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Novel electrochemical sensing of galactose using GalOxNPs/CHIT modified pencil graphite electrode

Mamta Sharma, Pooja Yadav, Minakshi Sharma*

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

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ABSTRACT

For the construction of galactose biosensor, chitosan was electropolymerised onto the pencil graphite electrode. This chitosan modified pencil graphite electrode acts as good matrix for immobilization of enzyme nanoparticles of galactose oxidase. Development of this nanocomposite was further confirmed by Fourier transform infrared spectroscopy and scanning electron microscopy. The presence of chitosan makes the present galactose biosensor more efficient, reproducible and stable. The sensitivity was reported 7×10^{-3} mA/mM/cm^2 with linear range from 0.05 to 25 mM and better detection limit of 0.05 mM. When the solution of galactose was spiked with 0.5 mM and 1 mM, the analytical recoveries were found 98.6% and 97.6%. A better storage stability was achieved (90days) when compared to earlier reported biosensors.

1. Introduction

The measurement of galactose level is important for the clinical significance of galactosemia and galactose intolerance [1]. Galactose is present at a maximum concentration of 0.28 mM in normal subjects and 1.11 mM in neonates less than 5days old [2]. In neonatal infants, value higher than 1.11 mM consider as fatal galactosemia [3]. The galactosemia is an inherited disorder analyzed by inability of human body to utilize galactose due to insignificant amount of enzymes responsible for galactose metabolism such as GALT [4]. Untreated patients in the newborn period manifest poor feeding, failure to thrive, jaundice, liver disease, cataracts, *E. coli* sepsis, and neonatal deat [5]. Even after treatment some may develop central nervous system problems in both sexes [6] and primary ovarian insufficiency (POI) in women [7,8]. Treatment of galactosemia involves restriction of dietary galactose that includes milk and milk products [9]. Previous methods for galactose determination include colorimetric [10], mass spectrometric [11], thin layer chromatography [12] and high-performance liquid chromatography [13,14]. However, these methods were costly, tedious, time consuming and not sufficiently specific with high interference, therefore to overcome these problems biosensors are attained enormous attention in last few years [15]. Various bioanalytical problems such as sensitivity, specificity, reproducibility and reliability have been resolved by applying nanostructure based electrochemical biosensors [16]. A biosensor is an analytical devise that combines a biological sensing element with a transducer to produce a signal proportional to the analyte concentration [17]. Enzyme-based electrochemical biosensors have potential applications in our life including health care, food safety and environmental monitoring [18]. Chitosan, being a natural high biopolymer and a product of deacetylation of chitin, used to improve the sensitivity of enzymatic biosensor due to its excellent biocompatibility, biodegradability, non-toxicity, high mechanical strength, good adhesion and cheapness properties [19]. Various galactose biosensors were developed to measure galactose concentration in food, blood and urine samples for clinical as well as nutritional purposes. Therefore, the present work described the novel improved amperometric determination of galactose in different baby feed products by preparing nanoparticles of galactose oxidase and their direct immobilization onto chitosan modified pencil graphite electrode. Galactose oxidase catalyses the reaction between D-galactose and oxygen as follows:

$$\text{D-galactose} + O_2 \xrightarrow{\text{galactoseoxidase}} \text{galactohexodialdose} + H_2O_2$$

$$H_2O_2 \rightarrow O_2 + 2e^- + 2H^+$$

2. Materials and method

2.1. Materials

Galactose oxidase (GalOx) from Dactylium dendroides (E.C.1.1.3.9), horseradish peroxidase (HRP), lactase enzyme were purchased from

* Corresponding author.
E-mail address: sminakshi.2007@rediffmail.com (M. Sharma).

