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Electrochemical DNA biosensor for the detection of sanguinarine in adulterated mustard oil

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ABSTRACT

PANi/ClO_4 doped films were developed electrochemically to immobilize DNA as biosensing platform to detect sanguinarine from adulterated mustard oils. The principle of technique was based on the interaction/intercalation of sanguinarine with dsDNA using electrochemical method. Further, it was suggested that sanguinarine intercalates with DNA strands forming complexes, results in the decrease of redox peak currents. In addition, the decrease of the peak current is proportional to the concentration of sanguinarine. The results based on the voltammetric signals decreased in concomitant increase of sanguinarine concentrations due to base pairing in dsDNA. The bioelectrode exhibited the detection limits 2–64 μM. The recovery experiment results found between 89% and 121% from spiked edible mustard oil sources. The correlation found between the current vs. concentration of SA with a correlation coefficient of (r^2) of 0.995 at 95% confidence limit. UV–VIS, CV, DPV, AFM, and SEM. characterized the bioelectrodes. Thus, the proposed electrochemical DNA biosensor detected SA and promising for real-time analysis of small molecules of environmental interest. Copyright © 2010 VBRI press.

Keywords: Double stranded deoxyribonucleic acid; polyaniline; biosensor; sanguinarine

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Introduction

Sanguinarine (SA), a benzophenanthridine alkaloid derived from plant species, with multiple biological and pharmaceutical effects, like anti-proliferative agent, anti-

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bacterial, anti-tumour, anti-oxidative activity, anti-gingivitic, anti-periodontic, commercial tooth paste and oral ringes for their anti-plaque effect, DNA-intercalater, antifungal properties, hepatotoxic, mullucicidal [**1-6**]. Recently, a discussion re-opened regarding its toxicity, which focused on Epidemic Dropsy (ED) in India. Edible mustard oil is the source of essential fatty acids, which maintain cell membrane integrity [**7**]. Several epidemics reported in different cities of India and other countries including Bangladesh, Fiji Island, Madagascar, Mauritius, Nepal, and South Africa. However, 1998 epidemic dropsy in Delhi was possibly the largest so far wherein over 3000 persons hospitalized and 65 lost their lives. Sanguinarine and dihydrosanguinarine are the toxic etiological agents in Argemone oil [**8**]. The main symptoms of the ED include vomiting, diarrhea, anorexia, nausea, dyspnea, palpitation, hyper pigmentation of body parts, burning sensation of eyes, bilateral pitting edema of lower limbs, erythema, breathlessness, tachycardia, hepatomegaly, Glaucoma, cardiac and respiratory failure, oxidative stress, crepitations in the lungs and gallop rhythm [**9-12**]. Recently, *in vivo* studies have shown that AO and isolated sanguinarine alkaloids possess genotoxic and carcinogenic potential and this implicated to DNA damage in blood of dropsy patients, which might be the reason for the incidence of hepatobiliary/gall bladder cancers in the North and Eastern regions of India [**13**]. AO or isolated Sanguinarine alkaloid shown to cause DNA damage in liver, bone marrow, and blood cells using alkaline comet assay in mice [**14**]. AO intoxication in rats have revealed that toxicity is mediated by excessive production of reactive oxygen species (ROS), which may induce lipid peroxidation (LPO) leading to the disintegration of hepatic biomembranes including microsomal membrane thereby causing destruction of cytochrome P-450 protein [**15**] and even as low as 0.01% Argemone oil in diet can induces toxic manifestations [**16**].

Polyaniline (PANi) is a good conducting polymer with electrochemical activity, chemical stability, environmental stability, proton doping/dedoping, and redox properties. These unique features have been widely used in different type of electronic applications like DNA biosensor with few advantages like low temperature synthesis and tunable conductivity [**17-20**].

