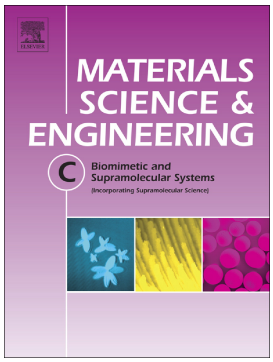

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Detection of alprazolam with a lab on paper economical device integrated with urchin like Ag@ Pd shell nano-hybrids

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ABSTRACT

We present results of the studies relating to fabrication of a microfluidic biosensor chip based on urchin like Ag@ Pd shell nano-hybrids that is capable of sensing alprazolam through electrochemical detection. Using this chip we demonstrate, with high reliability and in a time efficient manner, the detection of alprazolam present in buffer solutions at clinically relevant concentrations. Methylene blue (MB) was also doped as redox transition substance for sensing alprazolam. Nano-hybrids modified EμPAD showed wide linear range 1 – 300 ng/ml and low detection limit of 0.025 ng/l. Low detection limit can further enhance its suitability for forensic application. Nano-hybrids modified EμPAD was also employed for determination of drug in real samples such as human urine. Reported facile lab paper approach integrated with urchin like Ag@ Pd shell nano-hybrids could be well applied for the determination of serum metabolites.

Keywords*:* Micro fluidic paper-based analytical device (EμPAD); Alprazolam; silver nanoparticles; methylene blue; nano-hybrids; palladium shell; urchin like Ag@ Pd shell nano-hybrids

1. Introduction

Micro fluidic paper-based analytical devices (EμPADs) correspond to a newly budding podium for nano-biosensing [1]. Major advantage in paper based devices is they are much economical as compared to metal or carbon electrodes based biosensors and can be used with effortlessly in resource-restricted backgrounds [2]. These virtues make paper based devices capable for advancement of health in remote areas where electricity and expensive equipment are not always available [3]. Moreover, the low cost and ease of fabrication makes paper based devices a best candidate for many applications [4]. Nowadays researchers have integrated paper based devices in many techniques such as Raman [5, 6], UV−Vis [7], fluorescence [8, 9], mass spectrometry [10] and electrochemiluminescence [11, 12]. PAD assay is also linked to electrochemical methods [13-17] that offer low power requirements, quantitative analysis, ease to miniaturize and requires no expertise. Researchers have also introduced or integrated nano-materials for enhanced or amplified sensing signal for diagnostic or environmental applications [18, 19]. The advantageous features of nano-materials for biosensing mainly arise from their unique physical/chemical characteristics such as high surface to volume ratio, superior electrical conductivity and biocompatible [20, 21]. However nano-materials could become reason for increased cost of μPADs but their advantageous features enhance the performance of device. Therefore, present research focused on nano-hybrids based μPADs for electrochemical sensing of alprazolam drug.

Alprazolam is drug from benzodiazepine group, is a short-acting psychotropic drug which are characterized by a high potency and used for oral administration [22]. Alprazolam is widely used as an anti-depressive. Alprazolam are subsequently used therapeutically as anxiolytics, tranquilizers, hypnotics, and anti-epileptic and muscle relaxants [23]. The extensive use of this class of drugs has increased apprehension for recreational abuse. The misuse of this drug is internationally widespread which means that any forensic laboratory may encounter a range of these compounds. Overdose of this drug causes acute drowsiness, muscle numbness, coordination problem, fainting and lethal if heavily overdosed [24]. Thus, monitoring of this drug is of utmost importance to clinicians and forensic toxicologists.

Earlier methods used for the determination of alprazolam in biological fluids and pharmaceutical formulation are UV spectroscopy [25], high performance liquid chromatography (HPLC) [26-28], GC-MS [29] and LC-MS [30, 31]. All these methods are conventional and time consuming. Thus, the present research reported the first nano-hybrid array based μPAD for electro-sensing of alprazolam in biological fluids for clinical and

forensic trial. The reported nano-hybrid array based μPAD is really quick, has a wide linear response and can be applied for detection of other intoxicated drugs.

In the current report, the electrochemical sensing of alprazolam was done using methylene blue doped silver core shell palladium nano-hybrids (Ag@ Pd shell nano-hybrids). Electroanalysis of alprazolam is characterized by the redox transition of the methylene blue. Alprazolam was reduced to corresponding dihydro derivative on the methylene blue Ag@Pd modified surface. The electrochemical tests indicated that urchin like Ag@ Pd shell nano-hybrids can generate a strong electrochemical signal in phosphate buffered saline (PBS) without any other substrates.

2. Experimental

2.1 Materials

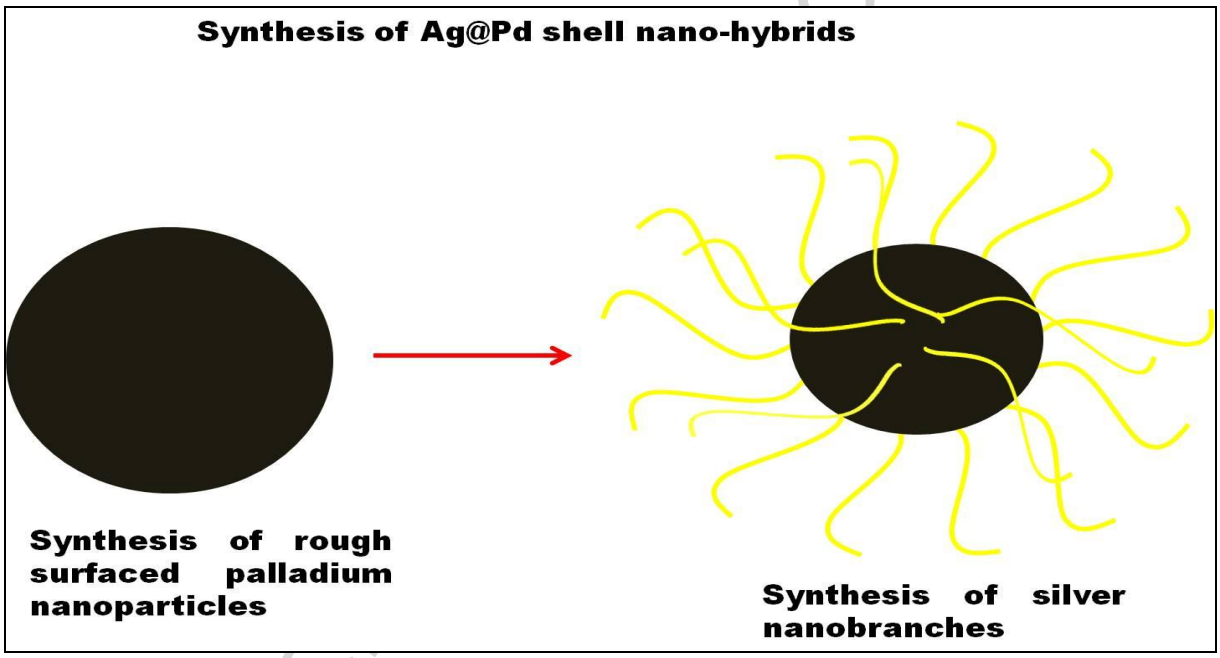
Alprazolam were purchased from Pharmaceutical Co. (India). All solutions were prepared with doubly distilled water. Silver nitrate ($AgNO_3$) and Potassium hexachloropalladate (K_2PdCl_6) were procured from Sigma, India. Triton-X-100 was obtained from Thermo Fischer Scientific, India. All other chemicals were of analytic reagent grade. Stock standard solutions of alprazolam were prepared by dissolving the appropriate amount in water.