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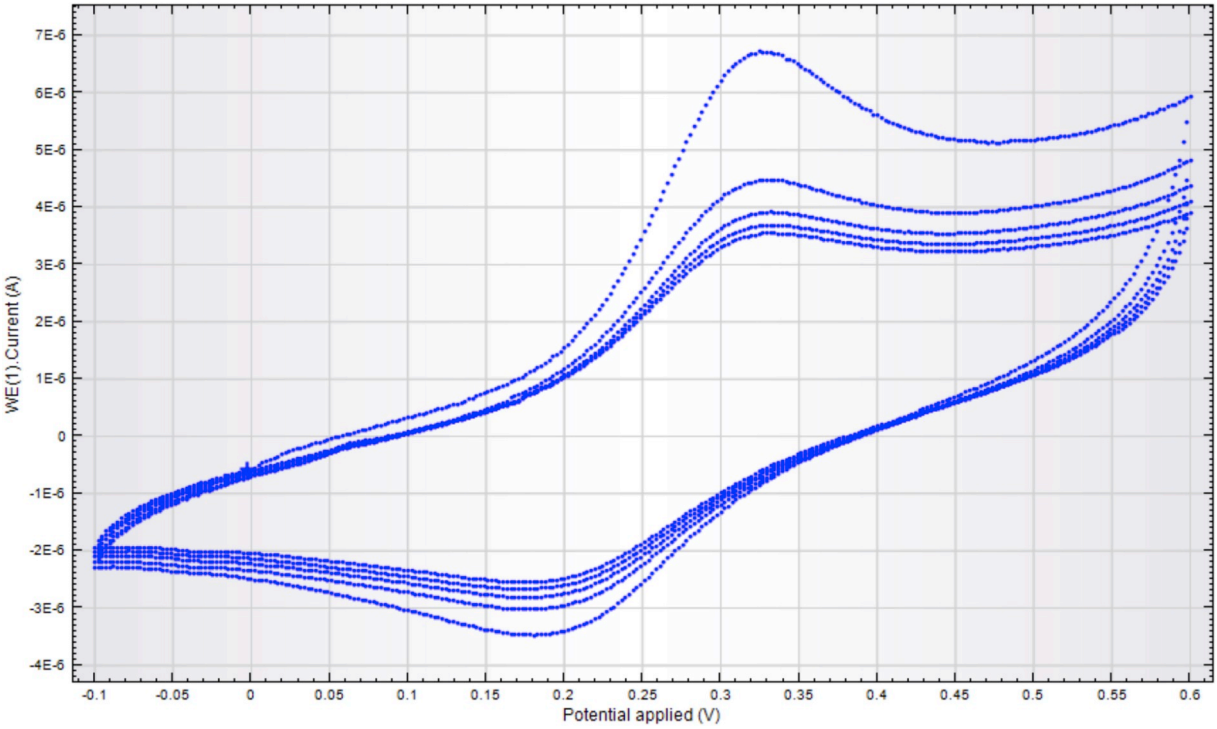

Fig. 1. Cyclic voltagram of chitosan deposition by applying 20 deposition cycles at −0.1V to 0.6V with scan rate of 50 mV/s.

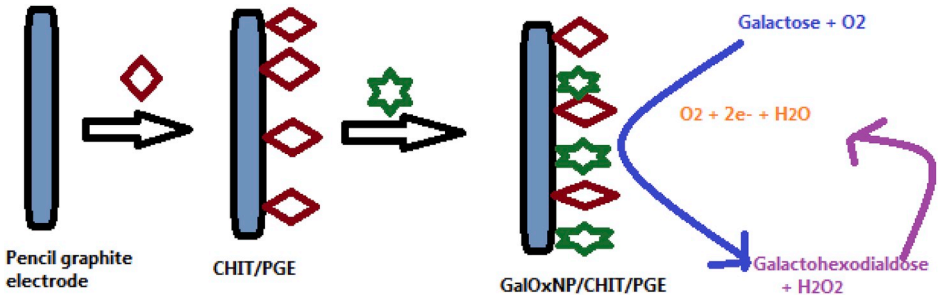

Fig. 2. Scheme of construction of GalOxNPs/CHIT/PGE based bionanosensor.

Sigma Aldrich St.Louis, USA, 4-aminophenazone, phenol, D-galactose, silica gel powder, absolute ethanol, sulphuric acid (H_2SO_4), hydrogen peroxide (H_2O_2), glutaraldehyde (25%), cysteamine dihydrochloride, potassium chloride (KCl), chitosan (CHIT) were purchased from Hi Media Pvt. Ltd, France. Baby food products of different brands were purchased from local market. Double distilled water was used in all experiments. All other chemicals were of analytical grade and used as such without any purifications.

2.2. Preparation and characterization of galactose oxidase nanoparticles (GalOxNP)

The GalOx nanoparticles (NPs) were prepared by desolvation method with some modifications [20]. 6 ml of absolute ethanol was added to 3 ml of enzyme solution (1 mg/ml) at a dropping rate of 0.1–0.2 ml/min under constant stirring of 500 rpm at 4 °C. Further, 1.8 ml of glutaraldehyde (25%) was added for the cross linking of the enzyme nanoparticles with continuous stirring of 500 rpm at 4 °C. Later, 0.12g of cysteamine dihydrochloride was added to the suspension to introduce the thiol (-SH) groups and left the suspension for 5–6 h under continuous stirring. The suspension was then cold centrifuged at 12000 rpm for 10mins to separate out the GalOxNPs from the remaining suspension. Pellet was dispersed in 1 ml of 0.1 M potassium phosphate buffer (pH 6.0). These prepared enzyme nanoparticles were further characterized by Fourier transform infrared spectroscopy (FTIR Thermo scientific, USA) and Transmission electron microscopy (TEM JEOL 2100F) to study the morphology and size.

