Original Research Article **Biochemistry**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

GALACTOSE OXIDASE NANOPARTICLES BASED NANOSENSOR FOR AMPEROMETRIC DETERMINATION OF GALACTOSE

MAMTA SHARMA[1] AND MINAKSHI SHARMA[1*]

[1]*Department of Zoology, Maharshi Dayanand University,Rohtak-124001,Haryana,India.*

ABSTRACT

A method is described for the construction of an amperometric biosensor for measurement of galactose in serum. The nanosensor comprises galactose oxidase nanoparticles immobilized directly on the surface of gold (Au) electrode as working electrode, Ag/AgCl as reference electrode and platinum (Pt) wire as an auxiliary electrode connected through potentiostat. The nanosensor showed an optimum response at pH 6.0 and temperature 30°C, when operated at a scan rate of 50 mv/s. The working range of the sensor was 0.1 to 20 mM, with a detection limit of 0.16 mM. The enzyme electrode was used many times over a period of 90 days, when stored at 4°C.The Nanosensor has an advantage over the earlier enzyme sensor in that the enzyme nanoparticles are more stable, sensitive and achieve higher self life during construction of nanosensor. The fabricated nanosensor was successfully employed for the determination of galactose in human blood serum.

KEYWORDS: **Galactose oxidase, nanoparticles, Biosensor, Au electrode, Galactose.**

MINAKSHI SHARMA

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

INTRODUCTION

Galactose is the principal product for clinical purpose, especially for the diagnosis of galactosemia and galactose intolerance.[1] Blood galactose monitoring is very important, as it is a powerful indicator of various galactose related disorders. The metabolism of galactose may affect some degree of regulation of blood glucose level in infants. The normal level of galactose in serum is up to 1.11mM in neonates and up to 7mM in adults. Value greater than 7mM considered towards illness.[2] Fruits, vegetables, grains, breads, fats, sugars do not have galactose containing contents .only few vegetables and fruits have a law amount of bound galactose which is not easily consumed by the human body; therefore it can raise the galactose-1-phosphate level in the blood.[3]Various disorders associated with impaired galactose metabolism are galactosemia,[4] hyperbilinirubinemia, premature ovarian failure,[5] cataract and mental retardation.[6,7] Methods for galactose determination are Chromatographic,[8-10] Colorimetric[11] and Mass spectrometric.[12,13] These methods are either tedious or time consuming or not sufficiently specific, therefore biosensor is emerging field to combat these drawbacks.[14] Direct adsorption of enzyme NPs onto a metal electrode usually results in its denaturation and loss of bioactivity, due to change in its physiocochemical properties.[15] To overcome the said problem, enzyme have been tried to cross-link within their self in controlled manner to make cross-linked enzyme nanoparticles.Various studies shown that by using enzyme nanoparticles the response time could be decreased to a few seconds.[16-17] The unique characteristics of nanoparticles such as small size (1-100nm), high surface to bulk ratio lead to an increase in their optical, catalytic, mehanical, physical, and electric properties. The enzyme nanoparticles have enhanced shelf life, operational stability and complete stability towards leaching in aqueous media.[18] Therefore the present work describes the improved amperometric determination of galactose in blood by preparing galactose oxidase NPs and their direct immobilization on Au electrode through gold-thiol bonding.

EXPERIMENTAL

Reagents

Glutarldehyde (25%), Galactose oxidase, horseradish peroxidase were from Sigma-Aldrich Co., St.Louis, USA. Gold wire(23 carat) was purchased from a local market (23 Carat), Dextrose, cysteamine dihydrochloride, ethanol, methanol were from Himedia laboratory chemicals, India. All other reagents were of analytical reagent grade. Double distilled water (DW) was used in all experiments. Fresh serum samples of healthy and diseased persons were collected from the Hospital of Pt. BDS University of Health and Medical Science, Rohtak and stored at -20°C until use.

Apparatus used

Cyclic voltammetry (CV) measurements were performed on Potentiostat (Autolab,PGSTAT 101) with three electrode system, GalOxNP electrodeposited Au wire as a working electrode, Ag/AgCl electrode as reference electrode and Pt wire as an auxiliary electrode at an ambient temperature (25° C). Fourier Transform Infrared (FTIR) Spectra was recorded at FTIR (Bruker) in the Pharmaceutical Science Department, M.D. University, Rohtak and Scanning electron microscopy (Zeiss EV040) was done at Advanced Instrumentation Research Facility (AIRF), J.N.U. New Delhi. Transmission Electron Microscopy (TEM) was carried out at PU, Chandigarh. All experiments were carried out at room temperature.

