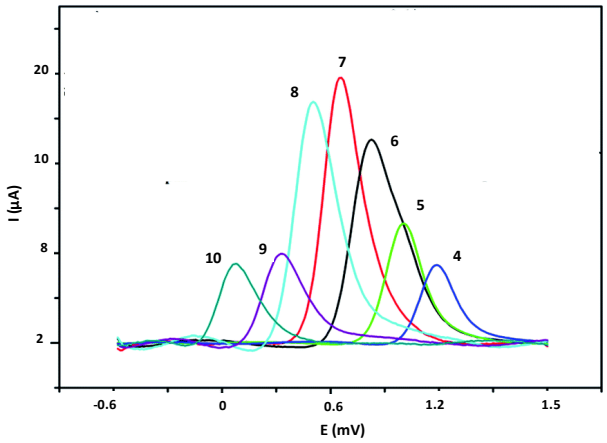

Figure 7. Current response of 5-Fluorouracil solution 1.0×10^{-6}molL^{-1} in the buffer solution at pHs 4 to 10.

Calibration curve and sensor characterization

For the measurements 10 mL of the drug solutions were introduced into the cell, and the FFTSW voltammogram was recorded. The standard deviations were estimated using both the calibration curves and standard addition methods. All data were obtained at room temperature. In this electrochemical detection method, the differential FFTSWV current of 5-Fluorouracilwas used for plotting the calibration curve, which is shown in figure 8.As can be seen in Fig. 8, the ΔIof 5-Fluorouracil solutions (from 1.0 to 800.0 nM in PB, (pH 7.4) increased gradually with increments of the concentration of the analyte and lastly leveled off.

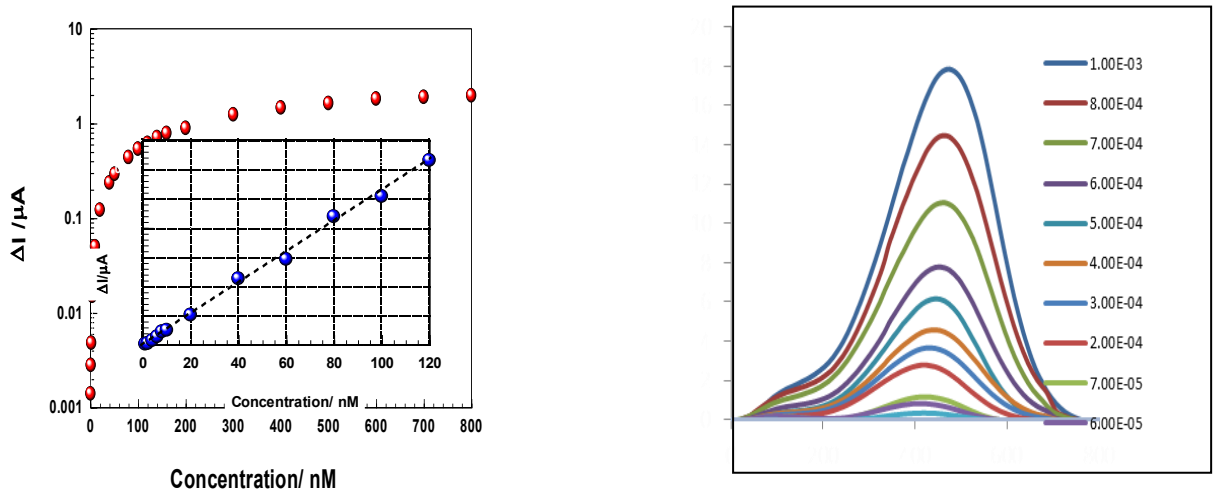

Figure 8. The calibration curve for 5-Fluorouracil standard solutions in the range of 0.1 nM to800 nM.The inset shows the linear relationship between ΔI and log(C) (nM) in the range of 0.1 nM to 120nM.Inset voltammogram,Response of thebiosensor upon the following addition of1nMof 5-Fluorouracilsolutions.

Results shown in this figure represent the integrated signal for 3 consecutive additions of the 5-Fluorouracil standard solutions

To obtain defection limit of the sensor for measurement of 5-Fluorouracil at first, a stock solution of 10 nM 5-Fluorouracil was freshly prepared, and then for making the other standard solutions, an aliquot was diluted to the appropriate concentration (see Figure 8 insets).The calibration equation was fitted line with a correlation coefficient of 0.996(n=5, SD=0.0067). A detection limit of 2.2×10^{-10} M, was estimated in evaluation, the performances of the fabricated biosensor is compared with some of the best previously reported.

In these measurements, the obtained recoveries for the spike milk samples were ranged from 98.5% to 101.1% and the contents of 5-Fluorouracil found are in good agreement with that specified by the manufacturers. The results are shown in Table 1. These results indicate that the FFTSW voltammetry method has acceptable precession and accuracy for rapid and sensitive determination of 5-Fluorouracil in pharmaceutical tablets. Also, in evaluation, the performances of the fabricated biosensor is compared with some of the best previously reported 5-Fluorouracil sensors based on the utilization of different materials as the working electrode and different detection techniques and it was confirmed that the presented biosensor for5-Fluorouracil with FFTSWV exhibited an excellent and reproducible sensitivity.

Table 1. Determination of 5-Fluorouracil in pharmaceutical tablets by standard addition method (concentrations in (nM)).

Samples	Detected	Added	Found	Recovery (%)
1	6.5	6.0	11.2	98.5
2	10.3	20.0	30.5	101.1
3	42.5	40.0	82.1	99.1
4	44.9	40.0	82.2	99.2

Stability and reproducibility of the electrode

The biosensor was evaluated by examining the analyte response, using FFTSWV technique over a long time period. It storage stability was investigated for 45 days at room temperature when not in used. The results showed that the sensitivity reduce only 4.7± 0.3% up that time, which gradually decreases afterwards might be due to the adsorption of impurities. In addition, after that time the biosensor response reaches to 95.2 of the initial current response.

Conclusion

A new determination technique was developed for the detection of 5-Fluorouracilin real samples based on the FFT SWV method, and DNA biosensor. The assay provides a much improved sensitivity for the detection of 5-Fluorouracil.Under optimal conditions, the designed biosensor exhibited a wide linear response to 5-Fluorouracil concentration, good sensitivity, repeatability and long term stability, 45days.Future experiments will be required to study the various factors in detail so as to lessen the influence of the environment, and investigate its application to FFTSWV detection.

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