2.3. Preparation of chitosan

1% chitosan solution was prepared [20]. 1% of acetic acid was prepared by dissolving 1 ml of acetic acid solution in 99 ml of distilled water. Further, 1g of chitosan was added to the prepared acetic acid solution and stirred using magnetic stirrer at room temperature for 24hr to get the soluble and fully dissolved chitosan solution (1%).

3. Fabrication of GalOxNP/CHIT modified pencil graphite electrode(PGE)

3.1. Cleansing of electrode

Before any surface modification, pencil graphite electrodes were polished with silica gel powder to make the surface smooth [21]. Electrodes were then dip in piranha solution (H_2SO_4:H_2O_2; 3:1 ratio) for 15mins followed by its washing with ethanol and distilled water. Electrodes were further sonicated using utrasonicator for 30 min to remove any adsorbed particles and then washed with distilled water three-four times. Electrodes were stored in 4 °C when not in use.

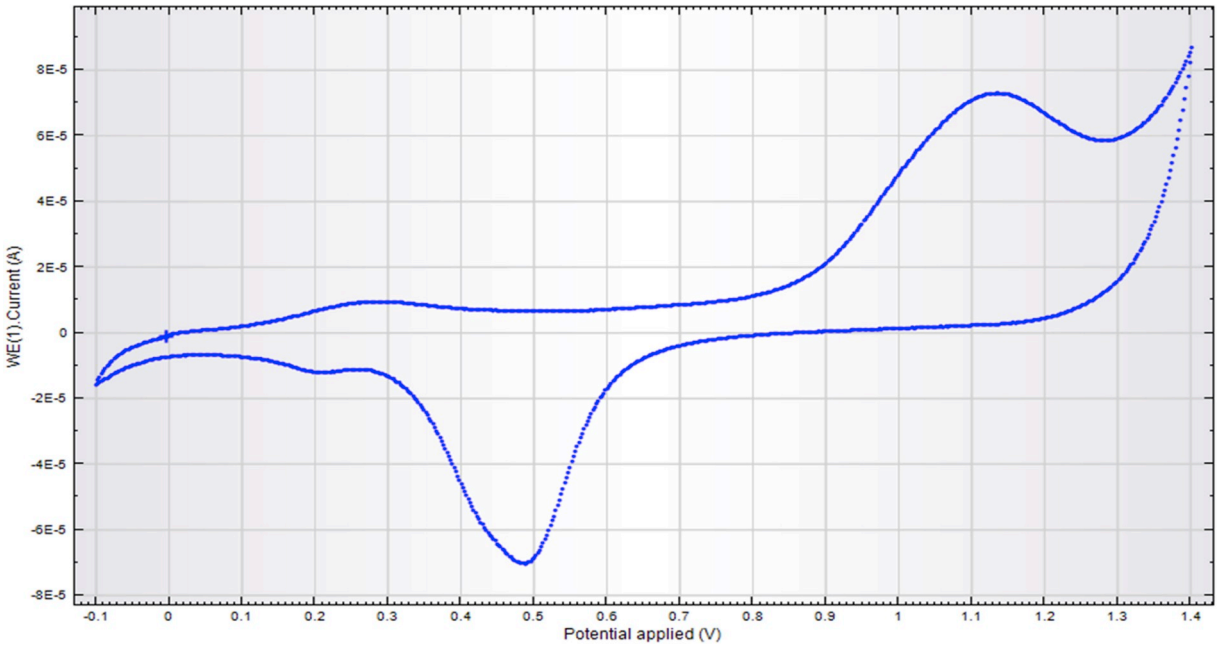

Fig. 3. Cyclic voltagram of GalOxNPs/CHIT/PGE in 0.1 ml of galactose (1 mM) in 0.1 M potassium phosphate buffer (pH 6.0) with scan rate of 50 mV/s.

