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Research Article

AMPEROMETRIC GALACTOSE BIOSENSOR BASED ON SILVER NANOPARTICLES/CARBOXYLATED MULTIWALLED CARBON NANOTUBES/POLYANILINE COMPOSITE FILM

Mamta Sharma and Minakshi Sharma*

Department of Zoology, Maharshi Dayanand University, Rohtak-124001, Haryana, India

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ABSTRACT

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A nanocomposite based galactose biosensor was prepared by covalent immobilization of galactose oxidase on to Silver nanoparticles/carboxylated multiwalled carbon nanotubes/polyaniline decorated gold electrode. Characterization of working enzyme electrode was done by Scanning electron microscopy (SEM), Electron impedance spectroscopy(EIS) and Fourier transform infrared spectroscopy(FTIR). Detection limit was found 0.5mM, within 2 sec at pH 6. The biosensor showed a working linear range upto 18mM. The prepared biosensor was used for detection of galactose in human serum.

Key Words:

Galactose, Biosensor, Detection,
Nanoparticles.

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INTRODUCTION

Galactose determination is of great value in many area of medical science like food science, human nutrition, medicine and fermentation industry (Charmantray *et al.*, 2013). Normally, Galactose concentration was reported 0.28 mM-7mM in adults and 1.11mM in neonates (Brahim *et al.*, 2002). Increased amount of galactose in blood serum and urine leads to the onset of a disorder like galactosemia and other metabolic diseases (Berry *et al.*, 2001; Khun *et al.*, 2012). If galactosemia is not treated early, infant may suffer from various health problems like cataracts, liver diseases, kidney problems, brain damage etc (Wen *et al.*, 2005). Principally three enzymes are involved in galactose to glucose transformation, a galactose kinase, galactose-1-phosphate uridylyl transferase and uridine diphosphate galactose 4 epimerase (Barisic *et al.*, 2008). Deficiency or lack of any of these enzyme results in higher concentration of galactose and the disease associated with it called as galactosemia (Reichardt *et al.*, 1992). 90% of the neonatal infants born with galactosemia could be cured at an early stage but if not properly cured than 70% of them resulted in death (Park *et al.*, 2007).

Various techniques have been reported for galactose determination like, chromatography (Hansen,1975;Yuh *et al.*, 1998; Silva, 2006; Chen *et al.*, 2002; Hu *et al.*, 1995;Chiesa *et*

al., 1999), florimetry and spectro photometry (Kim *et al.*, 2012; Li *et al.*, 2010; Frings and Pardue, 1964; Henderson and Fales,1980). Though these techniques are highly sensitive but still have some limitations as these are either tedious or time consuming. To overcome these problems biosensor are reliable and easy way to detect analyte in biological fluids. Electrochemically conducting polymers like PANI and cMWCNT has been studied for the construction of biosensor because of their easy and direct deposition on electrode surface, good redox conductivity of polymer and good electron transfer efficiency (Sudik *et al.*, 2010). Nanocomposites having different combinations of nanomaterials further improve the sensitivity, selectivity and other characteristics of the biosensor. Likewise various types of metal oxide nanoparticles are also reported for fabrication of biosensors among them AgNPs holds excellent property. Chemical resistance and surface alteration of these AgNPs simultaneously with functional polymers are of significant importance. using metal oxide nanoparticles response time, linear range, reproducibility can be enhanced (Ahuja and Kumar, 2009; Sistani *et al.*, 2014). A combination of silver nanoparticles-CNT has been also reported in biosensing area (Numnuam *et al.*, 2014).

Galactose oxidase have broad substrate specificity it converts alcohols into aldehydes with the production of H₂O₂ (Tkac *et al.*, 1999).

*Corresponding author: **Minakshi Sharma**

Department of Zoology, Maharshi Dayanand University, Rohtak-124001, Haryana, India



Electrochemical analysis of galactose concentration by the use of amperometric galactose biosensor are most advance technology because of simple ,rapid, sensitive, selective nature (Kanyong *et al.*, 2013). In this study we explore the development of economical galactose biosensor based on AgNPs/cMWCNT/PANI onto the Au electrode with immobilized galactose oxidase enzyme. A different combination of nanomaterials employed in galactose sensor has not reported yet, it depicts its novelty.