Assay for Galactose oxidase (GalOx)

GalOx assay was based on measurement of H_2O_2 generated in GalOx reaction using a color reaction with 4-aminophenazone, phenol and horseradish peroxidase as chromogen system.[19] It was carried out in 15ml test tubes wrapped with black paper. Assay mixture contained 1.8ml of 0.1M potassium phosphate buffer (PB, pH 6.0), 0.1ml enzyme (0.50units/ml) and 0.1ml galactose(360mg/ml) as substrate. After incubating the reaction mixture at 37°C for 2 min,1 ml of colour reagent was added and kept at room temperature for 15min to develop the color. A_{425} nm was read in spectronic -20 and H_2O_2 generated was determined from standard curve between H_2O_2 versus $A_{425.}$ The color reagent consisted of 50mg of 4-aminophenazone, 100mg phenol (solid), and 1mg horseradish peroxidase in 100ml of 0.4MPB, pH 7.0 and stored in amber colored bottle at 4°C.

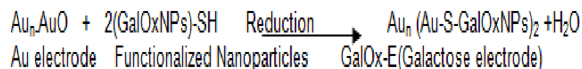
Preparation of Galactose oxidase Nanoparticles (GalOxNP) Aggregates

The GalOx-NP aggregate were prepared by desolvation with ethanol and subsequent cross linking with glutraldehde with modification.[14] In 1 ml galactose solution, 6 ml of absolute ethanol was added drop wise at a rate of 0.1 ml/min under constant stirring at 500 rpm, resulting into aggregation of enzyme/protein molecules into small particles. This was followed by an addition of 1.8 ml of 2.5% glutaraldehyde to these NPs suspensions under continuous stirring at 500 rpm at 4°C for 24 hr, thus ensuring the crosslinking of Nanoparticles and thus,forming enzyme nanoparticles. The resulting nanoparticles were purified by three cycle of differential centrifugation at 14000 rpm at 4°C. for 10 min, followed by redispersion of GalOxNPs in 0.1M potassium phosphate buffer (pH 6.0) and sonication for 5min.- SH groups were introduced on GalOxNPs by addition of 0.12 g of cysteamine with constant stirring for 5-6 h. At last GalOxNPs were separated from free enzyme solution by centrifugation at 12000 rpm at 4°C for 10 min, followed by dispersion in 0.1 M potassium phosphate buffer (pH 6.0) and stored at 4°C.

Preparation of Galactose NPs modified Au Electrode

The gold electrode was cleaned before immobilizing the the GalOxNPs. For cleaning the electrode was ultra sonicated in piranha solution (H_2SO_4:H_2O_2 in 3:1 ratio) for 30 minutes followed by its washing with distilled water. The Au electrode was then polished with alumina slurry. One millilitre suspension of GalOxNPs in 0.1M potassium PB, pH 6.0 was layered onto the surface of polished Au electrode and kept overnight at 4°C for its immobilization. The resulting GalOxNP/Au electrode was washed three –four times with buffer to remove unbound protein.The modified electrode was used as working electrode and stored at 4°C, when not in use.

The immobilization was confirmed by high resolution scanning electron microscope with and without the immobilized galactose oxidase nanoparticles. The thiol-functionalized cross-linked galactose NPs get bound to Au electrode through Au-thiolate bond through the process as follows:

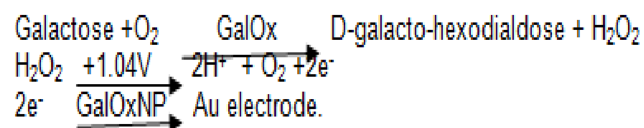


Characterization of enzyme electrode

The modified gold electrode was characterized by scanning electron microscopy (SEM)(Zeiss EV040,Model:JSM-6510, at Advanced Instrumentation Research Facility, Jawaharlal Nehru University, New Delhi), Fourier Transform Infrared Spectra in FTIR spectrophotometer (Bruker, Model -ALPHA),UV visible spectroscopy and electrochemical study in Potentiostat (Autolab 101) with three electrode system, GalOx/Au as working electrode, Pt as auxiliary electrode and Ag/AgCl (saturated 3M KCL) as reference electrode.To record FTIR spectra of hybrid material deposited onto Au electrode,the electrodeposited material was scrapped off the Au electrode, mixed with dried KBr and its pellet was formed by hydraulic pellet press. Then this pellet was kept into the socket of the FTIR spectrophotometer and its spectrum was recorded.