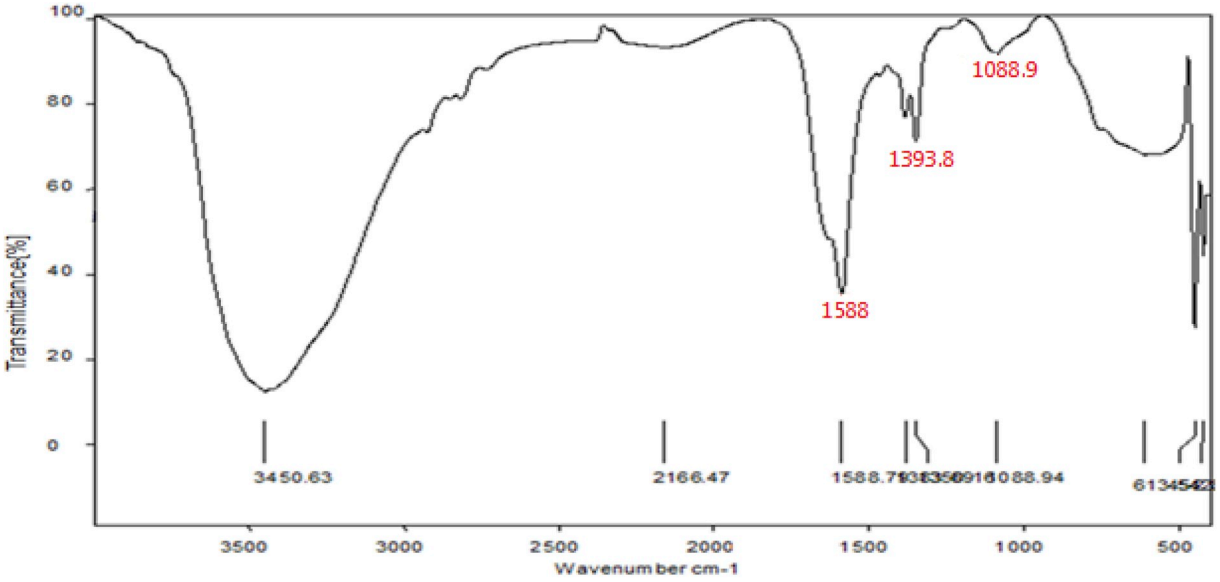

Fig. 4. FTIR image of prepared GalOxNPs.

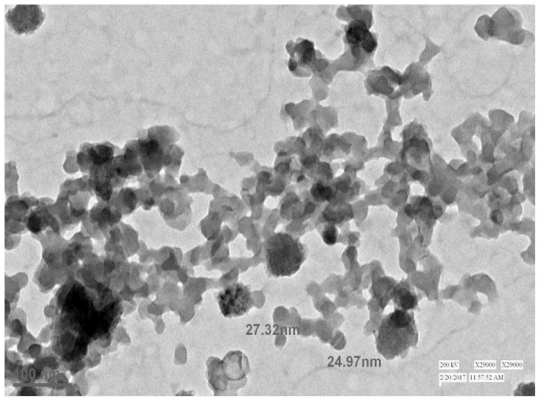

Fig. 5. TEM image of prepared GalOxNPs.

3.2. Electrodeposition of chitosan onto pencil graphite electrode (PGE)

The prepared chitosan solution (1%) was electropolymerised onto the Pencil graphite electrode using potentiostat [22]. Reaction mixture involve 25 ml of 1 M KCL having 1 ml of chitosan solution (1%). Chitosan was electropolymerised onto the pencil gephite electrode using potentiostat by applying 20 deposition cycles at −0.1V to 0.6V with scan rate of 50 mV/s (Fig. 1).

3.3. Immobilization of GalOxNPs onto chitosan modified pencil graphite electrode (CHIT/PGE)

For immobilization of GalOxNPs on to the chitosan decorated pencil graphite electrode, CHIT/PG electrode was immersed in 1 ml suspension of potassium phosphate buffer having 500 µl of GalOxNPs. Further this working electrode was kept overnight at 4 °C and left undisturbed.