The different types of electrode materials used for the investigation of the interaction study such as AuE [**21**], PGE [**22**], GCE [**23**] and SPEs [**24**] by using different electrochemical techniques like CV [**25**], SWV [**26**], and DPV [**27**] in three different ways, i.e. DNA modified electrode, drug-modified electrode and interaction in solution [**28**]. Gherghi et al., [**29-30**] studied electrochemical DNA biosensor, based on either carbon paste electrode (CPE) or hanging mercury drop electrode (HMDE) and used in the study of interaction among double stranded DNA (dsDNA) and single stranded DNA (ssDNA) and acridine orange, a well-known DNA intercalator. Wang et al. [**31**], Karadeniz et al. [**32**] showed the interaction of analyte with calf thymus dsDNA on the electrochemical DNA biosensor by using voltametric guanine and adenine oxidation signals, which greatly decreased because of analyte-DNA interaction attributed to the binding of analyte to these bases. Marin et al. [**33**] revealed the study of the interaction of analytes with DNA by electrochemical technique, which offers a very attractive route for converting the event into an electrochemical signal.

Until dates and to the best of our knowledge there has not been reported literature or work for the electrochemical detection of the interaction between sanguinarine and DNA by using DPV in combination with the PANi-ClO_4 film on ITO glass plate. The role of the DNA recognition layer is to detect the changes that occurred in the DNA structure during interaction with DNA-sanguinarine provides well evidence for the interaction mechanism and this interaction used for the quantification of sanguinarine in biological fluids as well as adulterated edible oil. The various analytical methods have been established for the detection of sanguinarine, including colorimetric [**34**], paper chromatography [**35-37**], TLC [**38-39**], HPLC [**40-41**], HPTLC [**42**], reversed-phase HPLC (RP-HPLC), capillary chromatography [**43**], LC-MS [**44-45**], etc. The best extraction methodologies without any cleanup procedures are required for the estimation of sanguinarine. It has remarkable oxidation-reduction properties, due to this, electrochemical methods targeted for the analysis. In this manuscript, the mechanism of the interaction between sanguinarine and DNA elucidated through electrochemical techniques like CV and DPV for the detection of sanguinarine extent, which is proportional to the decreased peak current response measured.

Experimental

Chemicals and Reagents

Sanguinarine procured from Aldrich, USA. The stock solution was prepared by dissolving known amounts of sanguinarine in autoclaved deionized water, and refrigerated at 4 ^{o}C. The sanguinarine concentrations of 1, 2, 4, 8, 16, and 32 uM made from the stock solution and used in the experiments. The dsDNA (Salmon testes) obtained from Sigma (USA). The dsDNA stock solution (1mg/ml) was prepared with TE solution (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and kept frozen at -20 ^{o}C. More diluted solutions of DNA were prepared with either autoclaved ultrapure distilled water. Other chemicals were used analytical reagent grade. 3,1-Ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysulfosuccinimide (NHS), procured from Sigma. All solutions were prepared using deionized water. Sodium acetate, acetic acid, sodium chloride, calcium chloride were obtained from Sigma and used as received. Double-stranded ds DNA in TE buffer solution (10 mM Tris–HCl/1 mM EDTA, pH 8.0) gave an A_{260}/A_{280} ratio more than 1.9 and suggested that the DNA was pure enough or free of protein. The supporting electrolyte of DPV experiments was 0.1 M phosphate buffer (pH 7.4). Edible mustard (*Brassica compestris*) oil obtained from the market of Itaewon, S. Korea. The edible mustard oil samples spiked with sanguinarine and treated as the adulterated sample with sanguinarine, which processed by the extraction procedure [**44**]. The concentrations of sanguinarine used to generate a calibration plot using 1, 2, 5, 10, 20, and 40 µg/ml. The interaction between DNA and sanguinarine

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carried out by applying cyclic voltammetry (CV) as shown in **Fig. 1A** and differential pulse voltammetry (DPV) using PANI/ClO_4 film electrode in solution containing PBS buffer in **Fig. 1B**.