Experimental Procedures

Reagents

Galactose oxidase was purchased from sigma-aldrich st. Louis, (USA), Carboxylated multiwalled carbon nanotubes (cMWCNT) (Functionalized) was purchased from Sisco research lab, Mumbai, India. Aniline, silver nitrate, ethanol, glutaraldehyde, N-ethyl-N-(3-dimethylaminopropyl Carbodiimide(EDC), N-hydroxy succinimide (NHS),D-galactose, potassium chloride(KCl), ascorbic acid, uric acid, glucose were purchased from Haryana scientific Lab, India. All other chemical were of analytical reagent grade. Double distilled water was used in all experimental studies.

Apparatus

Cyclic voltammetric studies were conceded in Potentiostat (Model: Autolab PGSTAT101, EcoChemie, Netherland) with three electrode system having Pt wire as auxillary electrode, Ag/AgCl as reference electrode and Au (diameter 1mm) as working electrode. All experiment were performed at room temperature. Transmission electron microscope (TEM) were recorded at SAIF, Punjab University, Chandigarh. Scanning electron microscope (SEM) (Zeiss) were performed at AIRF, JNU, New Delhi. Fourier transform infra red spectrophotometer (FTIR)(Bruker) carried out at pharmaceutical science department, M.D.University, Rohtak. UV spectrophotometer (systronics) and Ultrasonics (Rivotek), cold centrifuge (Remi), pH meter (systronics), water bath (Remi), Deep freezer (Super Tech) were also used.

Construction of GalOx/AgNPs/cMWCNT/PANI/Au modified electrode

Preparation of AgNPs

Preparation of silver nanoparticles were carried out by soft solution technique according to (Ngece *et al.*, 2011) with modification. Briefly, Solution I was prepared by adding 30mg of silver nitrate in 140 ml of ethanol and boil to 60-70⁰C for 30 min and solution II was prepared by adding 200mg of PVP in hot distilled water (1g/L).After this 10ml of solution II was added dropwise to solution I under vigorous shaking. After some time the colour become pale red, which indicate the completion of silver nanoparticles.

Electro-deposition of AgNP/cMWCNT/PANI onto Au electrode

Earlier than surface modification, Au electrode was surface-cleaned by alumina slurry with a polishing cloth then, by washing with distilled water, after that placed into ethanol solution and ultrasonicate for 30min to remove adsorbed

particles.To get the functionalized cMWCNT, 1 mg of cMWCNT was suspended in1 ml of a combination of concentrated H₂SO₄ and HNO₃ (3:1) and ultrasonicated for 2 hr to get a finally dispersed black coloured solution. The dispersed cMWCNT solution (0.1ml) was added into 0.5ml EDC and NHS mixture in 1:1 ratio and kept at room temperature for 1 hr. firstly the aniline was deposited onto the Au electrode by adding 500µl of aniline in 25ml KCL solution using cyclic voltammogram by exercising 20 deposition cycles at -1 to 1.5V at scan rate of 50mV/s (Fig 1A). After this 200µl solution of AgNPs and 500µl of activated cMWCNT were added to 25ml 1M KCL solution at -0.5V to 0.5V at scan rate of 50mV/s (Fig 1B). During the electrodeposition the exterior of Au electrode become green black pointing the deposition of AgNP/cMWCNT/PANI onto Au electrode. The resulting electrode was washed with distilled water and kept at 4⁰C in petri plate.

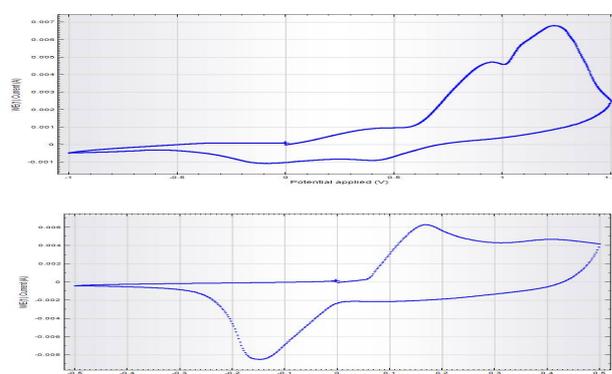
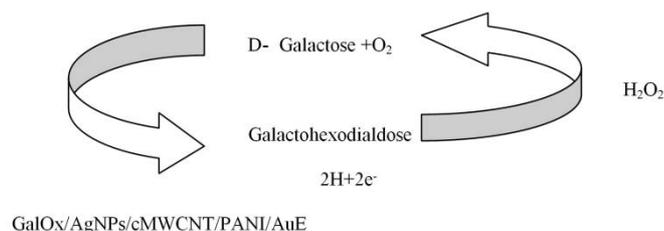