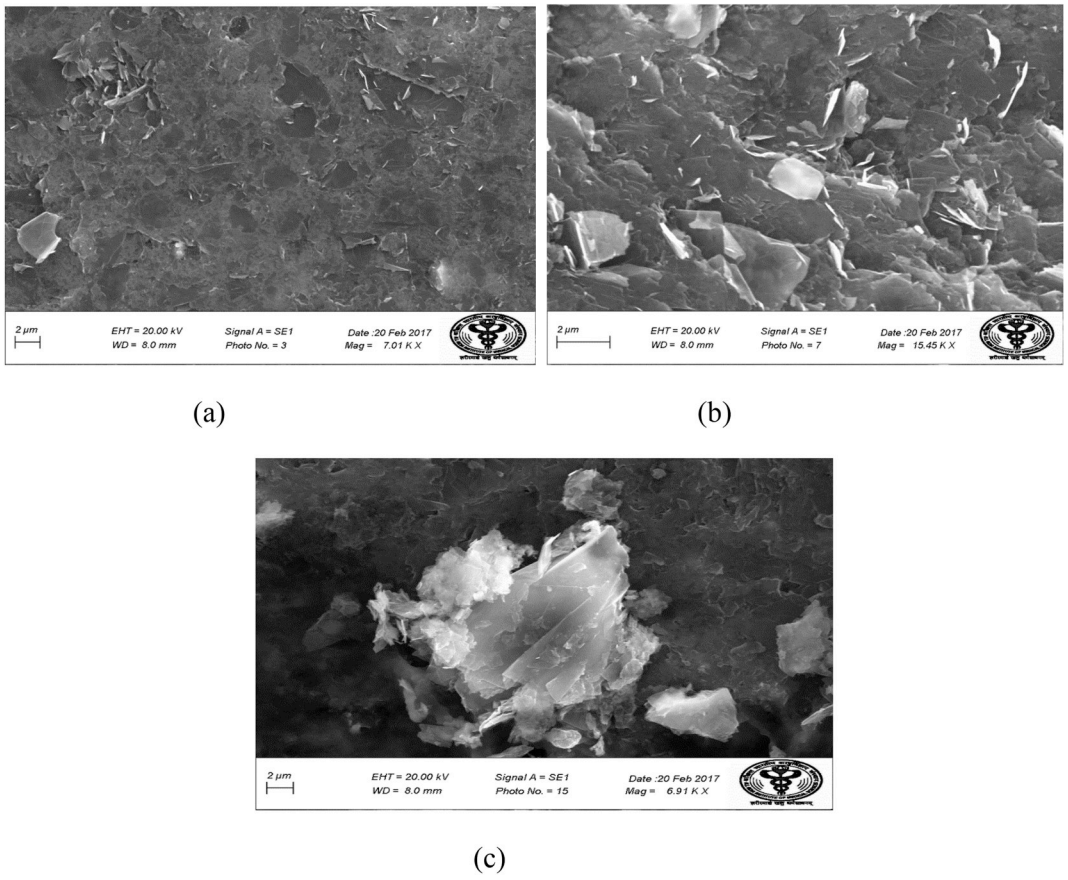

Fig. 6. (a) SEM image of bare pencil graphite electrode. (b) SEM image after electrodeposition of chitosan onto pencil graphite electrode (CHIT/PGE) (c) SEM image showing immobilization of GalOxNPs onto chitosan modified pencil graphite electrode (GalOxNPs/CHIT/PGE).

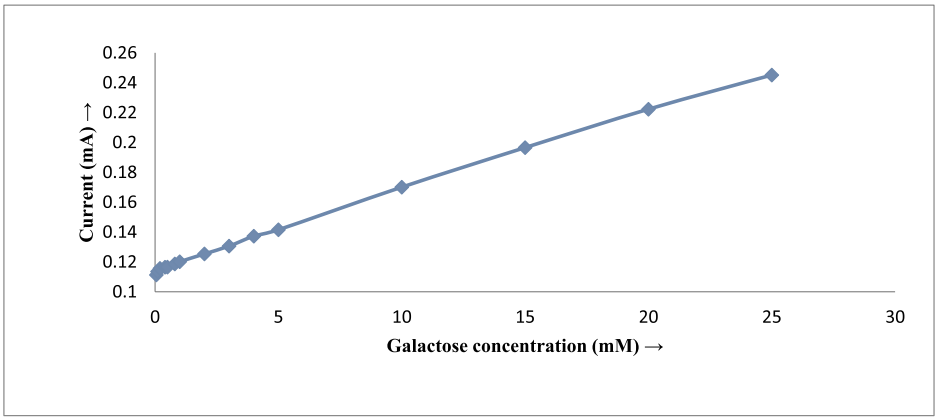

Fig. 7. Standard curve for galactose by GalOxNPs/CHIT/PGe electrode based biosensor.

Table 1
Recovery of galactose added to the galactose samples by current method.

Galactose added (mM)	Galactose found (mM)	%Recovery
–	0.25 mM	–
0.5 mM	0.74 mM	98.6
1 mM	1.22 mM	97.6

Recovery (%) = $C_{found}/(C_{original} + C_{added})$.

