# Nanocrystals of Zeolite Act as Enhanced Sensing Interface for Biosensing of Leviteracetum

**JAGRITI NARANG,[1] NIDHI CHAUHAN,[1] NITESH MALHOTRA,[2] C. S. PUNDIR[3]**

[1]Amity Institute of Nanotechnology, AMITY University, Noida, UP, India
[2]Amity Institute of Physiotherapy, AMITY University, Noida, UP, India
[3]Department of Biochemistry, M. D. University, Rohtak 124001, Haryana, India

*Received 26 June 2014; revised 7 August 2014; accepted 23 October 2014*

*Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24267*

**ABSTRACT:** In present work, nanocrystals of zeolites were employed as sensing interface for augmented electrochemical sensing of antiepileptic drug, that is, leviteracetum (LEV). Electrochemical sensing method was developed by depositing nanocrystals of zeolites and horseradish peroxidase (nanocrys zeolites–HRP) onto indium tin oxide (ITO)-coated glass surface for LEV detection. Various stages of biosensor fabrication were characterized by Transmission electron microscopy, X-ray diffraction, scanning electron microscopy, and electrochemical impedance spectroscopy. The fabricated nanocrys zeolites–HRP-modified sensor exhibited wide linear range (10–500 μM) and a low detection limit of 0.01 μM. The biosensor also showed a short response time (within 2 s). In addition, the biosensor exhibited high reproducibility, good storage stability, and anti-interference ability. The applicability of the nanocrys zeolites–HRP-modified sensor is to determine LEV level in spiked serum samples. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci
**Keywords:** leviteracetum; nanocrystals of zeolites; horseradish peroxidase; ITO electrode; bioanalysis; nanoparticles; physical characterization; enzymology; X-ray diffractometry

## INTRODUCTION

Surfaces play pivotal role in biosensing methods as biological reactions occur at surfaces and interfaces.[1–3] Among surfaces or interfaces, zeolite has captivated interest of many researchers for modification of sensing electrode as it also show synergistic effect with sensing interfaces.[4,5] Zeolites have many properties that makes them best suited for electrochemical sensing like its ion-exchange capacity, selectivity, and electrocatalysis.[6] Nanozeolites have unique properties like large surface area, tunable surface charge, and composition and chemical stability. These properties of zeolites can be exploited for electrochemical sensing as it can prove to be the best immobilization matrix of biomolecules. In the present report, we used zeolite as an immobilization matrix for binding of horseradish peroxidase (HRP) for electrochemical sensing of antiepileptic drug (AED), that is, leviteracetum.

Epilepsy is a neurological disorders characterized by epileptic seizures.[7] Approximately 50 million people worldwide are affected by epilepsy,[8] life-threatening neurological disorder that can prove to be fatal. Many AEDs are available to treat epilepsy. These drugs cause decrease in neuronal excitation and thus decreasing seizures. Among them, LEV [(S)-α -ethyl-2-oxo-1-pyrrolidine acetamide] is one of the newest drug that act as an antiepileptic agents, marketed worldwide only since 2000.[9] LEV has been accepted as a monotherapy treatment for epilepsy in partial seizures, or as an adjunctive therapy for partial myoclonic and tonic–clonic seizures.[10] This drug is also applied in the cure of many neurologic diseases for instance autism and Tourette syndrome.[11]

LEV levels detection in patient plasma is very important because there should be monitoring of drug dosage to decrease adverse effects[12] like drowsiness, weakness, unsteady gait, coordination problems, headache, pain, forgetfulness, anxiety, irritability or agitation, dizziness, mood changes, nervousness, and changes in skin pigmentation.[9] Different conventional methods are used for the determination of LEV and among them are gas chromatography,[13] LC–tandem mass,[14] HPLC,[15] and capillary electrophoresis.[16] These methods have many setbacks like low sensitivity, time consuming, need large volume of sample, and some of them require solid phase extraction.[17] Thus, an effortless and fast method is required for drug monitoring. In this study, we applied a simple, fast, and valid method for the determination of LEV.

In this report, we used electrochemical impedance spectroscopy (EIS) technique for fast and sensitive detection of LEV. We describe herein fabrication of LEV sensor based on nanozeolite decorated onto indium tin oxide (ITO)-coated glass surface for electrochemical sensing.

The exploitation of nanocrystals of zeolites provides high sensitivity and selectivity for electrochemical sensing of LEV. Improved sensitivity and a good stability of sensor indicating that zeolite is a good immobilization matrix for biomolecules.