2.2 Apparatus and methods

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were performed on a Potentiostat/ Galvanostat (Autolab, Eco Chemie, The Netherlands. Model: AUT83785) and driven by NOVA 1.8 software using a two electrode system. All the electrochemical experiments were performed at an ambient temperature ($25^{O}C$). Scanning electron microscopy (SEM) measurements were carried out at Department of Microbial technology, Amity University, Noida. Ultrasonication was performed on Misonix Ultrasonic Liquid Processors (mode XL-2000 series).

2.3 Synthesis of urchin like Ag@ Pd shell nano-hybrids and MB doped urchin like Ag@ Pd shell nanohybrids

The methylene blue doped urchin like silver core and palladium shell nano-hybrids were developed according to the method adopted by Wang et al. 2013 with certain modifications. $AgNO_3$ (0.2 g) and K_2PdCl_6 (0.1 g) were added into of 5% TritonX-100 (10 mL) aqueous solution. To this solution 1 ml of methylene blue (0.1 M) was also added. After 30 min stirring at room temperature, the mixture was heated to 70 ^{0}C under magnetic stirring until the color of the mixture changed to dark brown. TritonX-100 serves as both a reducing agent and as a protective agent during the reaction. The MB doped urchin like Ag@ Pd shell nanohybrids were obtained by centrifuging and washing, and then dried under high vacuum overnight [32]. The urchin like Ag@ Pd shell nano-hybrids were developed by the same procedure except that methylene blue was not added in this (Scheme 1).

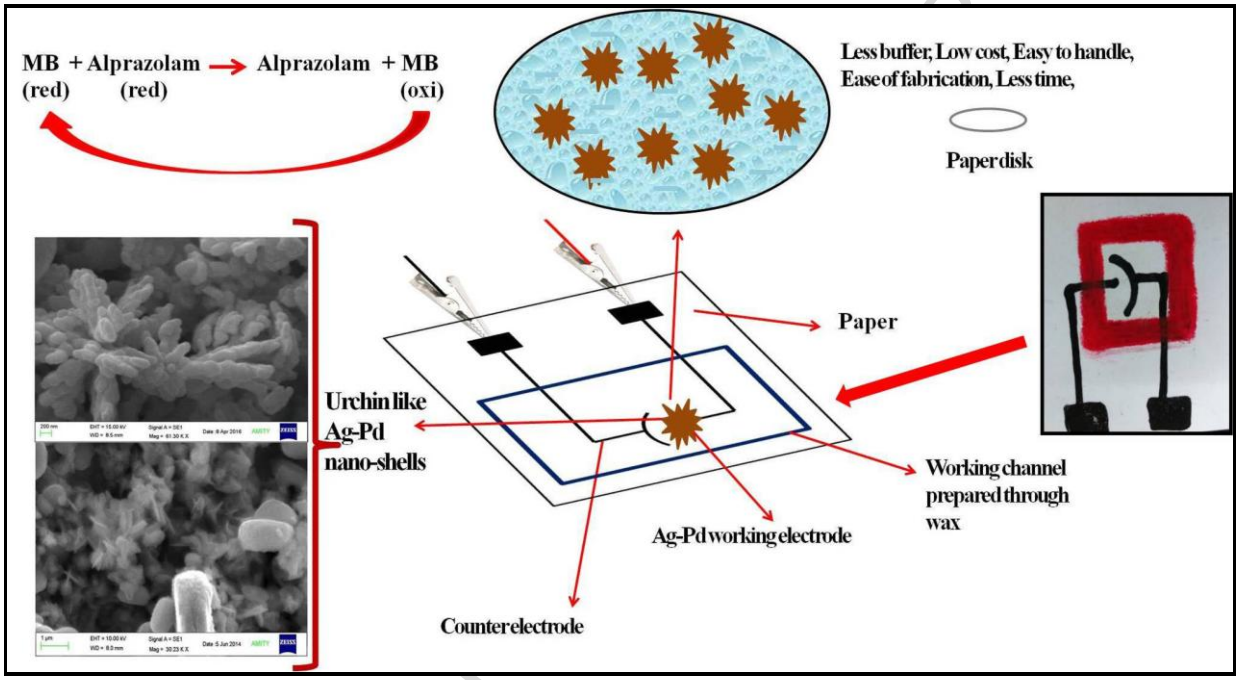


Scheme 1: Synthesis of urchin like Ag@Pd shell nano-hybrids

2.4 Fabrication of µPAD

The patterns of hydrophobic barriers were first designed using drawing software (turbocad). The patterns were printed on Whatman no. 1 chromatography paper using the high resolution laser printer set to the default parameters for photo-quality printing. The printed paper was then placed on a hot plate at 150 °C for 120 s, and the wax then melted and spread through the thickness of the paper. The patterned paper was ready for use after removing the paper from the hot plate and allowing it to cool to room temperature.

Wax deposition on paper is fast, inexpensive, and well suited method for batch processing of prototype analytical devices on paper. For fabrication of µPAD, the working (WE) and counter electrode (CE) was ornated on the paper using commercially available conducting carbon ink (Scheme 2). The prepared methylene blue doped silver core palladium shell nano-hybrids were drop casted on the circular WE followed by room temperature drying.



Scheme 2: Schematic representation of the fabrication and working principle of the microfluidic chip used for electrochemical sensing of alprazolam

2.5 Morphological characterization of nanomaterial

Nanomaterials silver core palladium shell nano-hybrids (Ag@Pd) were also characterized by scanning electron microscope (SEM) and Energy dispersive X rays (EDX). SEM study was done to depict the morphology of nano-materials. EDX was done for purity of sample.

2.5 Electrochemical characterization study of modified sensing electrode

Electrochemical study was done using cyclic voltammerty technique; the two electrodes of the EµPAD were connected to a potentiostat. 10 µl of PBS (50 mM, pH 7.0, 0.9% NaCl) was added into circular zone of working electrode for further study. Impedance study was also done using the same

electrodes dipped in PBS containing 5 mM $[Fe(CN)_6]^{3-/4}$, Frequency range: 0.01 Hz to 10 KHz at 0.20 mV s^{-1}. EIS and CV pattern of various stages of sensing electrode were taken. Various variable conditions like pH, incubation temperature and time were optimized. For evaluation of functioning of biosensor, parameters like precision and accuracy were also studied.

