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Research Article

An Improved Amperometric Lactose Biosensor Based on Enzyme Nanoparticles

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Abstract

A desolvation method was used for the synthesis of β-galactosidase (β-GAL) and glucose oxidase (GOD) nanoparticles (NPs) using ethanol. The enzyme nanoparticles (ENPs) were characterized by transmission electron microscopy (TEM) and Fourier transform Infra-red spectroscopy (FTIR) and were co-immobilized covalently onto surface of polycrystalline Au electrode. An improved amperometric lactose biosensor was constructed using ENPs (β-GALNPs/GODNPs) modified Au electrode as working electrode, Ag/AgCl as reference electrode and Pt wire as auxiliary electrode connected through potentiostat. Cyclic voltammetry (CV) and scanning electron microscope (SEM) were used for the study of working electrode before and after immobilization of ENPs. The biosensor showed maximum current at 0.25V within 5s, at pH 6.5 and 25ºC. The limit of detection (LOD) was i.e. 1mg/ml and working range was 1-10 mg/mL. The analytical recovery of added lactose (5 and 10 mg/mL) was 94.73±0.5% and 96.4±0.9%, respectively. Coefficient of variation (CV) within and between batch were <3.0 and <4.0 respectively. The biosensor exhibited a good correlation (R^2=0.91) between lactose level in milk as measured by standard (enzymatic colorimetric) method and present biosensor. The biosensor was employed for quantification of lactose in milk from human, cow, buffalo and goat. The working electrode lost 50% of its initial activity within 3 months, after its 120 uses, while being stored dry at 4ºC.

Keywords: Lactose; β-galactosidase nanoparticles; Glucose oxidase nanoparticles; Enzyme nanoparticles, Au electrode; Lactose biosensor; Milk

Introduction

The enzyme molecules in aggregated form are called enzyme nanoparticles (ENPs) within the dimensions of 1-100nm. ENPs provide increased surface area for immobilization, besides exclusive electronic, magnetic, optical, thermal, structural, and catalytic properties. The direct immobilization of enzyme onto metal may cause reduction in its bioactivity and stability. This difficulty was worked out by immobilizing ENPs onto Au electrode covalently (Pundir, 2015). It led to an evolution of simplified biosensors with improved analytic performance such as higher current response, lower detection limit, wider linearity and higher reusability. The ENPs of certain enzymes like horseradish peroxidase (Liu *et al*., 2005), glucose oxidase (GOD) (Sharma *et al*., 2010), uricase (Chauhan *et al*., 2014), cholesterol esterase (ChE) and cholesterol oxidase (ChOx) (Aggarwal *et al*., 2016), uricase, lipase, glycerol kinase (GK) and glycerol 3-

phosphate oxidase (GPO) (Narwal and Pundir, 2017), creatininase (CIN), creatinase (CII) and sarcosine oxidase (SOx) (Kumar *et al*., 2017), L-lactate dehydrogenase (LDH) (Narwal *et al*., 2018), sarcosine oxidase (Sox) (Kumar *et al*., 2017), urease (Jakhar and Pundir, 2018), pyruvate oxidase (PyOx) (Malik *et al*., 2019) have been prepared, immobilized/co-immobilized covalently over the Au electrode surface for the synthesis of improved amperometric biosensors for hydrogen peroxide, glucose, uric acid, total cholesterol, triglyceride, creatinine, lactate, glycerol, sarcosine, urea and pyruvate respectively. Hence ENPs have huge scope to be explored.