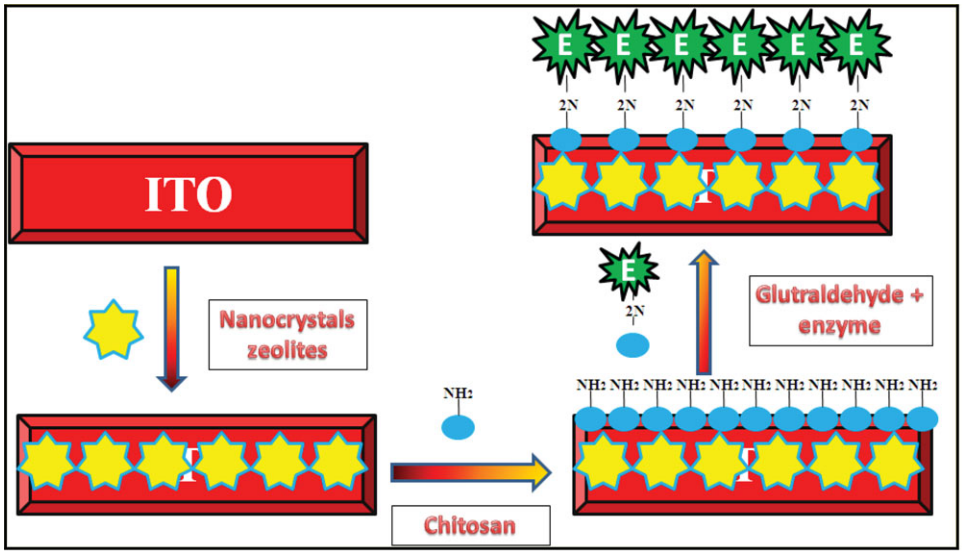
## EXPERIMENTAL

### Materials

Leviteracetum was procured from vendors of Piramal Healthcare Pharmaceutical Company, Rohtak, Haryana, India (name: Levesam; purity: 99%). HRP (source: Horse radish; purity: 99%), aluminum isopropoxide, tetramethylammonium hydroxide (TMAOH), tetraethyl orthosilicate (TEOS), sodium hydroxide solution (NaOH), silicon dioxide ($SiO_2$), aluminium oxide ($Al_2O_3$), and chitosan were procured from Sigma Chemicals

*Correspondence to*: Jagriti Narang (Telephone: +9811792572; Fax: +1262256700; E-mail: jags_biotech@yahoo.co.in)

Journal of Pharmaceutical Sciences




**Scheme 1.** Schematic representation of various stages of sensing electrode.

Company, St. Louis, Missouri. All other chemicals were of analytic reagent grade. Double distilled water (DW) was used throughout the experiments. Stock standard solutions of LEV were prepared by dissolving the appropriate amount in DW.

### Preparation of Nanocrystals of Zeolites

Nanocrystals of zeolites were prepared from a mixture containing 11.25 SiO2:1.8 Al2O3:13.4 (TMA)2O:0.6$Na_2O$:700 H2O according to the method of Kim et. al.[18] Tetramethylammonium hydroxide was mixed in DW with stirring until homogenous mixture is formed. Then, aluminum isopropoxide was added with continuous stirring until the solution gets transparent. Tetraethyl orthosilicate was also added with continuously stirring. Subsequently, sodium NaOH was added with overnight continuous stirring. Then, the solution was kept into oven at 100°C for 10 h. Then, there is formation of solid particles and was centrifuged at 18894.2 g for 30 min at 30°C, washed with deionized water, and dried at 80°C.

### Preparation of Nanocrystals Zeolites Modified Sensing Electrode

For preparation of thin film of zeolites nanocrystals on sensing electrode, the zeolites nanocrystals film was decorated by dip coating the surfaces with 5% zeolite suspension. Then, chitosan was dropped onto modified ITO electrode and was kept overnight for physical adsorption. Modified electrode (nanocrys zeolite/CHIT/ITO) was dipped into glutaraldehyde (2.5%; 1 mL) for cross-linking of enzyme. Electrode was dipped into enzyme solution (50 μL) and kept for 24 h at 4°C. The electrode was finally washed with 0.1 M phosphate buffer (pH 8.5) to remove any unbound enzyme. Then, there is a formation of modified sensing electrode (HRP/nanocrys zeolite/CHIT/ITO) and kept at 4°C, when not in use. This working electrode was characterized by scanning electron microscopy (SEM) and coefficient of variation (CV) at different stages of its fabrication (Scheme 1).