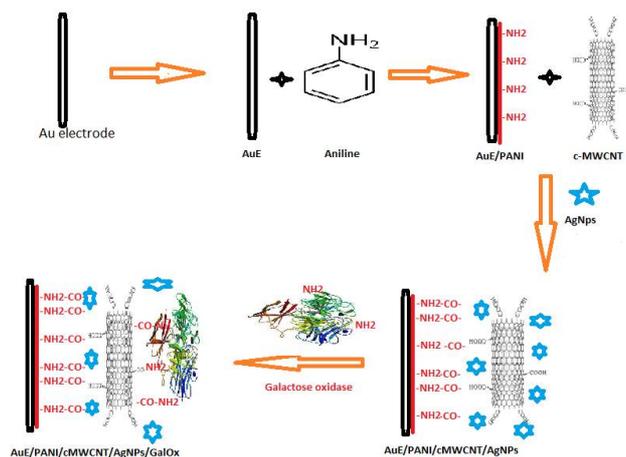
Figure 1 (A) cyclic voltammogram of electro-chemical deposition of PANI/Au (B) cyclic voltammograms of fabrication of AgNPs/cMWCNT/PANI on Au electrode in 0.1M KCL, Scan rate 50mV/s.

Immobilization of enzyme Galactose oxidase onto the AgNPs/cMWCNT/PANI/Au electrode

Immobilization of enzyme galactose oxidase onto the AgNPs/cMWCNT/PANI coated Au electrode was done through covalent linking by placing the electrode in potassium phosphate buffer (0.1M, pH 6.0) having 200µl of enzyme solution (1mg/ml) and kept it undisturbed overnight at 4⁰C. The principle behind the working of enzyme electrode was as follow. D-galactose was oxidized to galacto-aldehyde by enzyme electrode and then passing 2e⁻ from solution to working electrode through electrochemical reduction of Galactose. The current was calculated and it will be directly proportional to the amount of D-galactose as shown in Scheme 1.



Scheme 1 Scheme of generation of electron through GalOx/AgNPs/cMWCNT/PANI/AuE



Scheme 2 Graphic demonstration of chemical reaction involved in fabrication of GalOx/AgNP/cMWCNT/PANI/Au electrode.

Characterization study of GalOx/AgNPs/cMWCNT/PANI/Au enzyme electrode

The enzyme electrode was characterized by SEM, EIS and Fourier transform infrared spectroscopy (FTIR) methods at different steps of its modification. FTIR spectra was recorded by scrapping off the hybrid material from Au electrode and then mixed with potassium bromide (KBr) through hydraulic pellet press its pellet was formed and then put in a socket of spectrophotometer and its transmittance was recorded.

Response and Testing of GalOx/AgNPs/cMWCNT/PANI/Au

Electrochemical measurement were done via three electrode system with the GalOx/AgNPs/cMWCNT/PANI/Au electrode as working electrode, Pt as auxillary electrode and Ag/AgCl electrode as reference electrode in a cell containing 25ml of 0.1M potassium phosphate buffer(pH 6.0) having 1ml D-galactose(1mM). Current was measured by potentiostat using potential range of -0.4V to 1V at a 50mV/s scan rate. Utmost response was obtained at 0.59V (Fig 2). All further study were taken out at this voltage.

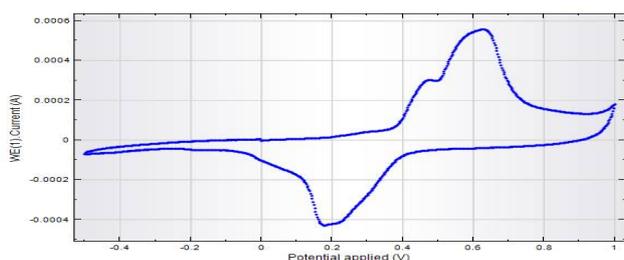


Figure 2 Cyclic voltammogram of GalOx/AgNPs/cMWCNT/PANI/Au electrode in 0.1M PB Buffer pH 6.0 contain 1ml of Galactose(1mM).

Optimization study of GalOx/AgNPs/cMWCNT/PANI/Au electrode.

The pH of the reaction buffer was made in range of 5 to 8 at an interval of 0.5 to study the optimal working state of enzyme electrode. Similarly to find out the optimal temperature, reaction buffer was stored at 15 - 45⁰ C at an interval of 5⁰ C. To check out the optimum response time, current was measured at an interval of 2 sec from 2-12 sec. The consequence of substrate concentration was also calculated by its different galactose concentration from 0.1 to 40 mM.