2.7. Sensing signal acquisition

For the electrochemical measurements, varying concentrations of alprazolam were made by dissolving an appropriate amount of compound in water. The electrochemical behavior of alprazolam was studied at urchin like Ag@ Pd shell nanohybrids EμPAD using cyclic voltammetry. A sample mixture to be analyzed (alprazolam) is transferred into circular region or chamber on the urchin like Ag@ Pd shell nanohybrids EμPAD. Redox profile of the drug suggests that there is one well-defined anodic peak and one peak observed in the reverse scan. Cathodic peak (Ip) is due to reducible group present in ring structure.

2.8. Employment of urchin like Ag@ Pd shell nanohybrids EμPAD for real samples

The fabricated ZnONWs PAD was employed for the detection of drug in real samples urine. Real samples were taken from volunteers and were not pretreated. These samples were evaluated for the determination of alprazolam.

3. Results and Discussions

3.1 Morphological characterization of urchin like Ag@ Pd shell nano-hybrids

Urchin like Ag@ Pd shell nano-hybrids were physically characterized by SEM observation at an acceleration voltage of 13 kV. Scanning microscopic images reveal the formation of urchin like Ag@ Pd shell nano-hybrids on 200 nanometer scale as shown in Fig. 1 (i). The formation of urchin like nano-hybrids might be due to balance of kinetic and diffusion rate control the growth of silver nano-branches on a rough aggregated Pd surface which coalesce to form solid sphere.

The Nano-hybrid modified electrode shows urchin morphology (with branches oozing out) as depicted from SEM image. An EDX spectrum (Fig. 1 (ii)) reveals the purity of sample, in which there are peaks of Pd and Ag in the spectrum.

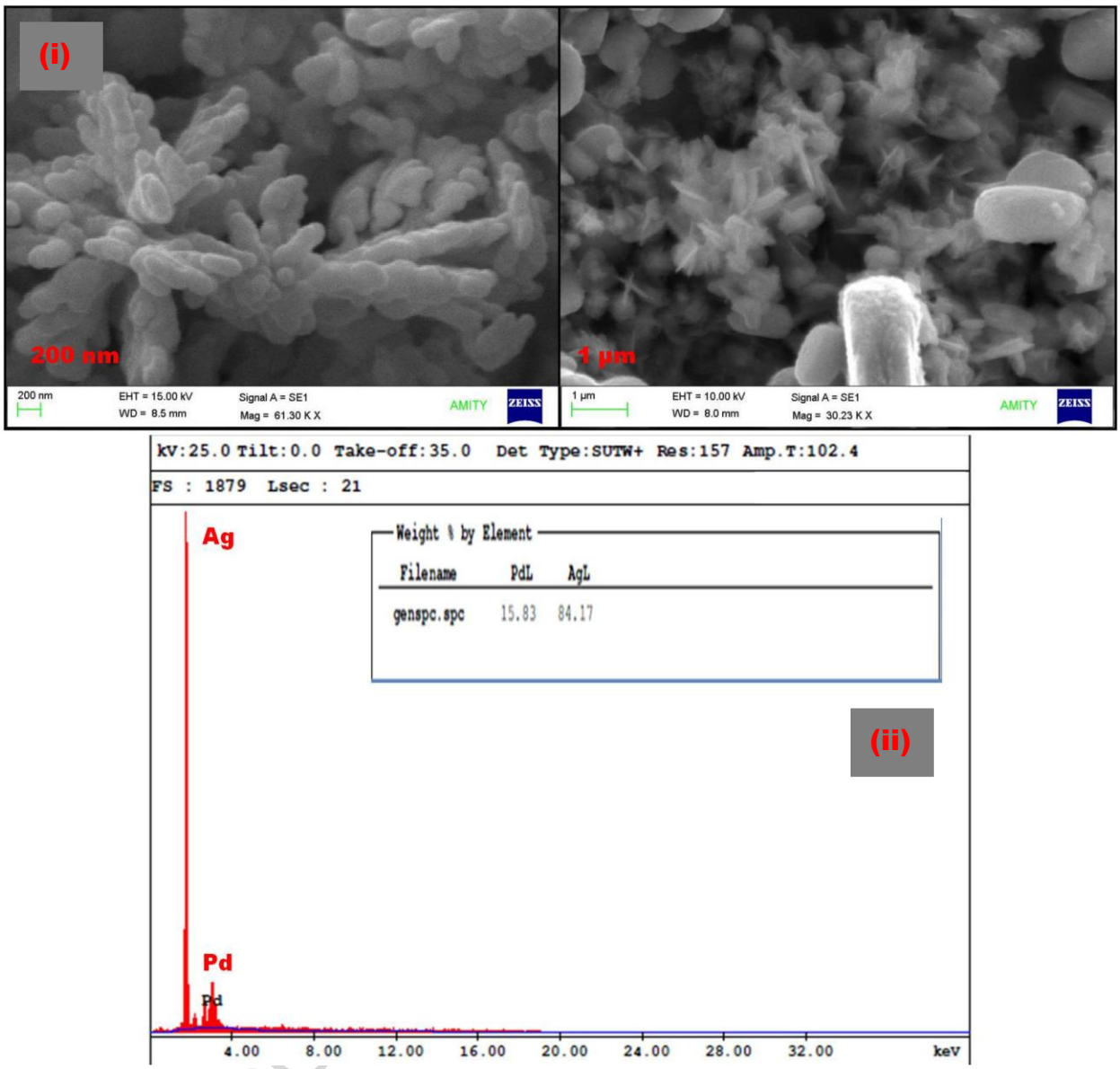


Fig. 1: (i) SEM analysis of Ag@Pd nanohybrids at various magnifications (ii) EDS analysis of Ag@Pd nanohybrids

Cyclic voltammograms (CVs) image was recorded in a sodium phosphate buffer 0.05 M (pH 7.0) at a scan rate of 100 mVs^{-1} in the presence of alprazolam (Fig. 2A). CV of WE without nano-hybrid showed insignificant sensing signal but when the WE was modified with nano-hybrids, there is an explicit amplified sensing signal was observed (0.1 mA). Thus, urchin like Ag@ Pd shell nano-hybrids was providing favorable environment to the electrode and promotes sensing signal. While upon doping

of methylene blue (MB), an oxidation peak was observed at 0.1 V and a reduction peak centered at -0.5 V. There is shifting of potential upon doping of methylene blue. MB is also acting as an electron mediator in the modified electrode. These anodic/cathodic peaks arise due to the presence of the methylene blue which provides confined pathway for the transportation of electrons from the alprazolam to the electrode. Furthermore Ag@ Pd shell nano-hybrids with a larger surface area facilitate higher mass diffusion efficiency due to the electrochemical activation of the methylene blue resulting in enhanced redox current. Synergistic association between nano-hybrids and methylene blue is promoting fast and increased conductivity as well as oxidation and reduction mechanism towards drug.