### Characterization Study of Nanocrystals Zeolites

X-ray diffraction (XRD) study of nanocrystals zeolites was carried out at Physics Department G. J. University, Hisar, India, using X-ray diffractometer (make: Rigaku Mini Flex II; Americas Corporation) and SEM studies have been used to characterize nanocrystals zeolites film-modified sensing electrode. Electrochemical study was performed using EIS and cyclic voltammetry technique, cell composed of HRP/nanocrys zeolite/CHIT/ITO as sensing electrode, Ag/AgCl as reference electrode, and Pt wire as auxiliary electrode were recorded in phosphate-buffered saline (PBS) (50 mM, pH 6.5, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4}$. Frequency range: 0.01 Hz to 10 KHz. EIS and CV patterns 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. Serum samples were acquired from University of Health Sciences (UHS), Rohtak, India. Various concentrations of LEV were spiked into serum samples. The measurements were performed after successive additions of spiked samples. After each addition, the potential between −1.00 and +1.25 V $s^{-1}$ at a scan rate of 50 mV $s^{-1}$ was recorded by cycling. To evaluate the data of LEV level obtained by present methods, following statistical formula was used: SD, mean, CVs, and correlation coefficient ($r$).

## RESULTS AND DISCUSSIONS

### Characterization of Various Stages of Fabrication of Nanocrys Zeolite-Modified Sensor

First step involved in the fabrication of biosensor was preparation of nanaocrystals of zeolites. Evidence for confirmation of preparation of nanocrystals of zeolites are shown below. Figure 1a reveals the microscopic image of nanocrystals of zeolites by transmission electron microscope. Image depicts that nanocrystals of zeolites was with size 50 nm. Figure 1b illustrates the XRD pattern of nanocrystals of zeolites. All peaks were consistent with the peaks of nanocrystals of zeolites (JCPDS card). Furthermore, no characteristic peaks were observed for other added impurities. Results revealed that pure nanocrystals of zeolites were formed.[19]

Second step involved in the fabrication of nanocrys zeolite-modified sensor was modification of sensing electrode

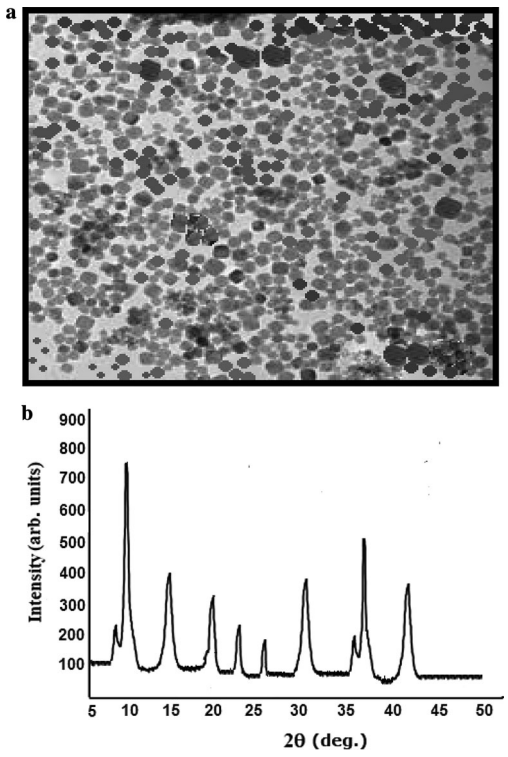



**Figure 1.** (a) Transmission electron microscope (TEM) image of nanocrystals of zeolites and (b) XRD pattern of nanocrystals of zeolites.

(HRP/nanocrys zeolite/CHIT/ITO). Modification of electrode was confirmed by SEM images. The surface morphologies of the (a) bare ITO, (b) nanocrys zeolite/CHIT/ITO, and (c) HRP/nanocrys zeolite/CHIT/ITO were studied. SEM image of the unmodified electrode reveals uncovered surface (Fig. 2a). SEM image of the nanocrys zeolite/CHIT/ITO-modified electrode showed rough surface (Fig. 2b) but after bioconjugation of enzyme, electrode morphology was altered into the smooth surface (Fig. 2c).

Coefficient of variation pattern was also carried out in order to validate the modification of sensing electrode by using the HRP/nanocrys zeolite/CHIT/ITO as the working electrode in the scanning potential range of $-1.00$ to $+1.25$ V $s^{-1}$ at the scan rate 50 mV $s^{-1}$ in 0.1 M phosphate buffer pH 7.5 (Fig. 3a) in the presence of leviteracetum. For comparative study, first bare ITO electrode was scanned in the potential range of $-1.00$ to $+1.25$ V $s^{-1}$ but no sensing was observed in the form of current, whereas electrochemical signal was observed when nanocrystal of zeolite (nanocrys zeolite/CHIT/ITO) was decorated onto bare electrode, which proved that nanocrystal of zeolite provides fast electron transfer kinetics (curve b). However, when enzymes gets conjugated with the sensing electrode (HRP/nanocrys zeolite/CHIT/ITO), signal was augmented as nanocrystal of zeolite provides biocompatible microenvironment for enzyme (curve c). Moreover nanocrystal of zeolite provides large and effective surface for immobilization of enzyme, which makes the reaction fast between enzyme and analyte.