The quantification of lactose, a disaccharide, in milk or other biological fluids is very important due to its relationship with nutritional value of milk, diagnosis and medical management of diarrhoea, nausea, abdominal bloating, vomiting and flatulence. The inability of adults and children to digest lactose is known as lactose malabsorption. Lactose is hydrolysed by lactase (beta-galactosidase) into glucose and galactose, which undergo further metabolism. The cells of intestinal villi produce lactase. The production of lactase declines mostly in the children of ages 2-5 years (Paige *et al*., 1975; Unger and Scrimshaw, 1981). The people in whom lactose is not metabolized, it acts as substrate for gas-producing gut flora, which convert it into acetic acid, butyric acid, propionic and other short chain fatty acids, which can lead to diarrhoea, bloating, flatulence, and other gastrointestinal symptoms. A gassy and nauseous feeling is caused by CO_2, methane and hydrogen as by-products (Friedl 1981; Suarez *et al*., 1995). Thus, lactose containing milk should be avoided by lactose intolerant people. The incapability to consume this milk sugar by humans has vast fitness issues especially between the ordinary people of the world, where the only source of nutrition is milk (Shapiro *et al*., 2002). Compared to tedious, time consuming, expansive traditional methods such as spectrophotometry, infrared spectroscopy, titrimetry, and chromatography (Harris, 1986; Folin and Denis, 1918; Amamcharla and Metzger, 2011; Druzian *et al*., 2005; Scrimshaw and Murry, 1988), biosensing methods are more simple, sensitive, specific and rapid for determination of lactose. Four types of lactose biosensors have been reported so far: (i) Amperometric (Sharma *et al*., 2007; Sharma *et al*., 2004; Goktug *et al*., 2005; Ammam and Fransacr 2010; Jasti *et al*., 2014; Portaccio and Lepore 2017) (ii) conductometric (Campos *et al*., 2014; Marrakchi *et al*., 2008) (iii) third generation (Soldatkin *et al*., 2013) and (iv) combined thermometric & amperometric biosensors (Stoica *et al*., 2006; Yakovlena *et al*., 2012). In these-biosensors, native β-galactosidase and galactose oxidase/glucose oxidase or cellobiose dehydrogenase have been immobilized onto the electrode directly, which may cause denaturation of enzyme(s) and thus reduction in its activity. This problem was overcome in the present work by preparing nanoparticles of lactase and glucose oxidase and co-immobilizing them covalently onto polycrystalline gold electrode (Liu *et al*., 2005; Sharma *et al*., 2010; Chauhan *et al*., 2014; Aggarwal *et al*., 2016; Narwal and Pundir, 2017; Kumar *et al*., 2017; Narwal *et al*., 2018; Kumar *et al*., 2018; Jakhar and Pundir, 2018; Malik *et al*., 2019). To the best of our knowledge, this is the first report on developement of a lactose biosensor based on enzyme nanoparticles.

Materials and methods

Chemicals

β-Galactosidase (β-GAL) or lactase from *E. coli* (500 units/mg) and Triton X-100 from Sigma-Aldrich, USA, glucose oxidase (GOD) from *A. niger* (125 units/mg), lactose, horseradish peroxidase and glucose from Sisco Research Lab (SRL), Mumbai were used. The dimension of gold wire was (2 x 20 mm, diameter x height, 24- karat) and purchased from local stationary market. All analytic reagent (AR) grade chemicals and double distilled water (DW) were used during all experimental works.

Instruments Used

Potentiostat/Galvanostat (Autolab, model AUT83785, Eco Chemise BV, The Netherlands) with the GPES 4.9 software consisted of ENPs modified Au as functional electrode, a KCl-saturated Ag/AgCl as standard electrode and a Pt as counter electrode in all experiments, a transmission electron microscope (TEM; model JEOL 2100F, Japan), Fourier transform infrared (FTIR) spectrometer, spectronic-20 (Thermo Scientific, USA) and a scanning electron microscope (SEM; model JSM-6510, JEOL) at AIRF, J.N.U, New Delhi. Scanning electron microscopy (SEM) of electrodes and transmission electron microscopy (TEM) of aggregates of ENPs were performed at advanced instrumentation research facility (AIRF), Jawaharlal Nehru University (J.N.U), New Delhi on commercial basis.

Assay of Free β-galactosidase (β-GAL)

β-galactosidase assay was performed according to ref. (Sharma *et al*., 2007). The reaction mixture was taken in 15 mL test tubes (covered with black paper) and consisted of 1.8 mL sodium phosphate buffer (0.05 M, pH 6.5) (PB), 0.1 mL dissolved β-GAL in PB, 0.1 ml dissolved GOD in PB, and 0.1 mL lactose solution (5 mg/mL) as substrate. The reaction mixture was incubated at 37°C for 5 min and then 1.0 mL of color reagent (consisting 50 mg 4-aminophenazone, 100 mg phenol and 1 mg horseradish peroxidase in 100 mL 0.4M sodium phosphate buffer, pH 7.0) was added and kept at room temperature for 15 min to attain pink color. Absorbance at 540nm was recorded in Spectronic-20D against control and the content of hydrogen peroxide produced in the reaction was calculated from standard curve between hydrogen peroxide versus absorbance at 540 nm.