Amperometric measurement of Total Galactose in blood serum

The biosensor was used for galactose measurement in blood serum level of healthy and diseased individuals suffering from various galactose related disorders. Various serum samples were collected from local hospital Pt. Bhagwat Dayal Sharma post graduate institute of Medical Science (Rohtak). The methodology was same as described earlier for response testing of electrode in optimal conditions with the replacement by serum. Substrate concentration was deduced from standard curve of substrate versus current (Fig 3).

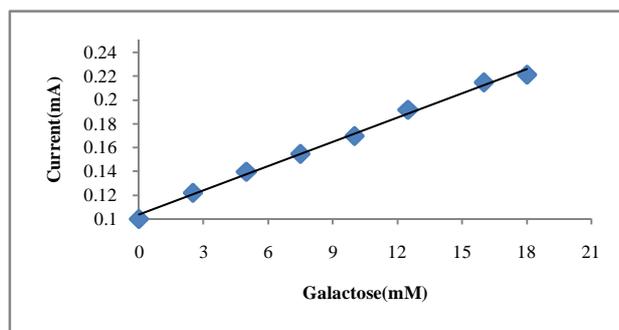


Figure 3 Standard curve of galactose by GalOx/AgNPs/cMWCNT/PANI/Au electrode based galactose biosensor.

Storage stability of GalOx/AgNPs/cMWCNT/PANI/Au electrode

The stability and resuability of working enzyme electrode was assayed for 6 months on weekly basis.

RESULTS AND DISCUSSION

Characterization of AgNPs

The prepared silver nanoparticles were characterized by TEM and FTIR spectra. TEM image of prepared silver nanoparticles showed the spherical shape (average size of 20nm) (Fig 4A). FTIR spectra showed the different peaks due to -OH stretching. After the reduction with silver nitrate shift in peak 456.66cm⁻¹ is attained confirming the formation of silver nanoparticles. At 1383.55cm⁻¹ stretching of -NO is observed. This is due to change and shift in peak of silver and silver nanoparticles (Fig 4 B).

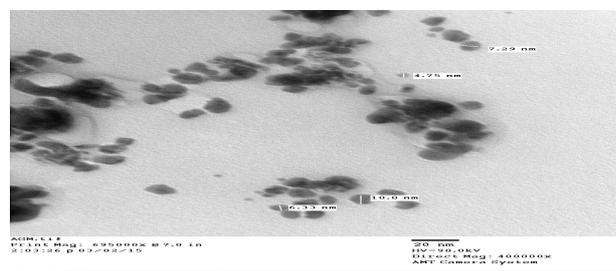


Figure 4 A TEM image of prepared Silver oxide Nanoparticle

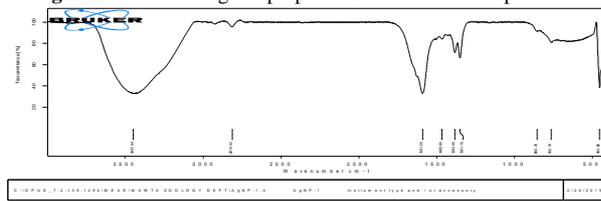


Figure 4 B FTIR of prepared Silver oxide Nanoparticle.

SEM Studies

SEM was done of working electrode at different steps of its preparation. The SEM images of bare Au electrode, PANI/Au, AgNP/cMWCNT/PANI/Au and GalOx/AgNPs/cMWCNT/PANI/Au electrode are shown in Fig 5 A-D. A smooth morphology was shown in case of bare electrode(Au) (Fig 5A) and fibrillar structure was observed in case of PANI/Au (Fig 5B). The SEM image of AgNP/cMWCNT/PANI/Au electrode exhibits its homogenous cable like morphology of nanocomposite film pointing towards its very well dispersion over the PANI layer in hybrid film (Fig 5C). Fig 5D shown the SEM image after immobilizing the GalOx enzyme. It indicates the look of sporadic globular beaded arrangement over the nanocomposite.

immobilization onto the AgNPs/cMWCNT/PANI/Au electrode. At elevated frequencies, diameter of semicircle suggest the electron-transfer resistance (R_{ct}). R_{ct} control the electron transfer kinetics at surface of electrode. Fig 7 display the Nyquist plot for EIS of GalOx/ AgNP/cMWCNT/PANI/Au in 5mM K₃Fe(CN)₆/K₄Fe(CN)₆ (1:1) as redox probe. It was found that R_{ct} value of GalOx/ AgNP/cMWCNT/PANI/Au electrode increased in comparison to R_{ct} value of AgNP/cMWCNT/PANI/Au electrode and PANI/Au. This increase in R_{ct} was due to poor electrical conductivity of enzyme at low frequency and it causes hinderance to electron transfer. The R_{ct} of AgNP/cMWCNT/PANI/Au was lower than PANI/Au due to high electron transfer efficiency and decreased resistance (Fig 7).