Electrochemical impedance spectroscopy was also performed at different phases of fabrication of working electrode in order to validate the modification of sensing electrode. EIS of

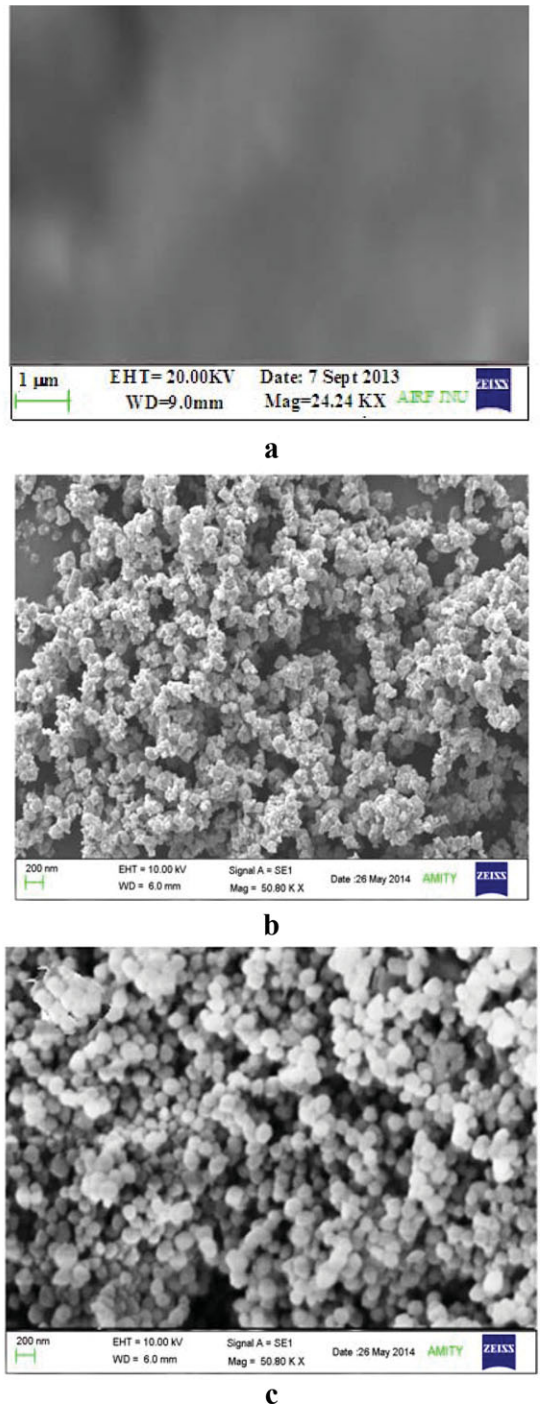



**Figure 2.** SEM image of (a) bare ITO, (b) nanocrys zeolite/CHIT/ITO, and (c) HRP/nanocrys zeolite/CHIT/ITO.

bare ITO (a), nanocrys zeolite/CHIT/ITO (b), HRP/nanocrys zeolite/CHIT/ITO (c) in the presence of LEV was taken. Sensing electrode modified with nanocrystals of zeolites produces small resistance charge transfer (1100 Ω) showing that the nanocrystals of zeolites enhances rate of electron transfer kinetics. After introduction of enzyme on the surface of sensing electrode in the presence of LEV, the Rct get further decreased (850 Ω) (Fig. 4b). The decrease in Rct value can be explained that after introduction of enzyme catalyzed redox