Instruments

Cyclic voltammetric experiments (CV) were performed on CHI 660A (CH Instruments Inc., TX, USA). A three-electrode configuration was employed. The working electrode was an ITO glass plate, Ag/AgCl (CHI, 3M KCl) electrode used as the reference electrode, and all potentials reported here referred to this electrode. A platinum wire electrode served as the counter electrode. ITO glass plates were ultra cleaned by using NH_4OH: H_2O_2: H_2O (1:1:4) and sonicated in acetone for 5 min and was deionized water and dried at N_2 gas prior to its us experiments performed at room temperature (25.0±0.5) °C. AFM performed by XE-100, Park Systems, S. Korea, and SEM by FESEM II, JSM 700F, Jeol Company, Japan.

Preparation of bioelectrode

ITO glass plate was ultra-cleaned using NH_4OH:H_2O_2:H_2O (1:1:5) at 80 °C for 30 min. After washing with H_2O immerged in acetone and sonicated for 5 min, then washed with H_2O_2 and dried with N_2 gas. The cleaned dried ITO plates subjected to electrochemically deposited PANi by NPV technique (25s) at 0.9 to 0 V, obtained thin film in comparison to CV and chronoamperometrically. Washed with deionized water, dried in desiccators and subsequently, dsDNA immobilized covalently using coupling reagent (EDC + NHS) for overnight incubation and washed with desired buffer (Buffer PBS pH 7.0) to removed unbound matrix. Subsequently, CV taken in PBS pH 7.0 containing 20 mM NaCl for the confirmation of immobilized DNA or PBS buffer pH 7.0 containing 20mM NaCl.

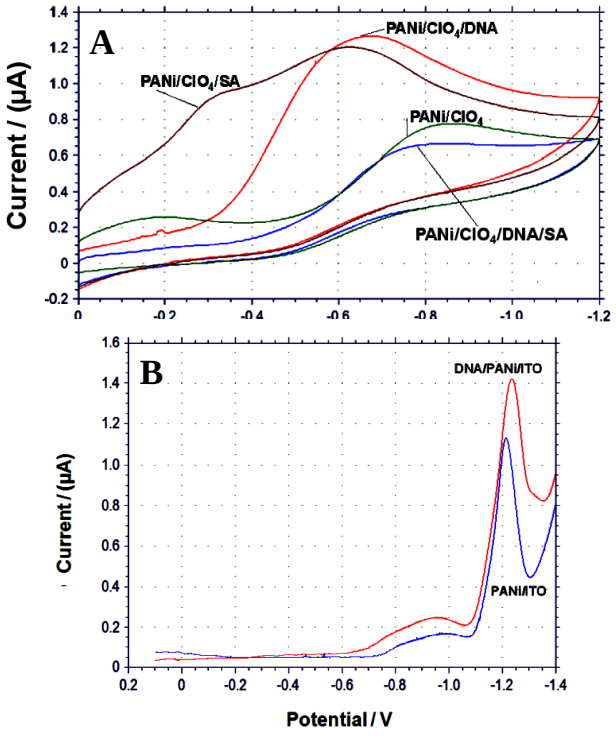

Fig. 1. (A) Cyclic Voltammograms show interaction study of sanguinarine with and without dsDNA using sanguinarine (16 uM) alone and sanguinarine (16 uM) with covalently modified DNA onto the film of PANi in 0.1 M PBS buffer, pH 7.0 and (B) Differential Pulse Voltammetry shows the PANi/ITO film and DNA/PANi/ITO film trend in 0.1M PBS solution, pH 7.0.

Results and discussion

Electrochemical behavior of sanguinarine

The CV measurements demonstrated the ability of SA to undergo oxidation/reduction in PBS buffer pH 7.4 (scan rate of 50 mV/s), obtained a peak anodic potential 700 mV for SA. SA has the polar $C=N^+$ bond to nucleophilic attack and existence of two forms in pH medium as positively charged cation and a neutral base. The electrochemical properties of sanguinarine (SA) on electrochemical oxidation on solid electrodes in buffered aqueous medium investigated. In addition, the detailed study of electro oxidation of SA results in the formation of redox active electro polymerized films, the electro polymerization was possible via formation of alkaloids ortho-benzoquinone derivatives [**46**].