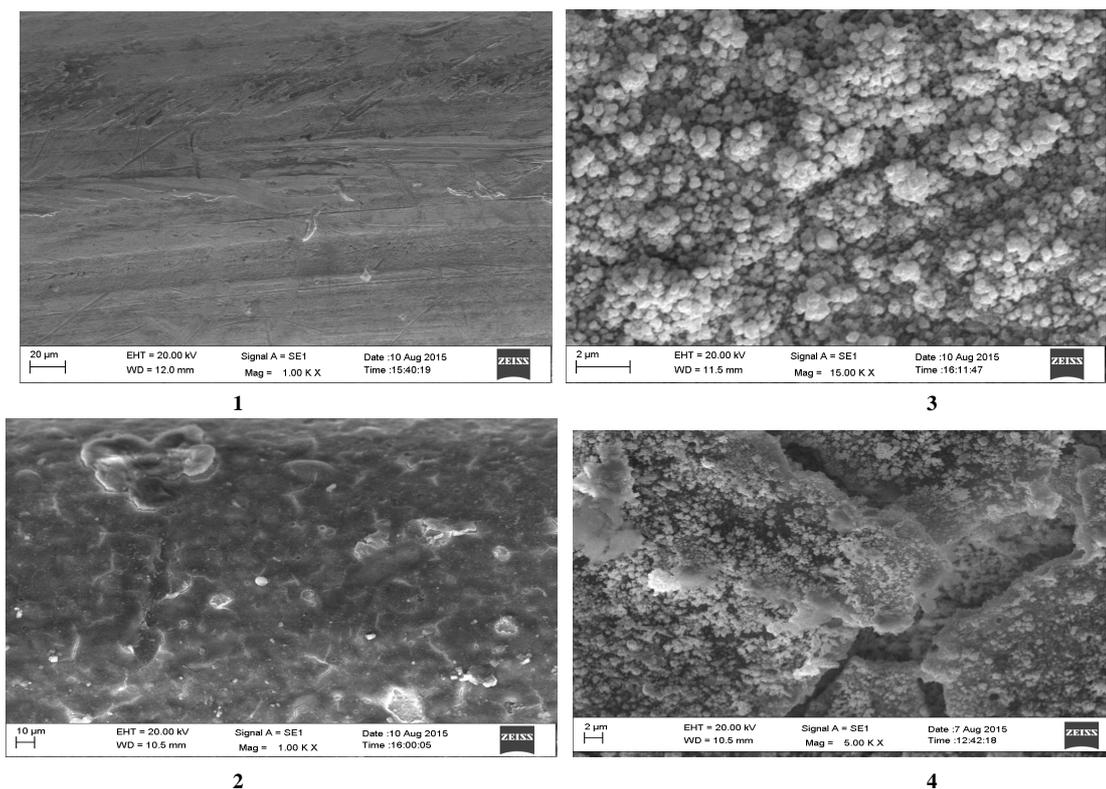


Figure 5 SEM images of (1) bare AuE (2) PANI/AuE (3) AgNP/cMWCNT/PANI/AuE (4)GalOx/ AgNP/cMWCNT/PANI/AuE

FTIR Studies of Au electrode

FTIR spectra of pure PANI exhibit a clear occurrence of benzoid ring at 1491cm^{-1} and quinoid ring vibration at 1548.23cm^{-1} due to oxidization of PANI. A very weak wide band around 3435cm^{-1} indicates the N-H stretching mode of salt of PANI (Fig 6A). FTIR spectra of AgNPs/cMWCNT/PANI showed a broad band around 3399.3cm^{-1} corresponds to $-\text{OH}$ group on cMWCNT (Fig 6B). No new absorption peak was observed in case of cMWCNT and PANI composite but there is slight change in peak shapes due to interaction between PANI and cMWCNT and AgNPs. A N-H stretching also observed due to its interaction. An additional absorption peaks was observed at 1502cm^{-1} due to enzyme binding (Fig 6C).