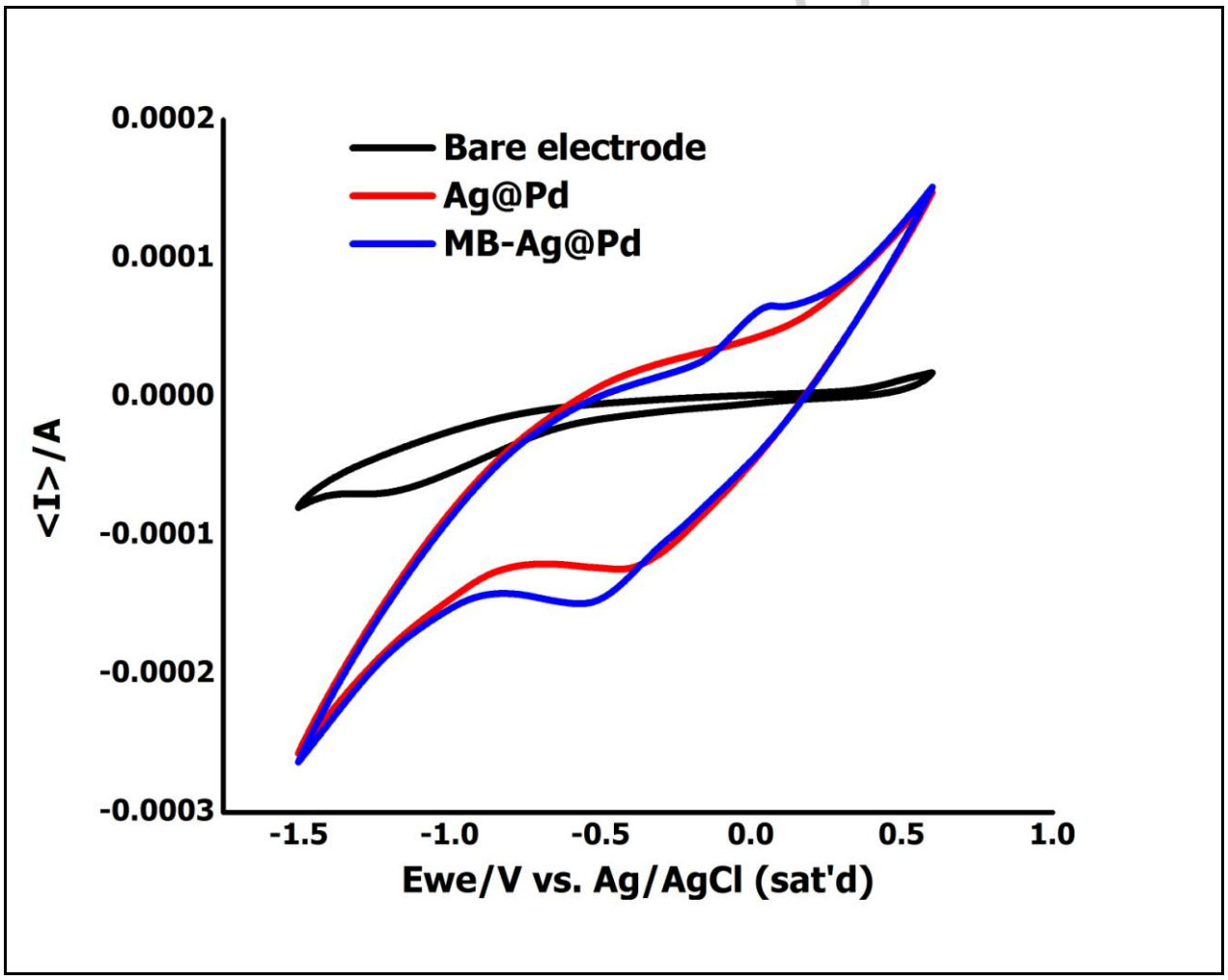


Fig. 2 (A): Cyclic voltammogram of bare electrode, Ag@Pd and MB-Ag@Pd in the scanning potential range of -1.5 to +1.5 V at the scan rate 100 mV s^{-1} in 0.1 M phosphate buffer (pH 7.0).

Evidence which confirms the formation of sensing interface is electrochemical impedance spectroscopy. The electrochemical impedance spectroscopy (EIS) technique was performed to detect the impedance of the electrode–electrolyte interface at various phases of electrodes. Change in impedance or resistance charge transfer (Rct) is indicative of the modification on the working area of PAD. The diameter of a semicircle plot in EIS spectra is measured value of RCT which determines the electron transfer kinetics of the electroactive species at the sensing interface. This resistance controls the electron-transfer kinetics of the redox probe at the electrode interface. EIS spectra was observed for WE without nano-hybrid, Urchin like Ag@ Pd shell nano-hybrids EμPAD and MB- Ag@Pd nano-hybrids in PBS containing 5 mM $[Fe(CN)_6]^{3-/4}$ at 0.20 mV s^{-1} (frequency range of 0.01 Hz –10 kHz) in the presence of alprazolam. Ag@Pd nano-hybrids EμPAD electrode observed smaller resistance charge transfer (1000 Ω) when compared with WE without nano-hybrid (Fig. 2 (B)) which shows almost linear spectrum. Upon doping MB, the Rct get further decreased (800 Ω). The MB- Ag@Pd nano-hybrids ascertain electronic pathway that decrease the channel distance for the diffuse ions (or electrons) between the alprazolam and the electrode. The above results depicts that electrode is more conductive in nature, which is in linet with results of the CV studies.