Construction and response measurement

Amperometric measurement was carried out using Ag/AgCl as reference electrode and the platinum as countercurrent and gold as working electrode. Current measurements were done with the potentiostat (AUTOLAB,101) at applied potential of -0.1V to 1.3V (vs Ag/AgCl) at scan rate of 50mV/s in 25ml of 0.1M potassium phosphate buffer (pH 6.0) containing 0.1ml of 10mM of galactose. The maximum response (current in mA) was observed at 1.04V (Fig.1) and hence further studies were carried out at this potential. To measure the response of enzyme electrode/biosensor, the three electrode system was immersed into 25ml of 0.1M Potassium phosphate buffer (pH 6.0) and the reaction was started by adding 0.1ml of galactose (10mM), which was oxidized to D-galacto-hexodialdose producing an electroactive H_2O_2(scheme-1). Formation of H_2O_2 was detected by its electro-oxidation under high voltage to generate electrons.The flow of electron i.e. current was measured in mA at +1.04V .The electrochemical reaction involved is as follows:



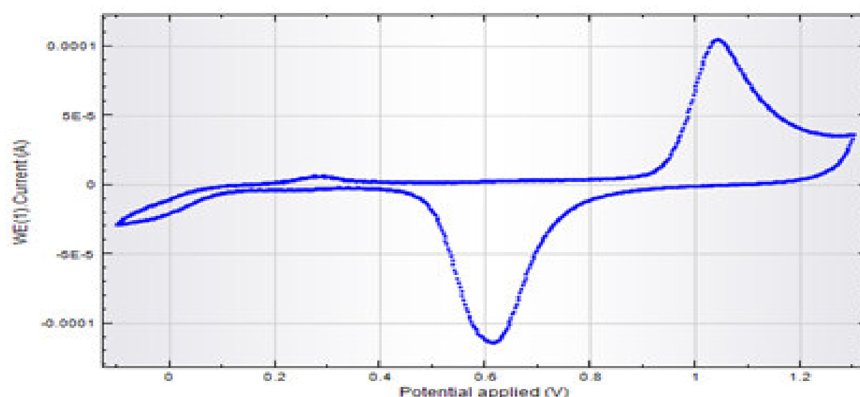


Figure 1
Cyclic voltagram for GalOxNPs/Au electrode 0.1ml of galactose in 0.1M potassium phosphate buffer(PB) (pH 6.0) with scan rate of 50mV/s.

Optimization of GalOx/Au electrode

To determine the optimum working conditions of the enzyme electrode, the pH of reaction buffer(0.1M) was varied from pH 5.0-9.0 at an interval of pH 0.5 using potassium phosphate buffer. Likewise, to determine optimum temperature, the reaction mixture was incubated at 20° C -50° C at an interval of 5°C.To determine the optimum response time ,the current was measured from 2 to 12 s at an interval of 2s. To study the effect of substrate concentration, different galactose concentrations ranging from 0.1 to 50mM were used.

Determination of Total Galactose in serum

Blood samples (1ml each) from healthy and diseased individuals suffering from hypo and hyper galactosemia, both male and female of different age groups were collected from Pt BDS University of Health and Medical Science, Rohtak, centrifuged at 5000rpm for 5 min and the supernatant (serum) was collected. Galactose in serum was determined by present biosensor in the similar manner as described above for its response measurement, under its optimal working conditions except that galactose solution was replaced by serum. The galactose content in serum was interpolated from standard curve between galactose concentration and current in mA.

Evaluation

The present biosensor was evaluated by studying its analytical recovery, detection limit, precision and correlation. Analytical recovery was studied using blood serum with two different standard concentrations (0.5 and 1mM) of galactose. Precision study was done to check the reliability and reproducibility of present biosensor by measuring galactose in six serum samples, five times on single day (within batch) and five

times again after their storage at -20°C for 1 week (between batch).Correlation studies was done by measuring galactose level in serum sample (both from healthy and diseased persons) by enzyme colorimetric method(x) and the present method(y) and their correlation (r) was studied using the regression equation.