DNA and sanguinarine interaction

The interaction of small molecules with DNA is very important as irreversible covalent and reversible non-covalent bindings. Covalent bonding involves base modification, alkylation, cross-linking of strands, strand breakage whereas non-covalent bonding or interactions are intercalation and groove binding. The DNA has several reactive sites (nucleophilic) depending on the sequence on the surface of the double helix. The DNA interacts with small molecule, complex stabilized by several non-covalent interactions such as van der Waals interactions, hydrophobic forces, and hydrogen bonding. Rarely, small molecule form nonspecific outside edge interactions with the DNA backbone, such a stacking onto the anionic DNA backbone to reduce the charge-charge repulsion between adjacent small molecules. Besides, this major and minor groove of DNA, which may plays a crucial role in binding. Minor groove showed affinity to A=T base pair, in this way the hydrophobic contacts between adenine C_2 hydrogen atoms and aromatic rings of analyte and acceptor C_2 carbonyl oxygen of thymine or N_3 of nitrogen of adenine interact with any hydrogen bond or amide group of analyte/drug. Similarly, hydrogen bonding exists with G≡C base pairs, the NH_2 group of guanine involving N_3 of guanine or cytosine C_2 atoms. In the minor groove, the N_2 amino group of guanine is susceptible to small molecules/drug. The structural perturbations in DNA due to intercalation occur around 1bp spacing, ~3.4A, or some unwinding of the DNA helix. These structural changes occurred on intercalation used for the analytical devices. In the interaction study of small molecule with DNA, water is very important component for the stability of the DNA structure. The water affects the conformation of DNA (NA) due to its high polarity and competition with intramolecular interactions such as hydrogen bonding and causes hydrophobic interactions.

Polyaniline properties

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PANi film on an ITO electrode is electroactive at neutral pH and extensively studied conducting polymers with wide applications due to its unique optical and electronic properties. We have prepared thin film by normal pulse voltammetry (NPV) technique, which is superior to cyclic voltammetry (CV) in respect to fine, uniform, and stable film with responding more rapidly than thicker films. PANi has the two partially oxidized forms one deprotonated emeraldine base (EB) and protonated emeraldine salt (ES). ES form is conducting in nature whereas EB non-conducting. Thus, the conductivity of electrode-supported films is sensitive to pH and electroactive at near neutral pH conditions investigated by several groups. The thin PANi film on an ITO electrode fabricated by NPV technique was nanoporous type, confirmed by SEM within the range of 60-500 nm. Also, our results confirmed that an ITO is the suitable substrate, which may be due to its ionizal 51 groups as counter ion formed thinner films of PAl[illegible].

The electro activity of PANi on an ITO electrode was due to hydroxylated indium species as ionizable counter ion which adsorbed PANi and maintaining redox activity, whereas Pt and Au electrodes show no redox activity at neutral pH i.e., electrochemically inert. PANi containing both amines and imines group acts as polyelctrolyte and adsorbed on an ITO surface. The surface of PANi Layer on ITO moderately hydrophilic like amine terminated SAM and showing consistent presence of amine and imino group at the surface of the film. Oxidation of PANi increases electrostatic repulsion between positively charge chains and alter chain conformation and film restructuring and migration of counter ions and solvents less affect film packing which lead counter ion migration to maintain neutrality and solvent migration into the expanded film and reduction of PANi Film.