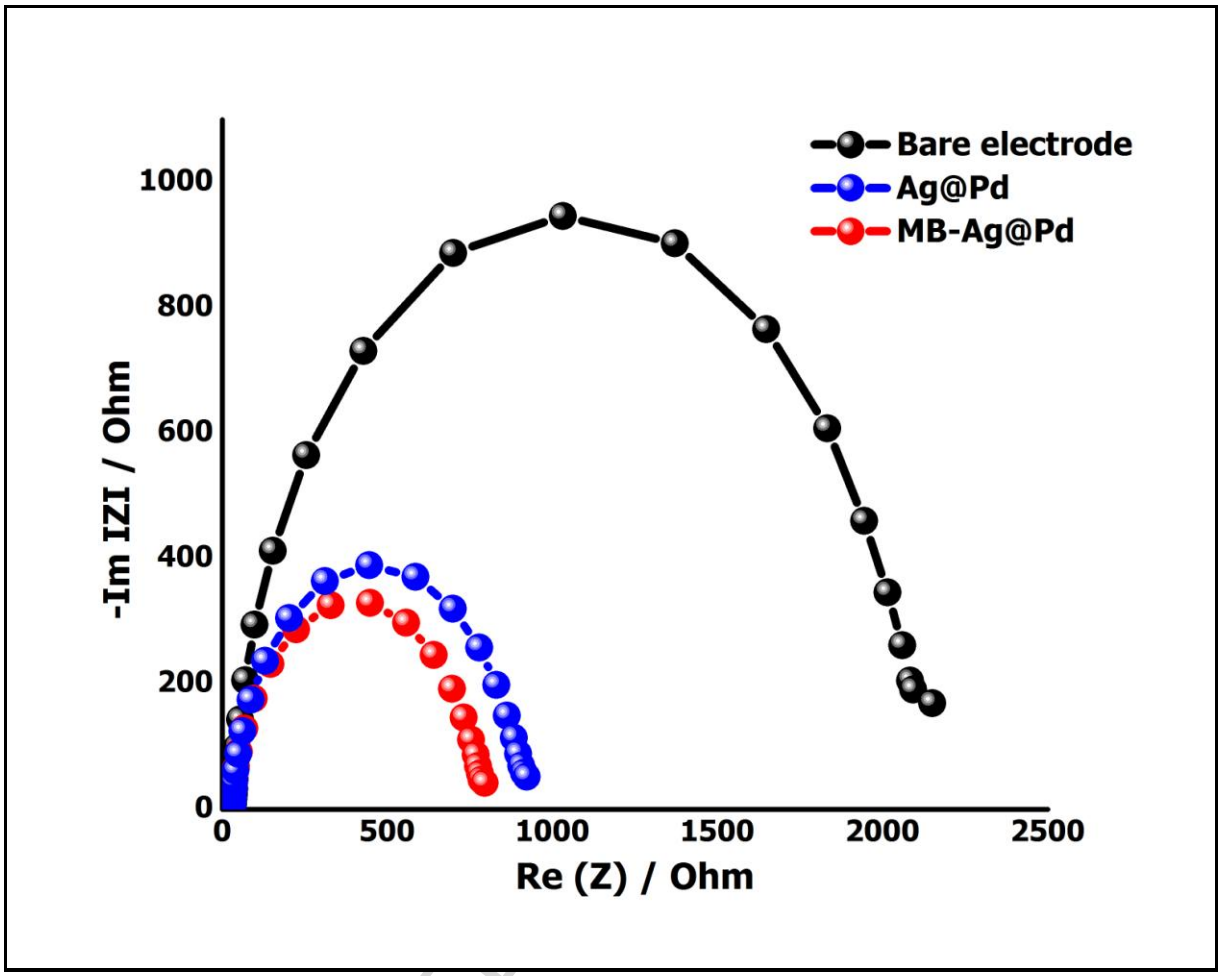


Fig. 2 (B): Electrochemical impedance spectroscopy of bare electrode, Ag@Pd and MB-Ag@Pd in PBS containing 5 mM $[Fe(CN)_6]^{3-/4}$, Frequency range: 0.01 Hz to 10 KHz at 0.20 mV s^{-1}.

3.2 Electrochemical principal of sensing alprazolam

Electro analysis of alprazolam is characterized by a relatively easily reducible group. Electrochemical reduction of the reducible group results in the corresponding dihydro derivative [23].

Alprazolam was reduced on the modified surface. In this process, the redox transition of the methylene blue was exploited for the determination of alprazolam drug. This reaction is coupled with reduction of alprazolam.

$$\text{MB (Ox)} \rightleftharpoons \text{MB (red)} \quad \text{(i)}$$

$$\text{MB (red)} + \text{Alprazolam} \longrightarrow \text{Alprazolam (red)} + \text{MB (Ox)} \quad \text{(ii)}$$

3.3 Figures of merit of the proposed electrochemical sensor for determination of alprazolam

For the electroanalytical determination of drug; CV was employed. Different concentrations of alprazolam (1 – 300 ng ml^{-1}) were exposed to the MB-Ag@Pd nano-hybrids. The cyclic voltammograms curves presented in Fig. 3 (a) show both oxidation and reduction peaks centered at 0.1 V and -0.5 V respectively which is due to the redox transition of methylene blue and reduction of alprazolam as mentioned above. During alprazolam detection, the MB directly accepts electrons from the reduced aplrazolam molecules in the presence of $[Fe(CN)_6]^{3-/4-}$ as the mediator. The sensing signal increased with an increasing concentration of alprazolam. This integrated paper based device showed dynamic response range between 1 to 300 $ngml^{-1}$ for alprazolam. The developed nano-sensor showed high sensitivity and is capable of detecting alprazolam at defined concentration in repeated experiments. A calibration graph of the oxidation (Fig. 3 (b)) and reduction (Fig. 3 (c)) peak current vs. log of alprazolam concentration resulted in linear behavior with alprazolam concentrations in the range of 1–300 $ngml^{-1}$, according to the following regression equation:

$$Ia = 0.764\ x + 0.45 \text{ with } r^2 = 0.95$$

$$Ic = -0.764\ x - 0.45 \text{ with } r^2 = 0.95$$

The detection limit is 0.025 $ngml^{-1}$ was calculated by using the relation 3Sb/m, where Sb is the standard deviation of the blank and m is the slope of the calibration curve, which was found to be 0.012 ng/mL.