Interference study

It was carried out by measuring the biosensor response before and after addition of physiological concentration of various interferants in 0.1M PB (pH 6.0) containing 0.1ml of galactose and the current was recorded.

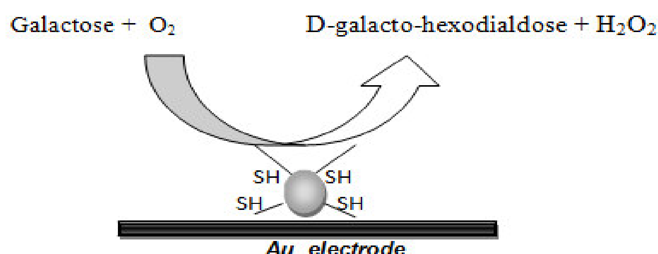
Resuability and storage stability of GalOxNPs/Au electrode

The long term storage stability of enzyme electrode was studied by measuring its activity weekly, storing it in 0.1M Phosphate Buffer, pH 6.0 at 4°C, when not in use.

RESULTS AND DISCUSSION

Preparation of GalOxNP nanoaggregates

GalOxNP aggregates were prepared by desolvation with ethanol and subsequent crosslinking with glutaraldehyde. There was a decrease of hydration layer around enzyme during dropwise addition of ethanol to GalOx solution, confirming the formation of aggregates. So, this doesn't reduces the enzyme activity. Forces like van der waal's, hydrophobic and electrostatic interactions exist between theses enzyme molecule. Thiol group was then introduced on to these NP aggregates by the addition of cysteamine (Scheme-1). These enzyme aggregates were cross-linked by reaction of reactive groups on the enzyme surface (e.g., free –SH groups) with a bi-functional cross-linking agent such as glutaraldehyde, rendering the aggregates permanently insoluble, while maintaining their pre-organized structures and hence, their activity. The activity of NP aggregates was increased as compared to the free enzyme, because these aggregates can orient themselves in such a way that their active site becomes more accessible to the substrate, without undergoing any structural change.This activation might be due to conformational changes induced in the enzyme during aggregation.



Scheme 1
Scheme of reaction mechanism of Galactose NPs/Au working electrode.

Characterization of GalOx NPs aggregates.
Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of GalOxNPs aggregates obtained a vibration band at 1634cm^{-1} due to C=N stretching formed after glutaraldehyde reaction, confirming the formation of GalOxNPs aggregates also with the vibration bands of amideI and amideII having its enzymatic activity. There is also a broadening of vibration band at 2000-2500cm^{-1} in case of GalOxNPs confirming the presence of more free amine (NH_2) groups, introduced by cysteamine dihydrochloride (Fig. 2).

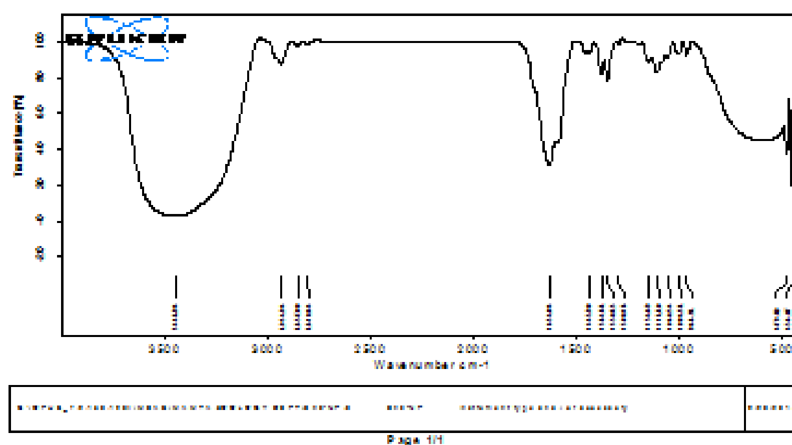


Figure 2
FTIR spectra of GalOxNPs

Transmission electron microscopy (TEM)

TEM image of synthesized GalOxNPs showed their spherical shape with diameter in the range of up to 20nm, revealing their synthesis (Fig. 3).