Assay of Glucose Oxidase (GOD)

GOD assay was based on measurement of H_2O_2 produced in GOD reaction using a color reaction with 4-

aminophenazone, phenol horseradish peroxidase as chromogen system (Sharma *et al*., 2004) and performed in 15 mL test tube wrapped with black paper. The reaction mixture contained 1.8 mL sodium phosphate buffer (0.05 M, pH 6.5), 0.1 mL of GOD (1 mg/mL) and 0.1 mL glucose (5 mg/mL) as substrate. After incubating the reaction mixture at 37ºC for 30 min, 1.0 mL color reagent was added and kept at 37ºC for 15 min to produce color. Absorbance was read at 540nm and the amount of hydrogen peroxide was interpolated from standard curve between hydrogen peroxide concentration and A_{540}.

Preparation of ENPs

The nanoparticles of β-GAL and GOD were prepared individually by desolvation method (Narwal and Pundir, 2017) with modification. First, 2 mL enzyme solution (2 mg/mL) was taken in a beaker and, then pipetting continuously 4 mL of ethanol drop wise at 0.1mL/min rate under continuous stirring at 500 rpm at 20ºC. The appearance of seemingly white colored precipitate in enzyme solution indicated the formation of ENPs. The addition of ethanol in enzyme solution withdrew the water molecules between the enzyme molecules. Then enzyme molecules interacted with each other by forces like van der Waals, hydrophobic and electrostatic interaction leading to aggregation of enzyme molecules to form ENPs (Chawla *et al.*, 2013). . After that 1.8 mL of glutaraldehyde (2.5 %) was added into ENPs and the mixture was stirred for 24 h for the proper cross linking between ENPs and thus ENPs suspension was formed. Purification of resulting ENPs was done by three cycles of differential centrifugation (14000g, 20 min) at 4ºC and the final pellet was re-dispersed in reaction buffer and subjected to ultra-sonication over 5 min, to the original volume in DW. Then 0.12 g of cysteamine was added to suspension of ENPs under constant stirring for 5-6 h, for introduction of thiol (–SH) groups on ENPs. At last, the centrifugation (1200 rpm, 4ºC, 10 min) was done to separate functionalized ENPs from free enzyme solution, followed by dispersion in 0.05 M sodium phosphate buffer (pH 6.5) and stored at 4ºC until used.

Characterization of β-GALNPs+GODNPs

The size and morphology of ENPs (β-GALNPs and GODNPs) was studied by transmission electron microscopy (TEM) at AIRF JNU, New Delhi on commercial basis and their chemical structure by recording FTIR spectra at Genetics Dept., MDU Rohtak.

Construction of ENPs modified Au electrode

The Au wire was cleaned with DW before its surface modification. Before surface modification, Au wire was cleansed with piranha solution ($3H_2SO_4$:H_2O_2) for 20 min and then washed with DW thoroughly. This polycrystalline Au electrode was polished with alumina slurry. Then electrode was dipped into ethanol for 2 min and again rinsed with DW. Then the electrode was dipped into 2mL mixture of thiol functionalized GODNPs and β-GALNPs in 1:1 (2.0 mg: 2.0 mg) at 4ºC under mild stirring for 48 hrs. The thiol functionalized ENPs got bound to polycrystalline Au electrode through Au-thiolate bond (covalent bond) with the release of one water molecule (Chauhan *et al*., 2014).

$$Au_n\text{-}AuO + 2(ENP)_2\text{-}SH \longrightarrow Au_n\,(Au\text{-}S\text{-}ENP)_2 + H_2O$$

About 55% of protein content of each ENPs was co-immobilized. The electrode with co-immobilized GODNPs+β-GALNPs was washed 3-4 times with the same sodium phosphate buffer to remove unbound protein and used as working electrode and stored dry at 4ºC.