The resulted GalOxNPs/CHIT/PG working electrode was washed 3-4 times with potassium phosphate buffer (PB, pH 6.0) and kept at 4 °C when not in use. The whole scheme of construction of working electron is shown in Fig. 2. The GalOxNPs/CHIT/PG electrode was further characterized by SEM technique to confirm the deposition of CHIT and immobilization of GalOxNPs over the PG electrode.

3.4. Construction and response measurement of GalOxNPs/CHIT/PGE based biosensor

All measurements were carried out through potentiostat by using Ag/AgCl as reference electrode, platinum as countercurrent electrode and GalOxNPs/CHIT/PGE as working electrode. Results were obtained by putting three electrode system in reaction mixture of 25 ml of 0.1 M potassium phosphate buffer(pH 6.0) having 0.1 ml of D-galactose as substrate (1 mM). Current was measured by applying potential at −0.1–1.4V with scan rate 50 mV/s in. The peak of maximum current

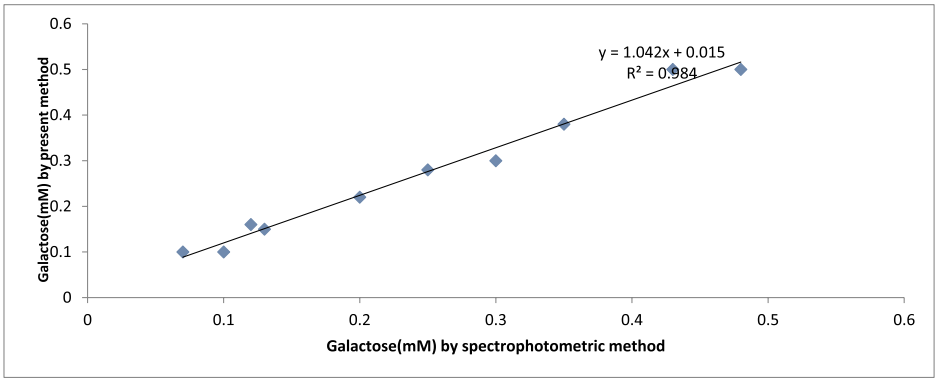

Fig. 8. Correlation between serum galactose value as obtained by chemical spectrophotometric method (x) and present galactose biosensor (GalOxNPs/CHIT/PGE) (y).

Table 2
Galactose concentration (mM/ml) in various baby food products.

s.no.	Sample	Concentration of galactose (mm/ml)
1.	Nestle Ceralac (8months+) wheat honey	0.3
2.	Nestle Ceralac (10months+) wheat-rice mixed fruit	0.48
3.	Nestle Ceralac (12months+) multigrain and fruits	0.43
4.	Amul infant formula	0.12
5.	Nestle Lactogen (upto 6months)	0.13
6.	Nutrica Dexolac Premium (upto 6months)	0.2
7.	Laktrum lactoferrin NFQ and colostrums powder	0.07

Storage stability of GalOxNPs/CHIT/PGE biosensor.

(mA) was observed at 1.1V as shown in Fig. 3. All subsequent amperometric studies were performed at this particular voltage.

3.5. Optimization study of GalOxNPs/CHIT/PGE biosensor

To determine the optimum pH for the present biosensor, the pH of reaction mixture was varied from pH 5.5 to 8.5 with regular interval of pH 0.5. Similarly, to determine the optimum temperature and time, the reaction mixture was incubated from 15 °C to 45 °C at an interval of 5 °C. Likewise to study time of response, the current was measured from 2 to 16s with interval of 2sec. The effect of galactose concentration on present biosensor response was determined by testing different concentrations of galactose from 0.05 mM to 40 mM.

3.6. Application and evaluation of GalOxNPs/CHIT/PGE biosensor

The present biosensor was tested for galactose detection in different baby feed products obtained from local markets. Further the present biosensor was evaluated by studying its detection limit, linearity, analytical recovery, precision and correlation. Analytical recovery was studied using two different concentration of galactose (0.5 mM and 1 mM) and percent recovery was measured. Precision study was also done to check the reliability and reproducibility of present biosensor by measuring galactose concentration in different baby food products, five times on a 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 baby food products by present method (GalOxNPs/CHIT/PGE biosensor) and standard enzymatic colorimetric method.

3.7. Storage stability of GalOxNPs/CHIT/PGE

The long term storage stability of GalOxNPs/CHIT/PGE was studied for 90 days by performing assay on weekly basis. The GalOxNPs/CHIT/PGE was stored in 0.1 M PB (pH 6.5) at 4 °C when not in use.