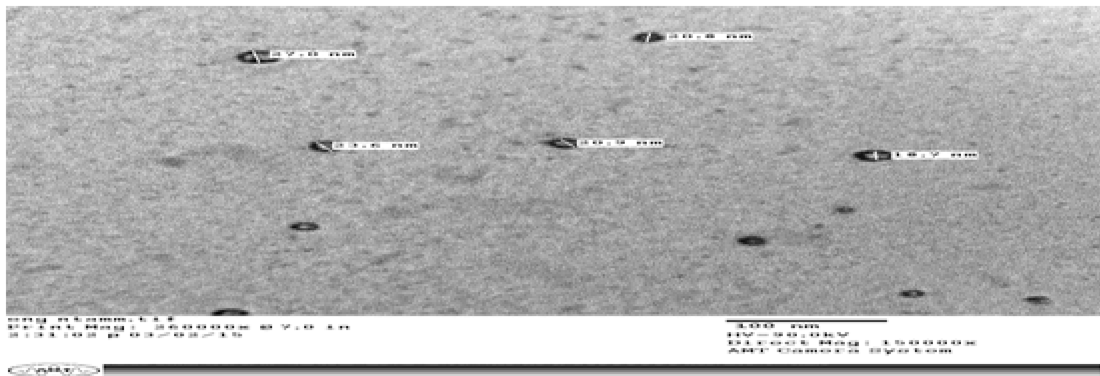


Figure 3

Transmission electron microscopic (TEM)image of GalOxNPs

Scanning electron Microscopy (SEM)

Surface morphology of both bare electrode[a] and GalOxNPs/Au [b] was recorded by Scanning electron microscopy (SEM). A homogenous surface was shown by bare Au electrode. The SEM image for GalOxNPs/Au showed globular structural morphology with cluster of aggregation of enzyme on the surface of Au electrode, confirming the immobilization of enzyme nanoparticles aggregates successfully (Fig.4).

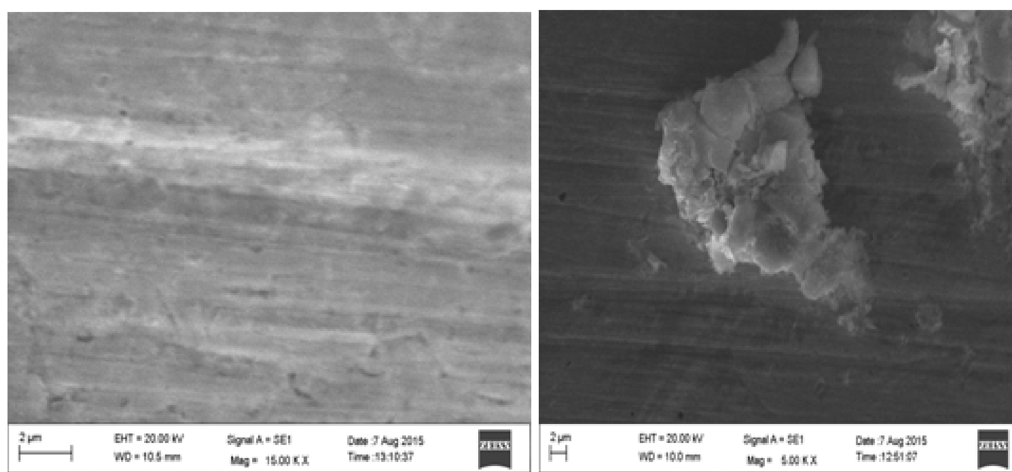


Figure 4

SEM image of Au electrode

[a] Bare Au electrode [b]with immobilized GalOxNPs aggregates

Optimization of the Biosensor

The optimum response was obtained between pH 5.5 and 6.5. The optimum temperature was obtained at 30^oC. when Galactose was added into potassium phosphate buffer, pH 6.0,the biosensor responded rapidly to the substrate Galactose and achieved 95% of current within 8sec.

Evaluation of Biosensor

1.Linearity

Linear relationship was found between current (mA) and Galactose concentration ranging from 0.1 to 20 mM (Fig. 5), with a sensitivity of 45 $mA/mM/cm^2$,which was better than earlier reported biosensors based on poly(N-glycidylpyrrole-co-pyrrole)(2-16mM),[20] thin film electrode(0.10mM-8mM),[21] eggshell membrane(0.1-8.5mM),[22] Chitosan matrix (1mM),[23] poly(3-hexyl thiophene)(0.05-0.5g/l).[24]