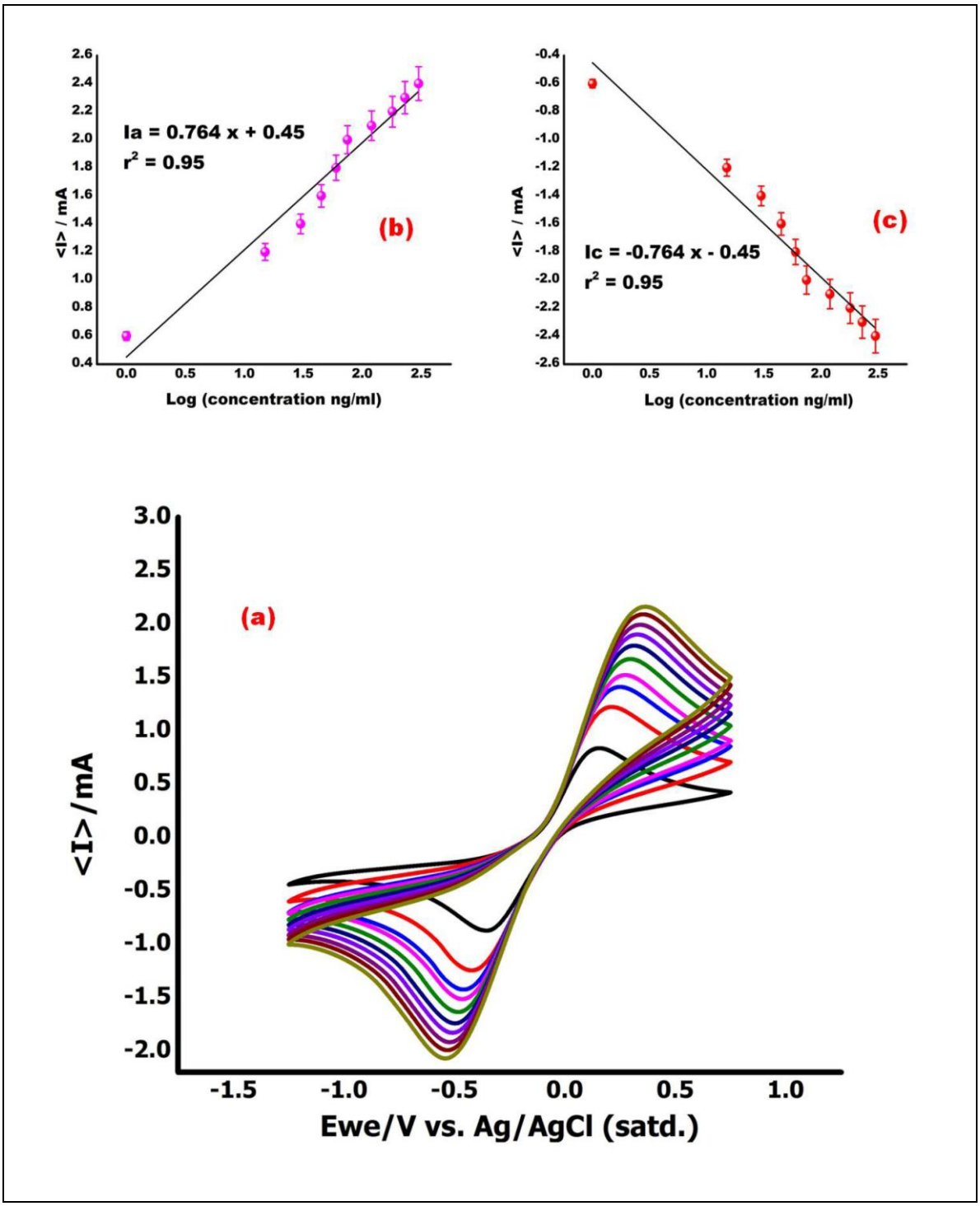


Fig. 3: (a) Cyclic Voltammograms of modified electrode using different concentrations of alprazolam (1 – 300 ng/ml) in the scanning potential range of -1.5 to +1.5 V at the scan rate 100 mV s−1 in 0.1 M phosphate buffer pH 7.0. (b) Calibration curve between log of substrate concentration and anodic current, Ipa (c) Calibration curve between log of substrate concentration and cathodic current, Ica

3.4 Optimization & Analytical performances of modified sensor

Cyclic voltammogram of MB-Ag/Pd EμPAD was recorded at different scan rates from 10 to 100 mV s^{-1} as well as from 100 to 1000 as mV s^{-1} as shown in Fig. 4 (a) and Fig. 4 (b) respectively. Figure depicts that the peak currents increased with the increasing scan rates with stable oxidation and reduction peaks in the range of 10 to 100 mV/s. However, at higher scan rates (100 to 1000 mV/s) similar increase in the peak currents was observed with increasing scan rates but the plot tend to level off at higher scan rates and neither any oxidation nor any reduction peak was observed. Thus, scan rates above 100 mV/s are not selected in CV studies.

Fig. 4 (c) displays that peak current, qualitatively, increases as a function of square root of scan rate, indicating slow electron-transfer kinetics on the CV time scale. Fig. 4 (d) further confirms the diffusion controlled behavior by plotting log I vs log v. All subsequent hybridization experiments employed a 100 mVs^{-1} for optimum scan rate.

The dependency of the peak current on scan rate can be expressed as:

$I\ (A) = 2.9 \times 10^{-6} \times (mV/s) + -5.09 \times 10^{-5}$ with a correlation coefficient of 0.96.

The plot between log Ip vs log v corresponds to the equation:

Log I (A) = 1.113 x (mV/s) – 5.50 with a correlation coefficient of 0.97.

CV results depicted that by using EμPAD (Fig. 3) the operating voltage of the sensor is less i.e. 0.1 V. Thus, in the subsequent experiments, 0.1 V is selected as working voltage for amperometric study. Due to this low voltage, many interfering species which are electro-active in nature such as ascorbic acid and other drugs can be eliminated.