4. Results and discussion

4.1. Characterization of GalOxNPs

Characterization of galactose oxidase nanoparticles were performed by Fourier Transform Infrared Spectroscopy (FTIR) and Tansmission Electron Microscopy (TEM).

4.2. FTIR

The FTIR of GalOxNPs was performed at Pharmaceutical Science Department, M.D. University, Rohtak. Two different vibration bands around 1090 and 1405 cm^{-1} predict the presence of amide I (C=O) and amide II (N-H and C-N) bands of peptide bonds in case of free galactose oxidase. An additional vibration band was observed at 1588 cm^{-1} in case of aggregates of galactose oxidase enzyme indicate the C=N streaching due to glutaraldehyde reaction. A broad band around 3500 cm^{-1} indicate the presence of more free amine groups formed due to cysteamine dihydrochloride (Fig. 4).

4.3. TEM

TEM was done at SAIF AIIMS, New Delhi. The TEM images revealed the hollow spherical shape of enzyme nanoparticles in 100 nm range as shown in Fig. 5.

4.4. Characterization study of GalOxNPs/CHIT/PGE

Scanning Electron Microscopy studies were performed at SAIF, AIIMS, New Delhi. The SEM image of bare PGE shows a smooth homogenous surface whereas net like crystal structure was observed after electrodeposition of chitosan over PG electrode. A globular structural morphology with cluster of aggregation of enzyme nanoparticles over the CHIT/PG electrode confirms its immobilization (Fig. 6a–c).

Table 3
Comparison of Current galactose biosensor with earlier reported biosensors.

Support	Correlation	Linear Range	Detection limit	Storage life	Response time	Precision	Analytical recovery	References
Poly(amphiphilic) pyrrole based platinum electrode	0.999	0.001mM-1.8 mM	5×10^{-4}mM	40 days	5s	-	-	[24]
Polypyrrole-hydrogel composite membrane	> 0.997	0.05–10 mM	25 μM	9months	70s	3.8 &4.4%	97%	[25]
Polypyrrole/polyanion/PEG/enzyme	0.98	0–24 mM	-	-	40sec	-	-	[26]
Polyacrylonitrile thin film-modified platin electrode	-	0.02–1.6 mM	-	30 days	-	-	-	[27]
Eggshell membrane	0.998	0.1–8.5 mM	-	3 month	100sec	-	94 &110%	[28]
Single walled carbon nanotubes/GCE	0.999	0–10 mM	0.025 mM	2.5hr	150injection/hr	1.15%	101.2 & 102.7%	[29]
Microfabricated thin film electrode	0.999	0.1–0.8 mM	-	> 1month	-	-	97.5%	[30]
Poly(glycidylmethacrylate-co-vinylferrocene)/platin electrode	-	2–20 M	0.1 mM	30 days	5sec	-	-	[31]
Conducting polymer microtubules modified ITO electrode	0.995	0.1–10 mM	0.01 mM	7 days	30sec	-	-	[3]
Poly(N-glycidylpyrrole-co-pyrrole)	0.990	2–16 mM	0.025 mM	10days	5sec	-	96.9 & 98.8%	[32]
Laponite clay film/platin electrode	-	0.001–1.6 mM	0.001 mM	30 days	5sec	-	-	[33]
Cobalt phthalocyanine modified SPCE	-	0.1–25 mM	0.02 mM	14 days	30sec	1.1–0.11%	99.9%	[34]
Co3O4 nanoparticles & MWCNT modified GCE	0.996	0.009–0.06 mM	90 μM	1month	20s	-	98%	[35]
AuNP modified graphene nanocomposite film	0.998	1.5μ-6mM	0.79 μM	10days	-	-	98–104%	[36]
Cellulose acetate modified screen printed carbon electrode	-	1.98–9.52 mM	0.2 mM	24hr	-	1.3%	-	[37]
GalOxNPx modified Au electrode	0.988	0.1–20 mM	0.16 mM	3month	8s	4.3% 4.8%	96.8 & 99%	[38]
GalOx/AgNP/cMWCNT/PANI/Au	0.993	0.1–18 mM	0.15 mM	6 months	2s	4.39 & 5.19%	96.2 & 98.4%	[39]
GalOxNPs/CHIT/PGE	**0.98**	**0.05mM-25mM**	**0.05 mM**	**90 days**	**2s**	**4.6 & 5.1%**	**98 & 97.6%**	**[Present work]**

4.5. Optimization study of GalOxNPs/CHIT/PGE biosensor

The optimum pH of current biosensor was obtained at pH 6.5. The optimum temperature for the present biosensor was reported 35 °C. The maximum time of response was obtained at 2sec, after which a regular decrease in the current was noted. For substrate concentration, the maximum current response of biosensor was depicted at 25 mM after which almost constant current value was noted.