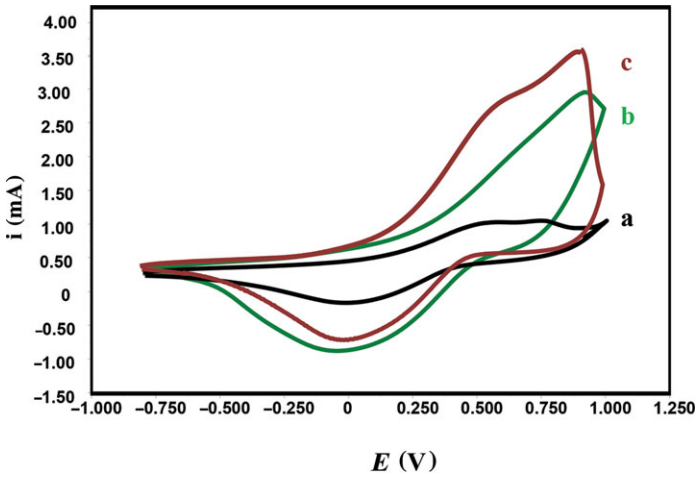



**Figure 3.** (a) CV pattern of (a) bare ITO, (b) nanocrys zeolite/CHIT/ITO, and (c) HRP/nanocrys zeolite/CHIT/ITO in a sodium phosphate buffer 0.05 M (pH 7.0) at a scan rate of 50 mV $s^{-1}$. (b) EIS of (a) bare ITO, (b) nanocrys zeolite/CHIT/ITO, and (c) HRP/nanocrys zeolite/CHIT/ITO in the background solution of phosphate buffer/KCl (0.1 M, pH 7.0).

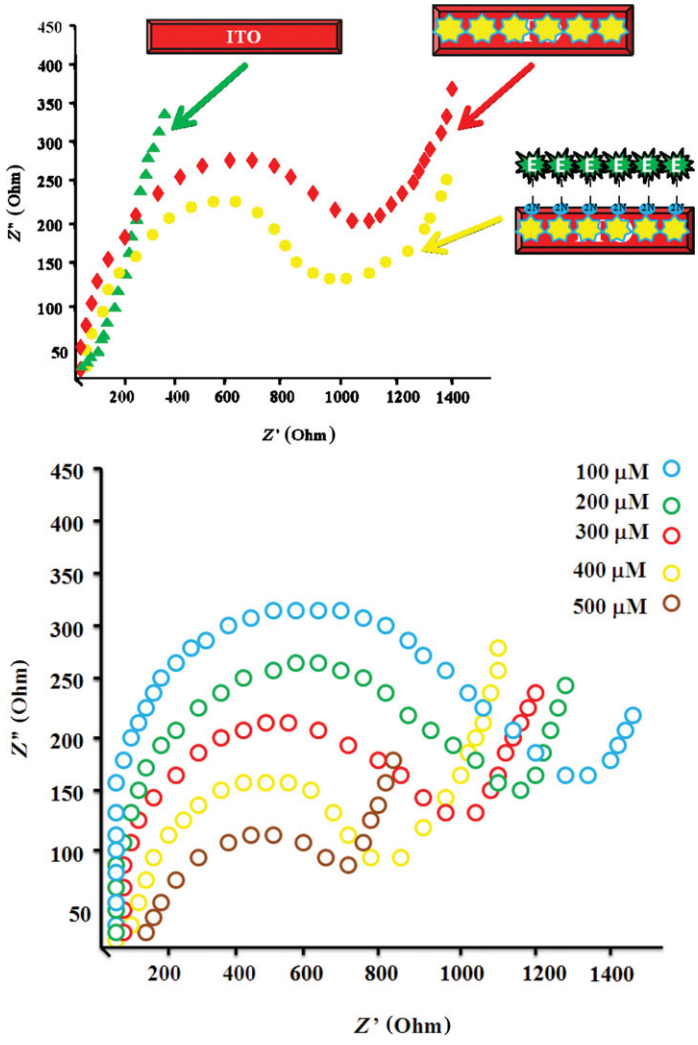
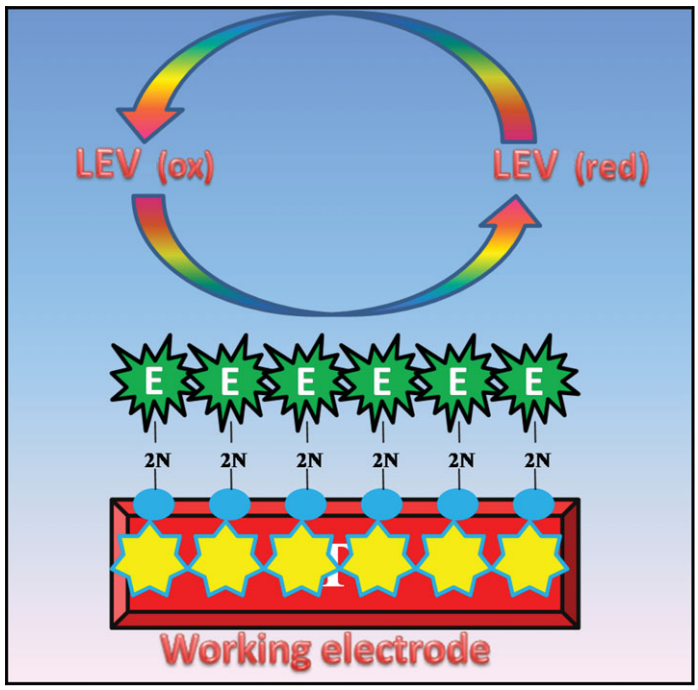



**Figure 4.** Impedimetric response of nanocrys zeolite sensor for LEV detection.




**Scheme 2.** Illustration of electrochemical sensing of LEV.

reaction was occurred and reaction rate become faster as LEV is a pyrrolidine derivative that can act as a substrate donor for the reduction of the HRP previously oxidized by $H_2O_2$ (Scheme 2). EIS studies are matching with the CV pattern of modified electrode.