Electrochemical impedance measurements

Information regarding changes in impedance over the electrode surface was provided by EIS. EIS confirm the enzyme

Creation of Galactose biosensor

Scheme 2 describes the preparation of galactose biosensor based on immobilization of GalOx onto AgNPs/cMWCNT/PANI ornamented Au electrode. Firstly the PANI was fabricated on to the bare Au electrode as the thickness can be controlled. After this, solution mixture of cMWCNT and AgNPs were electrodeposited on PANI decorated Au electrode simultaneously. Finally working electrode was prepared by immobilizing covalently galactose oxidase onto the AgNP/cMWCNT/PANI/Au electrode. NH_2 groups of GalOx helps in formation of amide bond ($-\text{CO}-\text{NH}$) with COOH group of cMWCNT with the help of EDC and NHS chemistry. Both EDC and NHS helps in activation of free $-\text{COOH}$ groups of cMWCNT. AgNP/cMWCNT/PANI/Au electrode in comparison to PANI/Au exhibit a strong current which indicates its large surface area and act as conducting matrix for faster reaction. It also helps in enhancing the sensitivity and response of galactose biosensor.

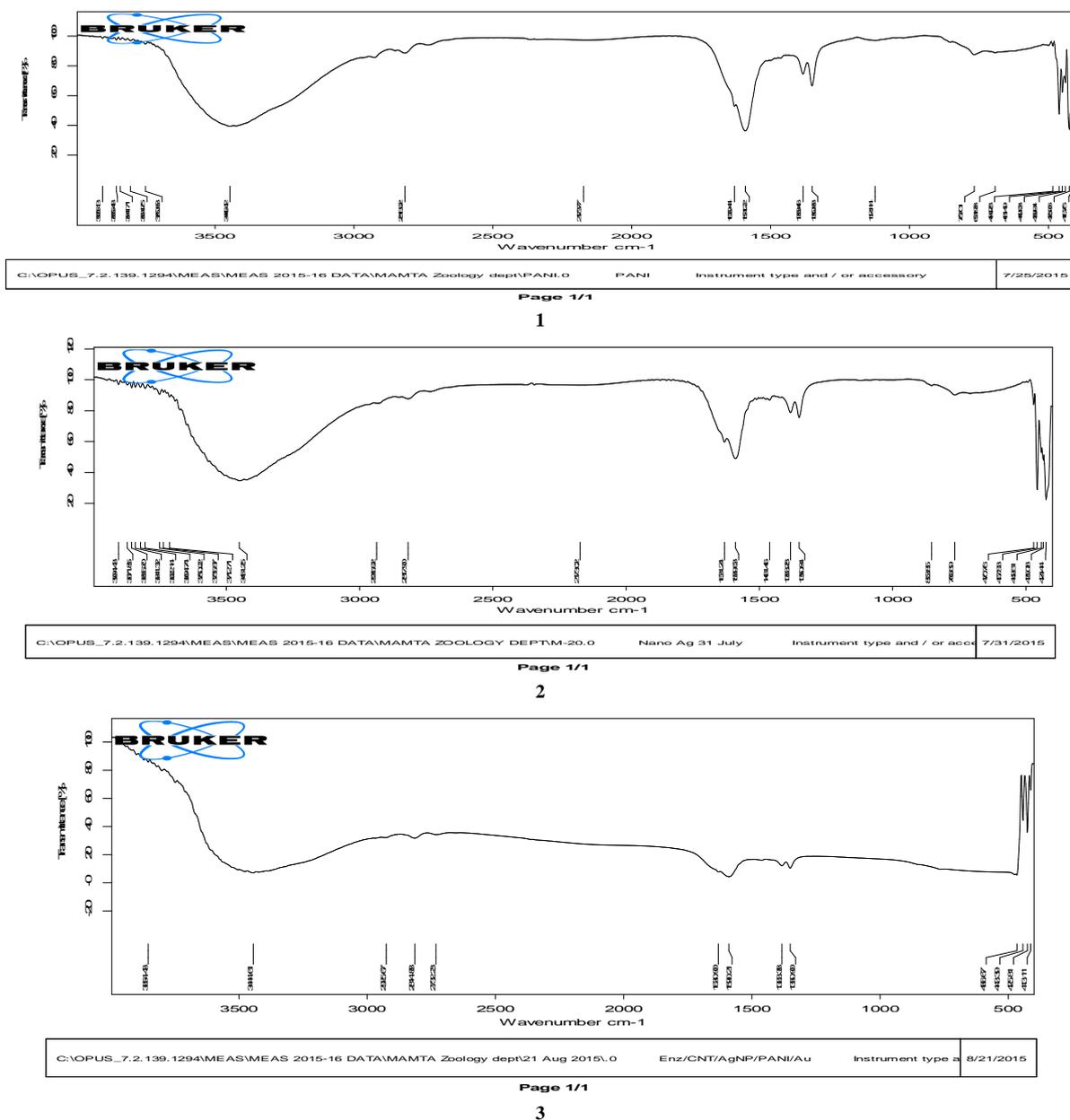


Figure 6 FTIR spectra of (1) PANI (2) AgNP/cMWCNT/PANI (3) GalOx/ AgNP/cMWCNT/PANI

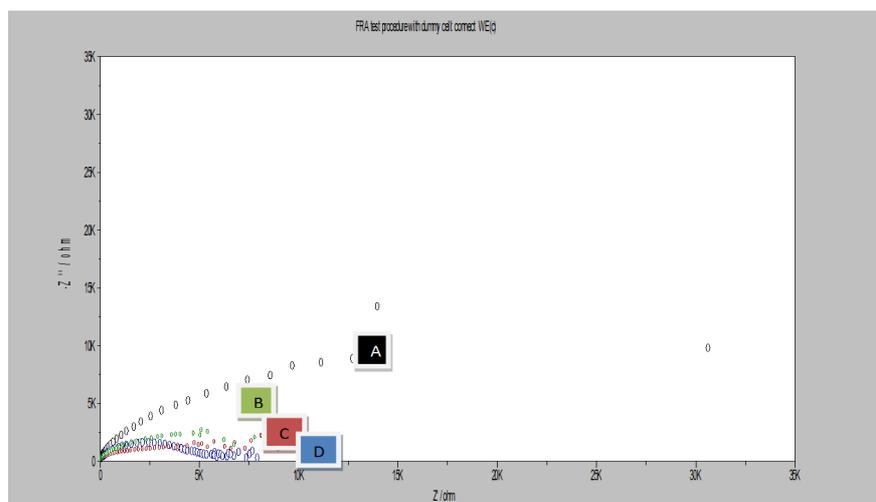


Figure 7 Nyquist plot of electrochemical impedance spectra(EIS) for Bare AuE (A) GalOx/AgNPs/cMWCNT/PANI/AuE(B) AgNPs/cMWCNT/PANI/AuE(C) PANI/AuE (D).

Table 1 Comparison of Current galactose biosensor with earlier reported biosensor

Support	Corelation	Linear Range	Detection limit	Storage life	Response time	Precision	Analytical recovery	References
Co3O4 nanoparticles and MWCNT modified GCE	0.996	9.0×10^{-6} - 6×10^{-4} M	90 μ M	1month	20s	-	98%	[Dalkiran <i>et al.</i> ,2016]