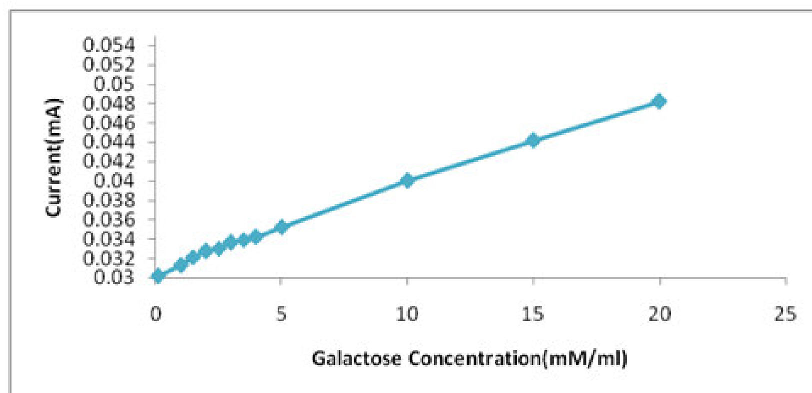


Figure 5
Effect of galactose concentration on the current response of the fabricated galactose biosensor based on GalOxNPs/Au electrode.

2.Detection limit
The detection limit (S/N=3) of the present biosensor was 0.16mM which is comparable with biosensors based on Cellulose acetate membrane (0.1m M)[2] and better/lower than those based on epidermis of solanum lycopericum (0.44mM)[26].

3.Analytical Recovery
Analytical recovery of added galactose in the serum samples was determined to check the accuracy. The mean analytical recovery of added galactose (0.5mM and 1mM) in serum (n=5) was 96.8 and 99.9% for present biosensor. This confirmed that the method is reproducible, precise and accurate for determination of galactose.

4.Precision
To check the reliability and reproducibility of the present biosensor, galactose content of the samples in one run (within batch) and after storage at -20°C for one week (between batch) were determined. The results showed that galactose value were agreed with each other and within batch and between batch coefficient of variation (CV) were 4.3 and 4.8% revealing the high reproducibility of the present biosensor.

5.Accuracy
In order to know the accuracy of the present method, the values of the total galactose in 10 serum samples were determined by standard colorimetric method(x) and compared with those obtained by present method(y). The serum galactose value by present biosensor showed a good correlation (r= 0.98)with those by standard enzymatic colorimetric method, with regression equation: y= (0.986x +0.054) (Fig.6) , revealing the high accuracy of the method.

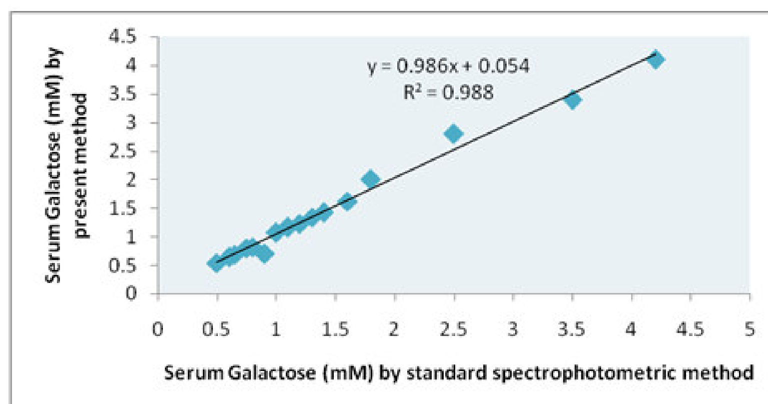


Figure 6
Correlation between serum galactose value as determined by spectrophotometric method (x axis) and present biosensor method (y axis) based on GalOxNPs/Au electrode.

Amperometric determination of total serum galactose.
The total substrate content of serum samples of healthy and diseased person suffering from various hypergalactosemic diseases in different age groups was determined by present biosensor and found in the range of 2.6 to 4.2mM/ml and 7.7 to 9.6 mM/ml respectively (Table 1).

Table 1
Galactose level of serum of diseased individual as measured by GalOxNPs/Au biosensor.

Age group (n=08)	Sex	Serum galactose level (Mean±SD)	
		Healthy	Diseased
<5	M	2.630± 1.171	9.025±1.194
	F	3.637 ± 1.441	9.650±1.252
6-10	M	4.278± 1.108	7.750±0.692
	F	3.975± 1.345	7.875±0.544
11-20	M	3.550± 1.073	7.852±0.448
	F	4.401±1.462	8.512±0.958
21-30	M	4.210±1.341	8.237±0.781
	F	4.225±1.229	8.712±1.343

Serum sample were collected from PGIMS hospital of apparently healthy and diseased individual suffering from various diseases related to galactose metabolism.