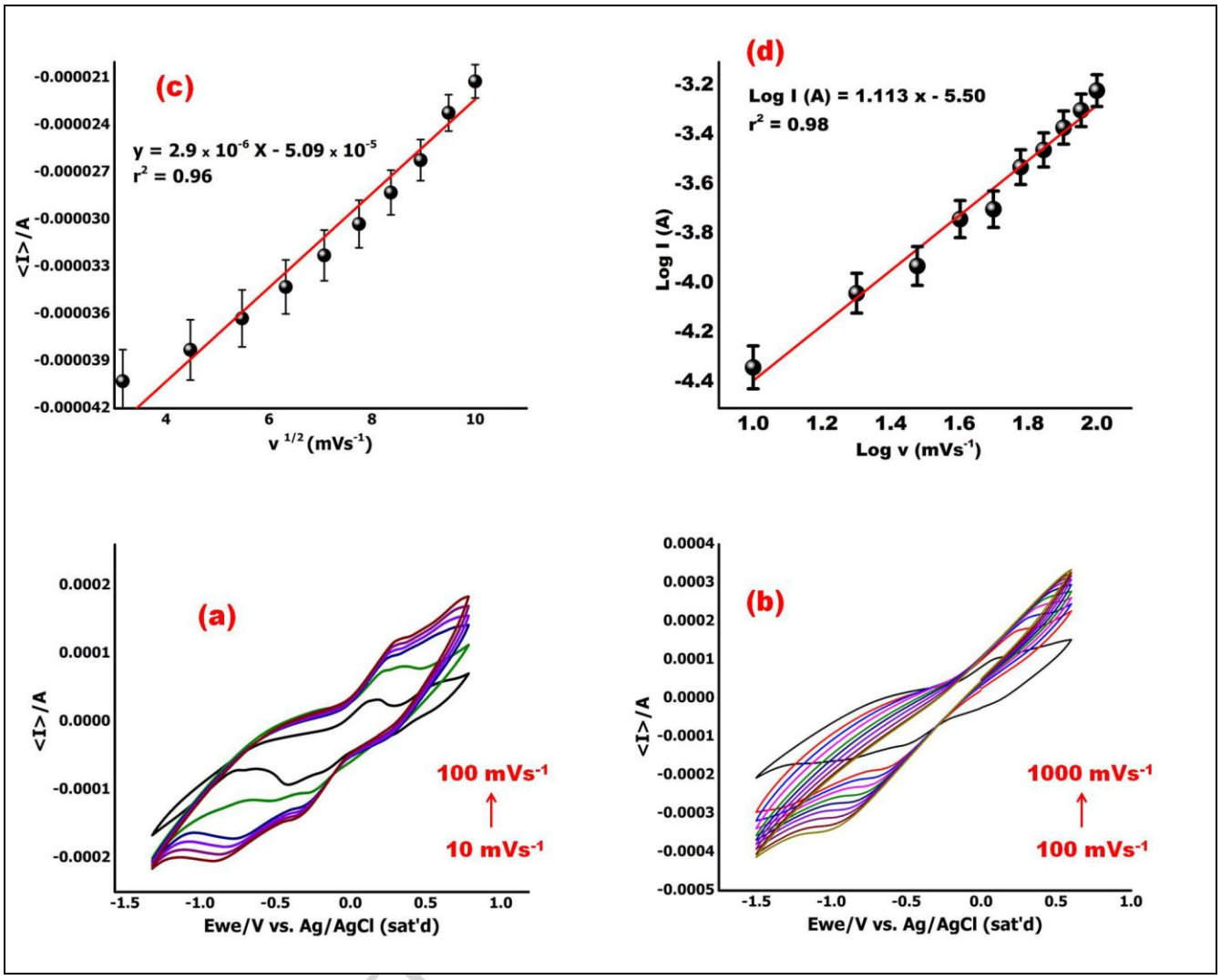


Fig. 4: Cyclic voltammograms of modified electrode at (a) scan rates ranging from 10-100 mV/s (b) scan rates ranging from 100-1000 mV/s in the scanning potential range of -1.5 to +1.5 V in 0.1 M phosphate buffer pH 7.0. (c) The dependency of anodic peak currents on the square root of potential sweep rates in a wide range of 10 – 100 mVs^{-1}. (d) Dependence of log of anodic peak current on log v ($mV\ s^{-1}$).

Various parameters were also optimized such as pH, temperature and response time. The pH varying from 5.8–8.0 was studied and found that there is slight shift in the peak potential to more negative values with an increase in pH which is due to reducible behavior of alprazolam. There is insignificant difference with increase in pH. Highest sensing signal was observed for pH 8.0. Sensing signal was also observed after variations in temperature. Temperature was varied from 20 to 40^0 C and the sensing signal was observed maximum at 35 ^oC, indicating that the developed sensor works best at room temperature (Fig. 5(b)).

The constant potential was applied for periods of 2 s to 60 s before performing the potential sweep runs. As incubation times are increasing, the electrochemical sensing generates insignificant current difference, but the evaporation of the solution with time also decreases the current level. An optimum incubation time was kept 10 s (Fig. 5 (c)), potential application time prior to the potential sweep runs.

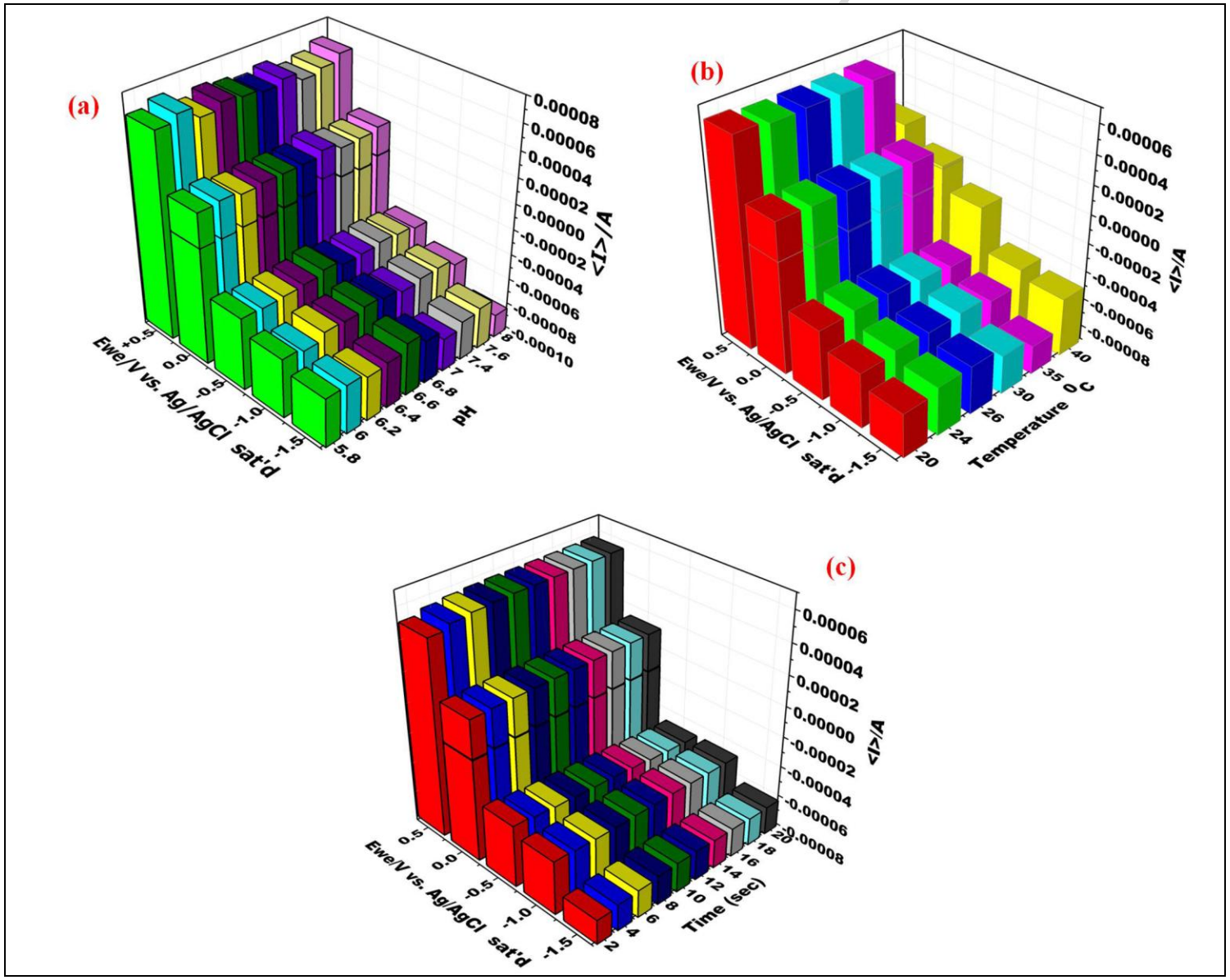


Fig. 5: 3D representation of the Cyclic voltammograms data of modified electrode at different (a) pH (b) Temperature (c) Time in the scanning potential range of -1.5 to +1.5 V at the scan rate 100 mV s−1 in 0.1 M phosphate buffer pH 7.0.