Gold nanoparticles modified graphene nanocomposite film	0.998	1.5 μ -6mM	0.79 μ M	10days	-	-	98-104%	[Xie <i>et al.</i> , 2016]
Cellulose acetate modified screen printed carbon electrode	-	1.98-9.52mM	0.2mM	24hr	-	1.3%	-	[Kanyoung <i>et al.</i> ,2016]
Cobalt phthalocyanine modified screen printed carbon electrode	-	0.1-25mM	0.02mM	14 days	30sec	1.1-0.11%	99.9%	[Kanyoung <i>et al.</i> , 2013]
Laponite clay film-coated platin electrode	-	0.001-1.6mM	0.001mM	30 days	5sec	-	-	[Charmantray <i>et al.</i> ,2013]
Poly(glycidylmethacrylate-co-vinylferrocene)-modified platin electrode	-	2-20M	0.1mM	30 days	5sec	-	-	[Cevik <i>et al.</i> , 2010]
Single walled carbon nanotubes-modified GCE	0.999	0-10mM	0.025mM	2.5hr	150injection/hr	1.15%	101.2 \$ 102.7%	[Tkac <i>et al.</i> ,2007]
Polypyrrole.polyanion/PEG/enzyme conjugate dopant modified	0.98	0-24mM	-	-	40sec	-	-	[Sung and Bae, 2005]
Polyacrylonitrile thin film-modified platin electrode	-	0.02-1.6mM	-	30 days	-	-	-	[Kan <i>et al.</i> , 2005]
Eggshell membrane	0.998	0.1-8.5mM	-	3 month	100sec	-	94 \$110%	[Wen <i>et al.</i> , 2005]
Poly(N-glycidylpyrrole-co-pyrrole)	0.990	2-16mM	0.025mM	10days	5sec	-	96.9 \$98.8%	[Senel <i>et al.</i> ,2011]
Microfabricated thin film electrode	0.999	0.1-0.8mM	-	>1month	-	-	97.5%	[Neng-Qin <i>et al.</i> ,2007]
Polypyrrole-hydrogel composite membrane	>0.997	0.05-10mM	25 μ M	9months	70s	3.8 \$4.4%	97%	[Brahim <i>et al.</i> ,2002]
GalOxNPx modified Au electrode	0.988	0.1-20mM	0.16mM	3month	8s	4.3% 4.8%	96.8 \$ 99%	[Sharma and Sharma, 2016]
Conducting polymer microtubules modified ITO electrode	0.995	0.1-10mM	0.01mM	7 days	30sec	-	-	[Lee <i>et al.</i> , 2011]
GalOx/AgNP/cMWCNT/PANI/Au	0.993	0.1-20mM	0.15mM	6 months	2s	4.39 \$ 5.19%	96.2 \$ 98.4%	[This work]