Storage stability and reusability
The GalOxNPs/Au electrode lost 40% of its activity after its regular use over a period of 90 days when stored in 0.1M phosphate buffer, pH 6.0 at 4° C (Fig.7).This storage stability is much better than earlier reported biosensor biosensors.

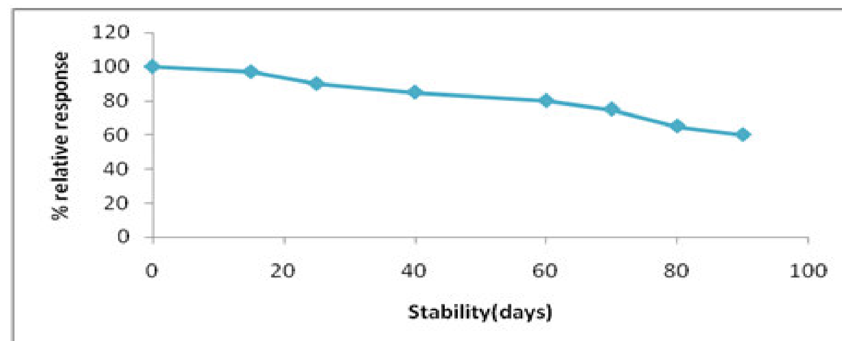


Figure 7
Storage stability of GalOxNPs/Au electrode when stored at 4° C.

Table 2
Comparison of various electrochemical Galactose biosensors

Support	Linear Range	Detection limit	References
Nafion thin film	0.10-8mM	-	[21]
Poly(3-hexyl thiophene)	0.05-0.5g/l	-	[25]
Chitosan matrix	Ns-1mM	25µM	[23]
Eggshell membrane	0.1-8.5mM	-	[22]
Poly(N-glycidylpyrrole-co-pyrrole)	2-16mM	25µM	[20]
Polypyrrole-hydrogel	0.05-10mM	25 µM	[27]
Cellulose acetate membrane	0.1-25mM	0.1mM	[2]
Epidermis of solanum lycopericum	0.44-7mM	0.44mM	[26]
Conducting polymer	0.1-1mM	0.01mM	[25]

CONCLUSION

GalOxNPs/Au electrode in the construction of an amperometric galactose biosensor has led to its improved analytical properties in terms of its short response time (8s), high storage stability (90 days), low detection limit (0.16mM) and wider working range 0.1-20 mM. Morever, the biosensor is mechanically robust, reliable and has been successfully used for detection of galactose in blood serum.

CONFLICT OF INTEREST

The author declare no conflict of interest.

ACKNOWLEDGEMENT

Author thanks to advanced instrumentation research facility (AIRF),J.N.U., New Delhi for scanning elelctrom microscopy and P.U. ,Chandigarh for transmission electron microscopy facility.Mamta Sharma thanks to M.D.University for providing university research fellowship.