Bioelectrode

The schematic presentation showed overall construction bioelectrode for the analysis of SA (**Fig. 2**). PANi/ClO_4 films prepared by NPV technique using three electrodes cell assembly, ITO as working, Pt as counter and Ag/AgCl as reference electrode in comparison to CV (current-voltage) and chronoamerometrically (current-time), found more thin films, and washed well with water and dry in desiccators for subsequent use. Covalent modification of dsDNA on the PANi film was carried out by using equimolar volume of EDC and NHS. Physical adsorption of dsDNA onto the modified electrodes carried out directly by gentle pouring method and kept for overnight prior to their use.

The *in vitro* interaction analysis of sanguinarine by UV-VIS spectroscopy and CV showed in the **Fig. 3A** and **Fig. 3B** respectively. Sanguinarine exhibits fine electrochemical response with a pair of stable redox peaks. However, significant decrease of its peak currents observed after the addition of dsDNA. With the presence of dsDNA, there is no appearance of new redox peaks and no shift of the peak potentials. Note that the peak currents decrease along with the increase of the dsDNA concentrations. The probable mechanisms responsible for the current peak attenuation with no significant change of peak potential suggested. (1) Adsorption between sanguinarine and DNA on the electrode surface, which may induce the peak currents, decreased. It depends on the molecules adsorbed

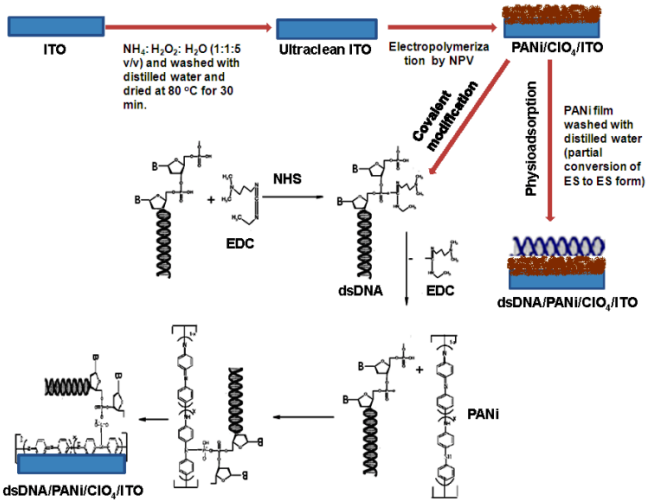

Fig. 2. Schematic presentation of proposed electrochemical DNA biosensor.

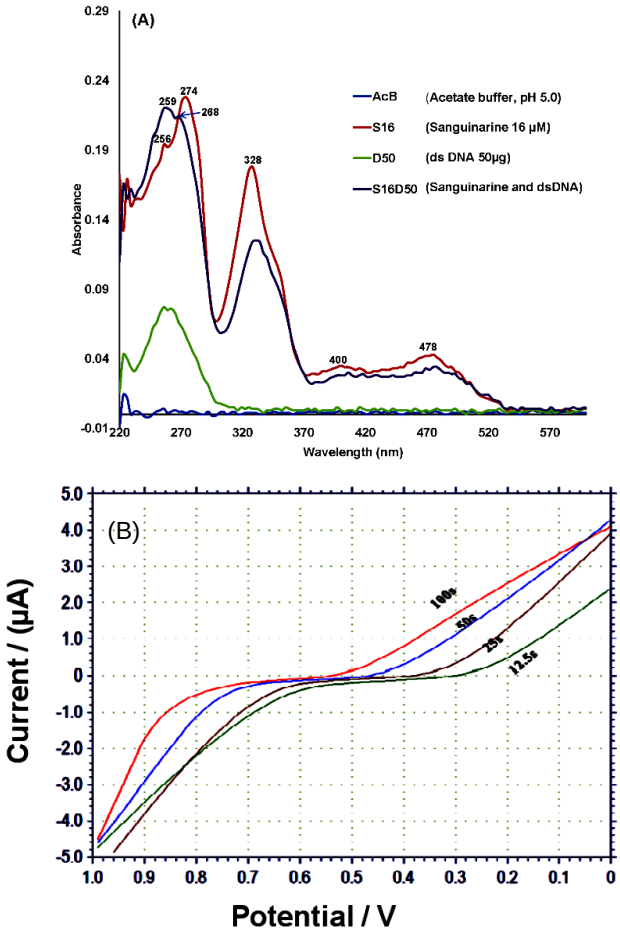

Fig. 3. (A) UV-Vis Spectra show the interaction of SA with dsDNA and **(B):** Figure shows electropolymerization of PANi onto ITO plate by normal potential voltammetry (NPV) in time dependent manner using 0.1 M aniline, 0.1 M Lithium perchlorate solution.