Response testing of GalOx/AgNP/cMWCNT/PANI/Au electrode

Cyclic voltammogram of PANI/Au and AgNP/cMWCNT/PANI/Au was recorded in 25ml of 0.1M KCL in absence of D-galactose. Whereas in case of GalOx/AgNP/cMWCNT/PANI/Au, CV was recorded in presence of 1ml of 1mM D-galactose at scan rate of 50mV/s. It was found that oxidation rate was increased after addition of D-galactose. Bigger redox peaks was observed in case of GalOx/AgNP/cMWCNT/PANI/Au which indicate the oxidation of galactose onto surface of working electrode results in enhancement of current. Both AgNPs and cMWCNT acts as electron mediator helps to enhance electron transfer. The biosensor responded within 2 sec producing 90% of current.

Evaluation and performance of biosensor

The linear working range was reported from 0.1 to 18mM between current and galactose concentration which is better/comparable to earlier reported biosensor. The detection limit of the current sensor was reported 0.5mM, which is lower/better than earlier reported biosensor. Six different serum sample was tested in a day (within batch) and 5 times again after their storage at -20°C for one week (between batch) in order to check the precision value. The results of within batch and between batch were calculated by coefficients of variation (CV) which was found 4.3 and 5.1% respectively. The higher precision value indicates the good reproducibility of the current biosensor. To test the accuracy of the present biosensor, recovery study was done by adding two different value for galactose (0.5mM and 1mM). The high accuracy was found to be 96.2% and 98.4% respectively. The galactose concentration

in human serum samples was measured by present biosensor(y) and those with values obtain by spectro-photometric method(x), there was a better correlation found (r=0.993) and a regression equation y=1.097x-0.037. The electrode lost 20% of its initial activity after 150 uses during the span of 6 months when stored at 4°C. The good repeatability and stability was found for current biosensor employ the better immobilization of enzyme onto AgNPs/cMWCNT/PANI/Au electrode (Fig.8). Galactose content in apparently healthy individual was found with a mean of 2.2 to 3.8mM in males and 2.9 to 3.6mM in females and in diseased individuals it was found 7.79 to 9.96mM in males and 7.71 to 8.51mM in females (Table 1).

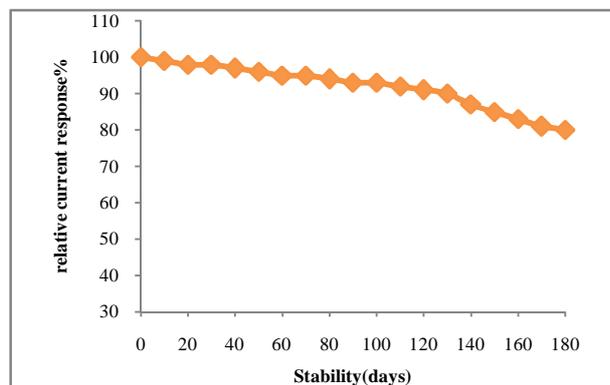


Figure 8 Effect of storage stability of present galactose biosensor.

CONCLUSION

The use of AgNPs/cMWCNT/PANI nanocomposites in the preparation of current biosensor helps to improve its analytical performance like its low working potential (0.59V), short response time (2s), low detection limit (0.5mM) and good

storage stability (6 months).Based on such observations, this nanocomposites could be further used for the improvement and preparation of other biosensors.

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