All above results confirmed the modification of sensing electrode with nanocrystals of zeolites and HRP enzyme. These depictions also confirmed that nanocrystals of zeolite act as an enhanced sensing interface for electrochemical sensing of AED, that is, LEV.

### Optimization and Analytical Performances of Nanocrys Zeolite-Modified Sensor

Various experimental variables were studied in order to detect the relationship between experimental variables and impedimetric detection of LEV. Experimental variables like pH and temperatures were optimized so that these conditions also enhanced the electrochemical signal with combination of sensing interface. Influence of the pH value on the electrochemical signal of HRP/nanocrys zeolite/CHIT/ITO was studied between 5.0 and 8.0 in 0.05 M PBS. The suitable pH with the maximum activity of the immobilized HRP was at pH 7.0 which was similar to the optimum pH of 7.0 for free enzyme.[20] Biosensor response was decreased at lower pH; it may be because of the acidic nature of zeolite. The current response reaches a maximum at approximately 60°C, and then goes down as the temperature turn higher. In order to maintain steady with the temperature of human body, 35°C is selected for this work. Effects of serum interferents such as glucose, uric acid, urea, and cholesterol were also studied in order to check the selectivity of the nanocrys zeolite-modified sensor. Serum interferents do not affect electrochemical sensing of LEV. So nanocrys zeolite-modified sensor is anti-interferents (Table 1).

For evaluating the sensor, various parameters like analytic recovery, precision, and accuracy of proposed method were

 DOI 10.1002/jps.24267

**Table 1.** Interference Effect of Various Compounds on Nancrys Zeolite Sensor

| Interferents | Relative Response (%) |
|---|---|
| Glucose | 100 |
| Ascorbic acid | 120 |
| Urea | 100 |
| Uric acid | 97 |
| Cholesterol | 98 |
| Bilirubin | 98 |
| Pyruate | 100 |

**Table 2.** Determination of LEV by Nancrys Zeolite Sensor in Human Spiked Serum Samples

| Serial Number | Spiked Serum Samples (μM) | Present Method (μM) |
|---|---|---|
| 1. | 10 | 10 |
| 2. | 20 | 21 |
| 3. | 40 | 42 |
| 4. | 60 | 60 |
| 5. | 80 | 80 |
| 6. | 100 | 102 |
| 7. | 200 | 205 |
| 8. | 300 | 310 |
| 9. | 400 | 400 |
| 10. | 500 | 500 |

studied. Analytic recovery of known amount of added LEV was determined by the present biosensor. The mean analytic recoveries of added 100 and 200 μM LEV were 98.5 ± 1.4 and 98.1 ± 1.1 (mean ± SD; $n = 5$), respectively. For checking the reproducibility and reliability of the present biosensor, LEV content in 10 spiked serum samples was estimated on single day (within batch) and five times again after storage at 4°C (Table 2). The accuracy of the proposed method was determined by spiking serum samples with different concentrations of LEV. The present method also showed a high correlation ($r = 99$; $n = 10$).

### Electrochemical Sensing of AED

The change in electron transfer resistance after the successive addition of AED (LEV) in the range of 10–500 μM was studied. EI spectra were recorded in 5 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ containing 0.1 M KCl with incubation time of 2 s (Fig. 4). The incubation time was kept 2 s, after that analytical signal was produced. The Rct decreased with increasing AED concentrations as can be seen in Figure 4. It is mainly because of the redox reaction of LEV, which helps in generating electrons. The generated electrons are directly proportional to LEV concentrations. Increase in electrons enhanced charge transfer rate leading to decreased in Rct value resulting in increased sensitivity of the sensor. The results of experiments carried out in duplicate sets reveal reproducibility of the system within 1%. Limit of detection was found to be 0.01 μM. Response time and linear range depicts that nanocrystal of zeolites provides high and effective surface area for conjugation of enzyme on its surface. It also helps in providing the close proximity of enzyme and substrate that helps in producing analytical signal within 2 s.

Calibration was also performed using square-wave voltammetry with increasing concentration of LEV. The peak current is linearly related to the various concentrations of LEV (Figs. 5a and 5b).