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on electrode at different accumulation times, surface area of electrode. (2) The presence of DNA changes the electrochemical kinetics of the electro active molecules. The major electrochemical kinetic parameters of sanguinarine, either in the absence or in the presence of dsDNA, can influence the electrochemical kinetics. However, the dsDNA does not significantly alter the kinetics of the sanguinarine redox reactions at electrode surfaces. (3) The electrostatic attraction between sanguinarine and DNA and found that the peak currents decreased with the addition of DNA under acidic, neutral and alkaline medium and electrostatic charge of sanguinarine does not affect its interaction with DNA. (4) The finally and most accepted mechanism for the binding and complexation between sanguinarine and DNA. In this context, further the UV-Vis absorption spectrum showed potential evidence for possible intercalation of sanguinarine [**45**]. The CV voltammogram revealed the electrochemical behavior of sanguinarine is different with the addition of dsDNA and ssDNA (data not shown). The peak currents of sanguinarine decreased more sharply with the ad 52 dsDNA than with ssDNA, due to the weaker po ssDNA has and not intercalated with sanguinarine and proved the discrimination between ssDNA and dsDNA (data not shown).

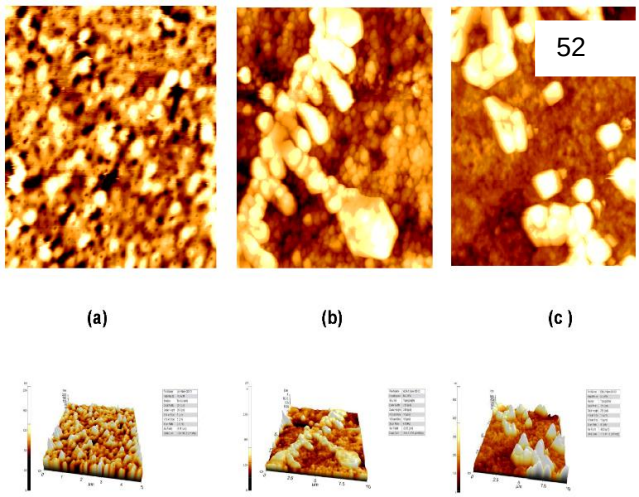

Fig. 4. Shows AFM images (a) ITO/PANi/ClO_4 film, (b) ITO/PANi/ClO_4/dsDNA film; physically adsorbed and (c) ITO/PANi/ClO_4/dsDNA film; covalently (EDC/NHS) modified.

Topographical analysis of bioelectrode by AFM and SEM obtained for the confirmation of desired DNA. The AFM image (**Fig. 4a**) ITO/PANi/ClO_4, physically adsorbed, revealed the morphological distinction and when compared with (**Fig. 4b**) ITO/PANi/ClO_4/dsDNA, covalently (EDC/NHS) modified (**Fig. 4c**) and in similar fashion SEM images as shown in **Fig. 5 (a), (b)** and **(c)**. The regular structures on the surface of electrode revealed the ITO/PANi/ClO_4 formation with uniform, dense whereas prepared bioelectrode surface revealed the change in the morphology attributed due to the presence of dsDNA. Thickness of PANi film was found to be ~5µm. The roughness values of the films before and after the immobilization of DNA were between 1-2 nm.