4.6. Evaluation study of GalOxNPs/CHIT/PGE biosensor

4.6.1. Linearity and detection limit

There was a linear relationship found between the current (mA) and galactose concentration (mM) ranging from 0.05 mM to 25 mM (Fig. 7) with detection limit of 0.05 mM and sensitivity of 7×10^{-3} mA/mM/cm^2, which was found better than those of earlier galactose biosensors (Table 3).

4.6.2. Analytical recovery

When the solution of galactose sample was spiked with 0.5 mM and 1 mM, the analytical recoveries were found 98.6% and 97.6% as shown in Table 1.

4.6.3. Precision

The coefficients of variation was determined within sample and between sample in different baby food products after their storage at −20 °C and it was found 4.6% and 5.1% respectively. It indicates good reproducibility and consistency of the present biosensor.

4.6.4. Correlation

The accuracy of the present biosensor was determined by comparing the measurements of galactose level in baby food products by present biosensor method (x-axis) with standard enzyme colorimetric method (y-axis). The values obtained by both methods showed a good correlation with r = 0.98 and with the regression equation, y = 1.042x + 0.015, which revealed high accuracy of present method (Fig. 8).

4.6.5. Applications of GalOxNPs/CHIT/PGE biosensor in baby food products

Applications of constructed GalOxNPs/CHIT/PGE biosensor were performed in various baby food products as described in Table 2. The samples were prepared in soluble form by dissolving 0.5g of each sample in 10 ml of Phosphate buffer (pH 6.5) [23]. Further, lactase enzyme softgels were added to release the free galactose in the sample. The reaction mixture contained 0.5 ml of sample in 10 ml of PB (pH 6.5). The current was measured at standard optimum conditions (Table 2).

The storage stability of GalOxNPs/CHIT/PGE biosensor was studied for 90days. After 90days, 40% decrease in its activity was observed as shown in Fig. 9. The GalOxNPs/CHIT/PGE biosensor was stored in 0.1 M PB (pH 6.5) at 4 °C when not in use.

5. Conclusion

In summary, the present biosensor was evaluated for its analytical performance. The linearity was found between 0.05 and 25 mM with detection limit of 0.05 mM. When the solution was spiked with 0.5 mM and 1 mM concentration of galactose, the analytical recoveries were found 98.6% and 97.6%. The storage stability of present biosensor was studied for 90days, after 90 days 40% activity was lost. The present biosensor had been successfully used for the detection of galactose in various baby food products, out of which, ceralac brand contain highest concentration of galactose and laktrum brand contain low concentration of galactose.

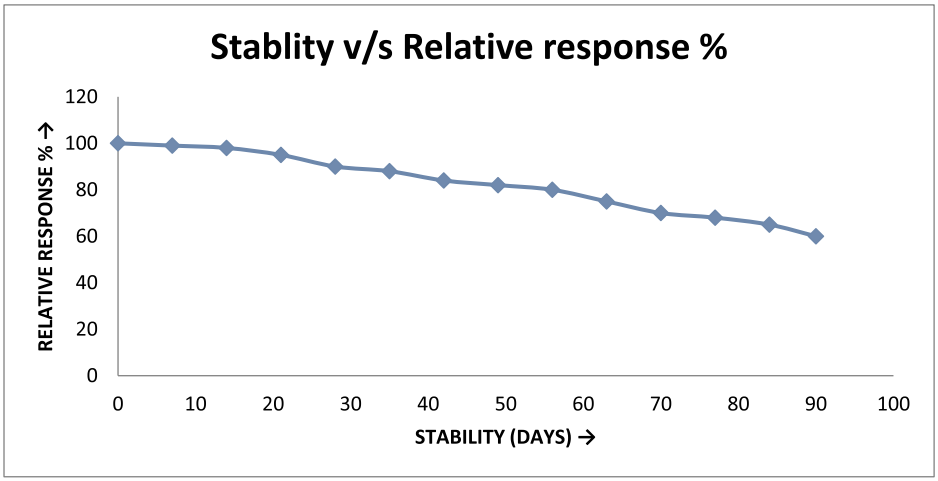

Fig. 9. Storage stability of GalOxNPs/CHIT/PGE biosensor.

Conflicts of interest

Authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carres.2019.107749.

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