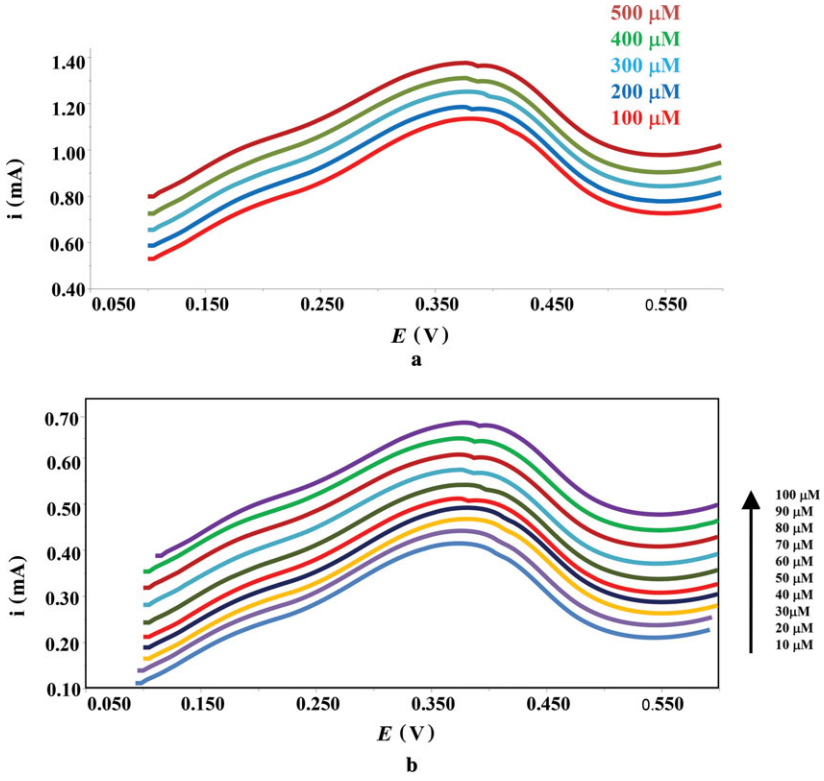



**Figure 5.** (a) SWVs response of modified electrode using different concentrations of substrate ranging between 100 and 500 μM and (b) 10 and 100 μM.

**Table 3.** Comparison of the Present Method with Other Biosensing Methods

| Matrix/Method | Enzyme | Response Time | Detection Limit (μM) | Linearity (μM) | Stability | References |
|---|---|---|---|---|---|---|
| HPLC–UV/chromatography | Nonenzymatic | NR | 0.1 | 1–75 | NR | 21 |
| Screen-printed carbon electrodes/cyclic voltammetry | HRP | NR | 17.5 | 100–830 | NR | 22 |
| Glassy carbon electrode/cyclic and square-wave voltammetry | Non enzymatic | 300 s | 0.005 | 0.006–0.5 | NR | 23 |
| Glassy carbon electrode/cyclic voltammetry | dsDNA-modified electrodes | NR | 0.1 | 0.5–5 | NR | 24 |
| HRP/nanocrys zeolites/CHIT/ITO/ impediometric and SWV | HRP | 2 s | 0.01 | 10–500 | 1 month | Present |

NR, not reported.

The developed nanocrys zeolite-modified sensor shows excellent reusability. The long-term stability is also tested after a month. It is revealed that the increase in the value of Rct has been found to be about 2% after 1 week, whereas Rct increases after 10 weeks resulting in about 40% loss in activity. This better shell life also depicts that zeolites are best sensing interface for biomolecules immobilization.

A comparison of analytic parameters of various nanoparticles-based biosensors for detection of LEV with the present biosensor is summarized in Table 3.

## CONCLUSIONS

Nanocrys zeolite-modified sensor acts as an amplified sensing interface for LEV and proved to be the best immobilization matrix for biomolecules. Applicability of this sensor is limited to electrochemical sensing of AED via EIS and SWV technique. LEV monitoring is necessary for mitigating its side effects. This developed technique can overcome the setbacks of other conventional methods. The nanocrys zeolite-modified sensor exhibits improved biosensing characteristics like linearity as 10–500 μM, fast response time of 2 s, specific and anti-interferents. The response of nanocrys zeolite-modified sensor with serum samples shows its implications toward the development of biosensor for commercial drug monitoring device.

## ACKNOWLEDGMENT

The present work was supported to one of the author (Jagriti Narang) by SERB, Department of Science and Technology (DST), India.