3.5 Application and Evaluation of sensor

Urchin like Ag@ Pd shell nanohybrids EμPAD was employed for the determination of alprazolam in real samples. Calibration results were obtained by adding spiked human urine samples, with the optimized parameters (0.1 V, 35 ^{o}C and 10 s of incubation time). The calibration experiments on spiked human urine show the performance of our devices for real sample detection in a complex environment that contains various other molecules. Accuracy of the present device was found to be 99%.

Nanohybrids EμPAD was further evaluated with analytical parameters obtained for the alprazolam (Table 1). The results of analytical recovery testing using the fabricated sensor are also presented in Table 1.

Table 1. Determination of recovery (n = 5) of alprazolam in urine sample by employing Urchin like Ag@ Pd shell nanohybrids EμPAD

Drug	**Amount added (mM) Mean ± SE**	**Amount found (mM) Mean ± SE**	**Recovery (%)**
Alprazolam	4 ± 1.25	3.9 ± 0.69	97.5

Table 2. Precision (n = 5) of of alprazolam in urine sample by employing Urchin like Ag@ Pd shell nanohybrids EμPAD

Drug	**Within batch (n=5) Mean ± SD mM**	**Between batch (n=5) Mean ± SD mM**
Alprazolam	5.60 ± **0.25**	5.40 ± **0.50**

3.6. Selectivity of the proposed Urchin like Ag@ Pd shell nanohybrids EμPAD

Possible interference for the detection of alprazolam was investigated by addition of various drugs and some other compounds to a phosphate buffer (pH 7.4) in the presence of alprazolam. A 100-fold excess concentration of the common drugs such as diazepam, chlordiazepoxide and oxazepam, which are some other benzodiazepines, did not show any measurable interference in alprazolam detection. As for the common interferences in biological samples for the determination of alprazolam, the influence of 100-fold excess concentration of ascorbic acid, glucose, tyrosine and cysteine were examined. In all cases, the change in alprazolam signal was found to be below 5%, suggesting the high selectivity of the proposed sensor towards the determination of alprazolam in the presence of the above mentioned potential interferences. Therefore, the procedure is able to alprazolam assay in the presence of excipients; hence, it can be considered selective.

Conclusions

Present study describes a novel paper based analytical device integrated with urchin like Ag@ Pd shell nano-hybrids for electro-sensing of alprazolam. Urchin like Ag @ Pd shell nano-hybrids was prepared through facile approach. Lab on paper device integrated with nano-hybrids showed amplified sensing response and lower working voltage. Urchin like Ag @ Pd shell nano-hybrids modified EμPADs was employed for the determination of different concentrations of alprazolam. Nano-hybrids modified EμPADs was also optimized in terms of working voltage, pH, response time and temperature for determination of alprazolam in real samples such as human urine. Nano-hybrids modified EμPADs showed wide linear range 1 – 300 ng/ml and low detection limit of 0.025 ng/l. Results depicted that biosensing performance is superior to that of other metal electrode based sensor for drug analysis. Nano-hybrids modified EμPADs has the potential for commercialization as it is facile, amplified sensing signal and lower working potential which eliminates interference from electro active species. This paper based sensing approach is valuable for clinicians and forensic toxicologists.

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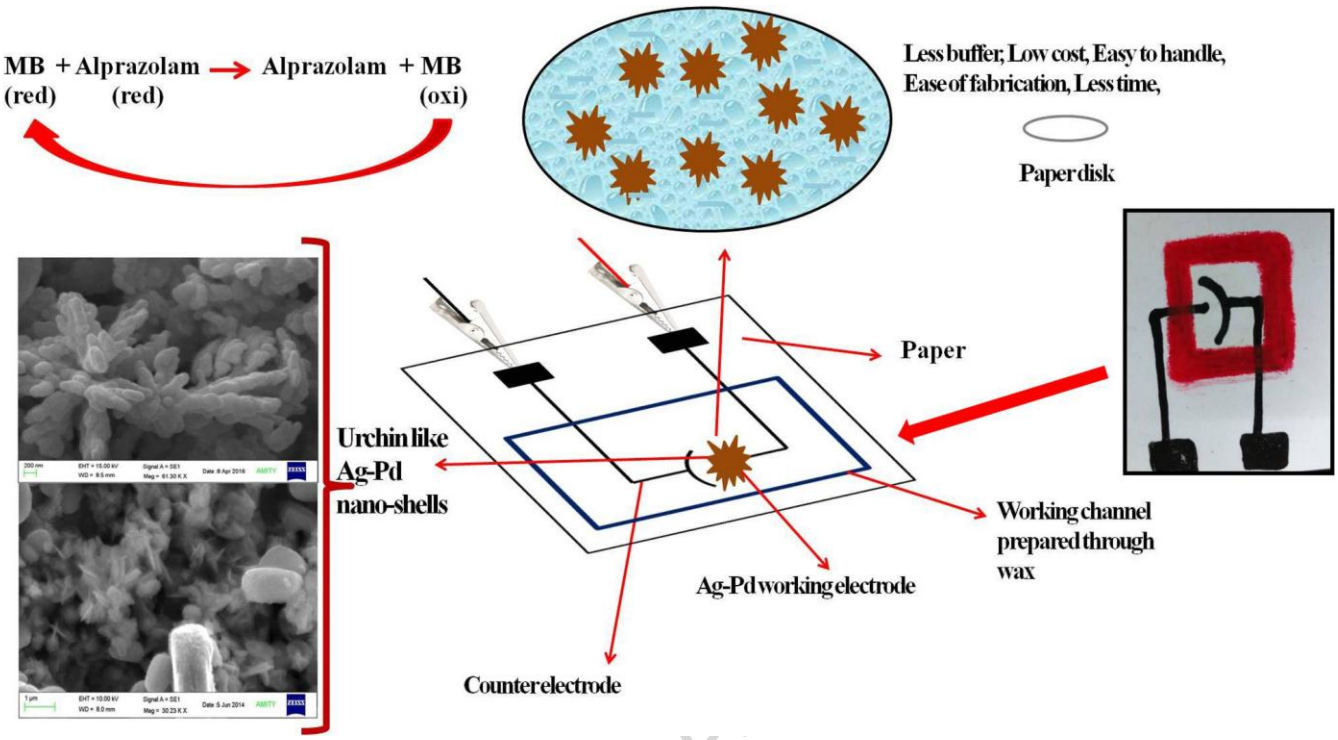

Graphical abstract

HIGHLIGHTS

- EμPAD offers many advantageous features such as facile approach, economical and have potential for commercialization.
- Extensive development can be made for industrial translation of this fabricated device.
- Ag@ Pd shell nanohybrids modified EμPAD showed wide linear range 1 – 300 ng/ml and low detection limit of 0.025 ng/l for alprazolam detection.
- The developed sensor was tested in real time samples like urine and serum and found good correlation (99%).