REFERENCES

1. Beutler E. Galactosemia:Screening and diagnosis. Clin. Biochem. 1991;24:293-300.
2. Kanyong P, Pemberton RM, Jakson K. Development of an amperometric screen –printed galactose biosensor for serum analysis. Anal Biochem. 2013; 435:114-119.
3. Prasad R, Singh R, Thapa BR. Biochemistry, molecular biology and molecular genetics of galactosemia. Transworld Research network. 2013:1-23.
4. Ohura T, Kobayashi K, Abukawa D. A novel inborn error of metabolism detected by elevated methionine and/or galactose in newborn screening: neonatal intrahepatic cholestasis caused by citrin deficiency. Eur. J. Pediatr.2003; 162:317–322.
5. Liu G, Hale GE, Hughes LC. Galactose metabolism and ovarian toxicity. Reprod. Toxicol. 2000;14: 377–384.
6. Lai K, Tang M, Yin Z, Klapper H, Wierenga K, Elsas LJ. ARRHI: a new target of galactose toxicity in classic galactosaemia. Biosci. Hypotheses. 2008; 1: 263– 271.
7. Yuh YS, Chen JL, Chiang CH. Determination of blood sugars by high pressure liquid chromatography with fluorescent detection. J Pharm Biomed Anal.1998.
8. Chen J, Yager C, Reynolds R, Palmieri M, Segal S. Erythrocyte galactose 1-phosphate quantified by isotope-dilution gas chromatography-mass spectrometry. Clin Chem. 2002; 48: 604-12.
9. Hu OY, Hu TM, Tang HS. Determination of galactose in human blood by high-performance liquid chromatography: comparison with an enzymatic method and application to the pharmacokinetic study of galactose in patients with liver dysfunction. J Pharm Sci.1995; 84:231-5.
10. Kim MI, Shim J. Colorimetric quantification of galactose using a nanostructured multi-catalyst system entrapping galactose oxidase and magnetic nanoparticles as peroxidase mimetics. Analyst .2012; 137:1137-43.
11. Frings. An Automatic Spectrophotometric Method for the Specific Enzymatic Determination of Galactose in Whole Blood and Plasma. Anal. Chem. 1964; 36: 2477–2478.
12. Li Y, Ptolemy AS. Quantification of galactose-1-phosphate uridyltransferase enzyme activity by liquid chromatography-tandem mass spectrometry. Clin Chem. 2010; 56:772–780.
13. Boller T, Meier C, Menzler S. EUPERGIT oxirane acrylic beads: how to make enzymes fit for biocatalysis. Org Process Res Dev. 2002; 6: 509–519.
14. Liu G, Lin Y, Ostatna V, Wang J. Enzyme nanoparticles based electronic biosensor. Chem Commun . 2005; 27:3481–348.
15. Zhang S, Wang N, Yu H, Niu Y & Sun C. Covalent attachment of glucose oxidase to an Au electrode modified with gold nanoparticles for use as glucose biosensor. Bioelectrochem. 2005; 67:15–22.
16. Huang L, Peng Z, Guo Y & Porter L . Identifying the emerging roles of nanoparticles in biosensors. Journal of Business Chemistry. 2010; 7: 15–30.
17. Zoua Y, Xiang C, Suna LX & Xu F.Glucose biosensor based on electrodeposition of platinum nanoparticles onto carbon nanotubes and immobilizing enzyme with chitosan-SiO2 sol–gel. Biosensors & Bioelectronics, 2008; 23:1010–1016.
18. Sheldon RA. Cross-linked enzyme aggregates as industrial biocatalysts. Org Process Res Dev. 2011;15: 213–223.
19. Arias EB, Cancho JC, Gonzalez, DL, Serrano, JM. Use of an enzyme assay in student classes. Biochem. Educ. 1992; 20:234–235.
20. Senel M, Bozgeyik I. A novel amperometric galactose biosensor based on galactose oxidase-poly(N-glycidylpyrrole-co-pyrrole). synthetic metal. 2011; 161:440-444.
21. Neng-Qin Jia, Zong-Rang Zhang , Jiang-Zhong Zhu , Guo-Xiong Zhang . A Galactose Biosensor Based on the Micro fabricated Thin Film Electrode.Analytical Letters.2003, 36.
22. Wen G, Zhang Y, Shuang S. Biosensor for determination of galactose with galactose oxidase immobilized on eggshell membrane. Analytical letter. 2005; 38:1519-1529.
23. Tkac J, Gemeiner P, Sturdik E. Rapid and sensitive galactose oxidase-peroxidase biosensor for galactose detection with prolonged stability. Biotechnology letters.1999; 13: 931-936.
24. Sharma SK, Suman, Pundir CS. Galactose sensor based on galactose oxidase immobilized in polyvinyl formal.Sensor and actuator B. 2005;119:15-19.
25. Lee KN, Lee Y, Son Y. Enhanced sensitivity of a galactose biosensor fabricated with a bundle of conducting polymer microtubules. Electroanalysis.2011; 23: 2125–2130.
26. Choi MM, Chan AYW, Wong ESC, Lee HHC. Development of a galactose biosensor with galactose oxidase-immobilized epidermis of Solanum lycopericum: potential point-of-care testing for citrin deficiency in highprevalence areas. Clin. Chim. Acta. 2011; 412: 391–392.
27. Brahim SI, Maharajh D, Narinesingh D, Guiseppi, Elie A. Design and characterisation of a galactose biosensor using a novel polypyrrole–hydrogel composite membrane.Anal. letter.2002; 35:797–812.