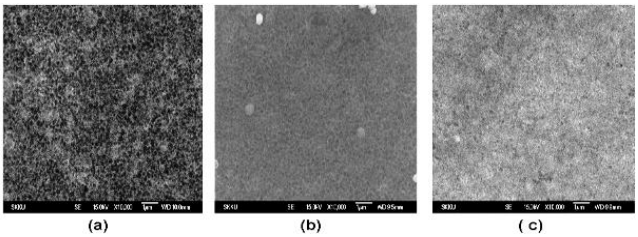

Fig. 5. Shows SEM image (a) ITO/ PANi/ClO_4 film (b) ITO/PANi/ClO_4/dsDNA film; physically adsorbed and (c) ITO/PANi/ClO_4/dsDNA film; Covalently (EDC/NHS) modified.

Differential Pulse Voltammetry of the PANi/ITO film and DNA/PANi/ITO film in 0.1M PBS, pH 7.0 revealed in the **Fig. 1B**. The DPVs show effect of SA with varying concentration in the range 2–64 µM vs. peak current (Fig. 6). The standard calibration plot was plotted (inset) which revealed as the deviation of all the data points within 5–12% (n = 3). The trend showed $r^2 = 0.918$ (good correlation coefficient) in **Fig. 6** and the detection range of SA exhibited 2–64 µM in Fig 6 and $r^2 = 0.996$ in **Fig. 7**.

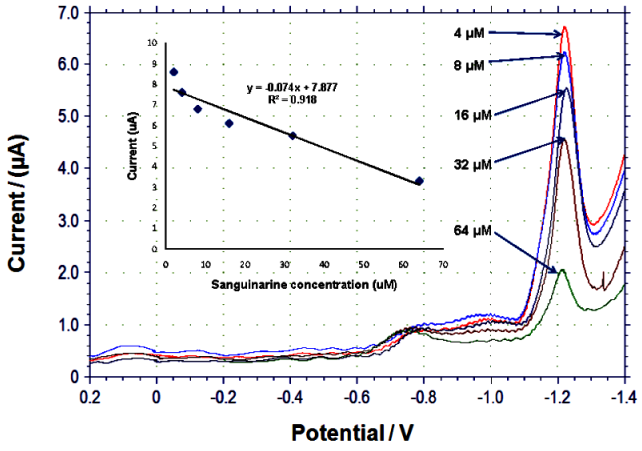

Fig. 6. DPV Shows the effect of sanguinarine at various concentrations using physioadsorbed dsDNA onto PANi/ClO_4 film in 0.1 M PBS buffer pH 7.0. Inset shows calibration plot of sanguinarine vs. current.

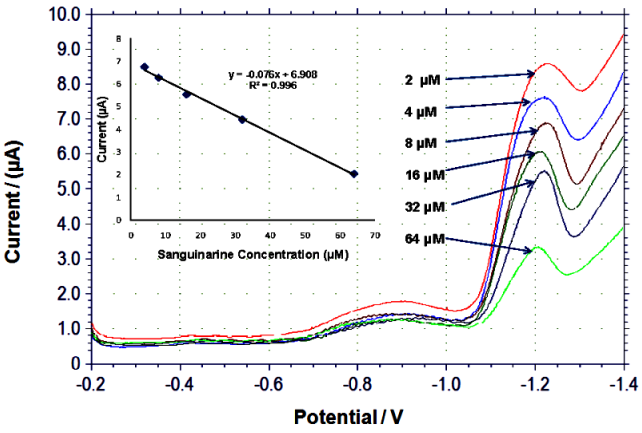

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Fig. 7. DPV Shows the effect of sanguinarine at various concentrations using covalently modified dsDNA onto PANi/ClO_4 film format in 0.1 M PBS pH 7.0. Inset shows calibration plot of sanguinarine vs. current.