## REFERENCES

**1.** Lvov Y, Lu Z, Schenkman JB, Zu X, Rusling JF. 1998. Direct electrochemistry of myoglobin and cytochrome P450cam in alternate layer-by-layer films with DNA and other polyions. J Am Chem Soc 120: 4073.

**2.** Thierry B, Winnik FM, Merhi Y, Tabrizian M. 2003. Bioactive coatings of endovascular stents based on polyelectrolyte multilayers. Biomacromol 125:7494..

**3.** Groth T, Lendlein A. 2004. Layer-by-layer deposition of polyelectrolytes—A versatile tool for the in vivo repair of blood vessels. Angew Chem Int Ed 43:926.

**4.** Walcarius A. 2006. Zeolite-modified paraffin-impregnated graphite electrode. J Solid State Electrochem 10:469–478.

**5.** Walcarius A, Rozanska S, Bessiere J, Wang J. 1999. Screen-printed zeolite-modified carbon electrodes. Analyst 124:1185–1190.

**6.** Valdés MG, Cordoves-Pérez AI, Díaz-García ME. 2006. Zeolites and zeolite-based materials in analytical chemistry. Trends Anal Chem 25:24–30.

**7.** Chang BS, Lowenstein DH. 2003. "Epilepsy". N Engl J Med18(3):1257–1266.

**8.** World Health Organization. 2005. Atlas: Epilepsy Care in the World.

**9.** Khalil BA. 2008. Levetiracetam in the treatment of epilepsy. Neuropsychiatr Dis Treat 4:507.

**10.** Gambardella A, Labate A, Colosimo E, Ambrosio R, Quattrone A. 2008. Monotherapy for partial epilepsy: Focus on levetiracetam. Neuropsychiatr Dis Treat 4:33.

**11.** Farooq MU, Bhatt A, Majid A, Gupta R, Khasnis A, Kassab MY. 2009. Levetiracetam for managing neurologic and psychiatric disorders. Am J Health Syst Pharm 66:541.

**12.** Brodtkorb E, Klees TM, Nakken KO, Lossius R, Johannessen SI. 2004. Levetiracetam in adult patients with and without learning disability: Focus on behavioral adverse effects. Epilepsy Behav 5: 31.

**13.** Isoherranen N, Roeder M, Soback S, Yagen B, Bialer V. 2000. Enantioselective analysis of levetiracetam and its enantiomer R-alpha-ethyl-2-oxo-pyrrolidine acetamide using gas chromatography and ion trap mass spectrometric detection. J Chromatogr B 745:325.

**14.** Matar K. 2008. Quantification of levetiracetam in human plasma by liquid chromatography–tandem mass spectrometry: Application to therapeutic drug monitoring. J Pharm Biomed Anal 48: 822.

**15.** Martens-Lobenhoffer J, Bode-Boger SM. 2005. Determination of levetiracetam in human plasma with minimal sample pretreatment. J Chromatogr B 819:197.

**16.** Shihabi ZK, Oles K, Hinsdale M. 2003. Analysis of the antiepileptic drug keppra by capillary electrophoresis. J Chromatogr A 1004: 9.

**17.** Narang J, Pundir CS. 2011. Construction of a triglyceride amperometric biosensor based on chitosan–ZnO nanocomposite film. Int J Biol Macromol 49:707.

**18.** Kim HC, Wallraff G, Kreller CR, Angelos S, Lee VY, Volksen W, Miller RD. 2004. Photopatterned nanoporous media. Nano Lett 4:7.

**19.** Kokotailo GT, Fyfe CA. 1995. Zeolite structure analysis with powder x-ray diffraction and solid-state nmr techniques. Rigaku J 12:3.

**20.** Pundir CS, Malik V, Bhargava AK, Thakur M, Kalia V, Singh S, Kuchhal N. 1999. Studies on horseradish peroxidase immobilized onto arylamine and alkylamine glass. J Plant Biochem Biotechnol 8:123–126.

**21.** Lobenhoffer JM, Stefanie MB. 2005. Determination of levetiracetam in human plasma with minimal sample pretreatment. J Chromatogr B 819:197.

**22.** Hernández-Martín A, Arcos-Martínez MJ, Alonso-Lomillo, Domínguez-Renedo O, Asunción M. 2009. Horseradish peroxidase covalent grafting onto screen-printed carbon electrodes for levetiracetam chronoamperometric determination. Anal Biochem 395:86.

**23.** Radi A, El-Sherif Z. 2002. Determination of levofloxacin in human urine by adsorptive square-wave anodic stripping voltammetry on a glassy carbon electrode. Talanta 58:319.

**24.** Radi A, El-Ries MA, Kandil S. 2003. Electrochemical study of the interaction of levofloxacin with DNA. Anal Chim Acta 495:61.