The calibration ranges of sanguinarine were set from 1-40 µg, which showed acceptable linearity. The logarithm calibration curves was constructed to determine the concentration of sanguinarine in spiked mustard oil samples most likely a suspected to be adulterated with argemone oil. The logarithm calibration plotted based on linear regression analysis, the logarithm standard curve for sanguinarine found to be linear with a correlation coefficient (r^2) of 0.995. The recovery studies carried out by spiking standard sanguinarine (1 and 40 µg/ml) to the edible mustard oil, showed the recoveries of sanguinarine to be 89% and 121%, respectively. A logarithm regression plots generated between the peak current vs. the concentration of sanguinarine in the extracted spiked edible mustard oil. There was a correlation between the current and concentration of sanguinarine with a correlation coefficient of (r^2) of 0.995 at 95% confidence limit.

Table 1. Shows recovery experiment and data was expressed as coefficient of variation.

*Standard SA concentration (µg)	Standard SA peak current obtained by DPV (µA)	*SA'Added in edible mustard oil (µg)	After extraction, peak current obtained by DPV (µA)	Amount of SA recovered (µg)	Coefficient of variation (CV, %)	Percent recovery (%)
1	18.12± 0.08	1	16. 20± 0.12	0.89	0.74	89.40
2	12. 10± 0.15	2	10.15± 0.21	1.68	2.07	83.90
5	9.31 ±0.21	5	8.70± 0.18	4.67	2.06	93.40
10	6.21 ±0.15	10	5.61± 0.23	9.03	4.07	90.34
20	4.0 ±0 21	20	4.18± 0.32	20.9	7.65	104.50
40	1.80 ±0.98	40	2.18± 0.20	40.4	9.17	121.11

*Data presented in the table, experiment carried out in triplicate

Edible mustard oil samples (in triplicate) spiked with sanguinarine. The concentrations of SA detected in all these samples shown in **Table1**. The recovery experiment was investigated (**Table 1**) to not only evaluate bioelectrode but also determine SA in edible mustard oil sample. Calculated from the peak current of immobilized dsDNA vs. SA interaction and final data obtained depicted in **table 1**. However, in edible mustard oil sample, recovery was found (89–121%), probably due to the interaction/intercalation (pH, ionic strength) when compared with standard SA without extraction of the spiked samples. To overcome this problem more advance clean-up procedure should be required. The reliability of data expressed as coefficient of variation in the range of 0.74–9.17 (**Table 1**).

Analytical performances of DNA biosensor were checked the effect of pH, interaction time, and applied potentials. The influences of experimental parameters like temperature, pH, and applied potential for optimum analytical performance to DNA biosensor are a pre-requisite (data not shown). The bioelectrode for single measurements, suggested shelf life might be 3-6 months under refrigeration at 4 ^oC.

Conclusion

The methodology developed for estimating sanguinarine by DPV and recovery of sanguinarine in the spiked edible mustard oil is not only simple, rapid and economical but also supported very well for the validation of our biosensing system. This newly analytical device used for the screening purpose in food laboratories to check food quality and in forensic investigations related to argemone oil poisoning. The bioelectrode exhibited the detection limits 2–64 µM. The recovery experiment results found between 89% and 121% from spiked edible mustard oil sources. The correlation found between the current vs. concentration of SA with a correlation coefficient of (r^2) of 0.995 at 95% confidence limit. Sanguinarine and ds-DNA form a complex that attenuated the electrochemical response, due to the sanguinarine binding to ds-DNA by intercalation with insertion between adjacent base pairs of DNA duplex strand. This mechanism is not only exhibit an ability to discriminate dsDNA from ssDNA towards anticancer drug activity potential but also, making a candidate for electrochemical indicator in detecting DNA hybridization signals. The electrochemical genosensor based on interaction study with sanguinarine is experimentally convenient, simple, sensitive, simple, rapid, cost-effective, and user-friendly and require very small quantity of materials with easy and very short detection time. This kind of biosensor is very promising for the future in medical and environmental monitoring; specifically DNA and DNA targeted small molecules for the designing of the specific molecule for application in diagnostic tests and development of drugs.

Acknowledgements

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