Fabrication of Lactose Biosensor and Its Response Measurement

All electrochemical measurements of lactose biosensor were carried out using a conventional three-electrode system having GODNPs+β-GALNPs/Au electrode as the working electrode, a Pt wire as the axillary electrode and an Ag/AgCl (saturated 3 M KCl) electrode as the standard electrode through potentiostat. Cyclic voltammetric (CV) measurements were carried out in a three-electrode cell containing 25 mL sodium phosphate buffer (0.05 M, pH 6.5) and 0.1 mL of lactose (5 mg/mL). Current measurements were performed applying CV in the potential range, -0.75 and +0.75 V. To record cyclic voltammograms, the following instrumental parameters were used: step potential +0.75 mV and scan rate 20 mVs^{-1}. All electrochemical measurements were carried out at room temperature. The oxidation peak was observed at 0.260V (vs.Ag/AgCl) for present electrode, corresponding to the oxidation of H_2O_2 arisen due to the following enzymic electrochemical reaction (Fig 1).

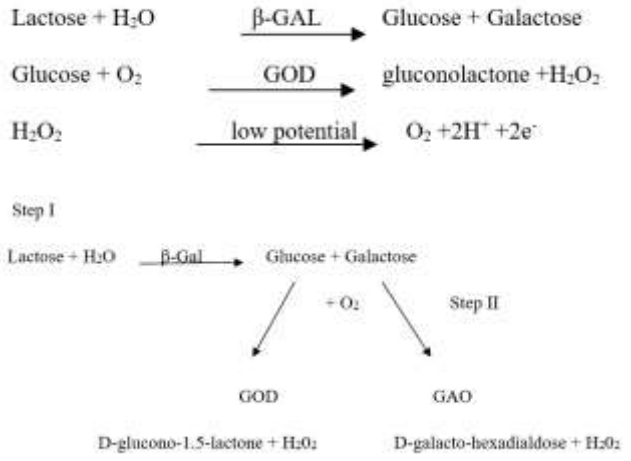


Fig. 1: Schematic representation of lactose biosensor and chemical reactions involved in Biosensor response measurement.

The increase in the value of the oxidation current with increase in lactose concentration resulted, due to the increased concentration of H_2O_2 during enzymatic reaction. This well-defined oxidation peak at 0.260V and reduction peak at -0.031V clearly indicated the catalytic properties of modified electrode (Fig. 2).

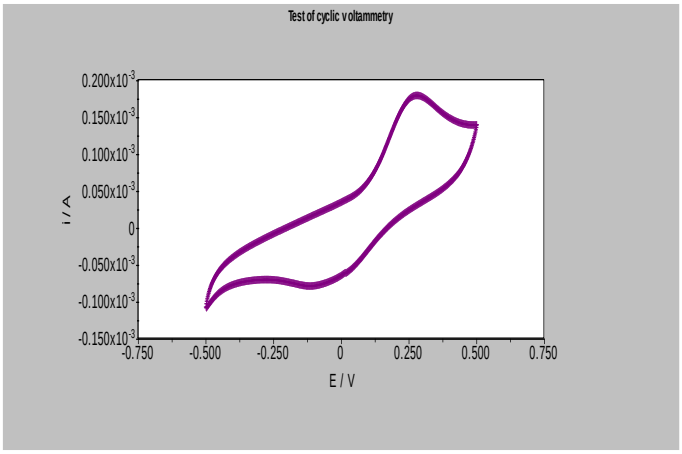


Fig. 2: Cyclic voltametric responses of a β-GALNPs/GODNPs/AuE 25 ml of 0.05 M sodium phosphate buffer (pH 6.5) containing lactose at scan rate of 20 m Vs^{-1} (β-GALNPs=β-galactosidase nanoparticles; GODNPs = Glucose oxidase nanoparticles; AuE= gold electrode)

Optimization of Lactose Biosensor

Lactose biosensor was optimized by studying various kinetic parameters. Firstly, to investigate optimum pH, the pH of reaction buffer (0.05 M), pH was varied from 6.0-7.5 at an interval of 0.5 using sodium phosphate buffer. Secondly, to determine optimal incubation temperature, the reaction mixture was incubated at various temperatures arising from 20-50°C. Thirdly, the effect of lactose concentration on biosensor response was studied by taking lower (1-10 mg/mL) and higher (10-100 mg/mL) concentration range of lactose.

Determination of Lactose in Milk Samples

All the milk samples (1 mL) from cow, hybrid cow, goat and human were taken in micro-centrifuge tubes and centrifuged at 2000 g for 5 min at room temperature. Pellets (insoluble proteins) and supernatant (milk sugars and fats) from sample tubes were collected. The clarified part of supernatant of milk was diluted suitably and stored at 4ºC until used. The procedure for milk lactose determination was the same as shown for biosensor response measurement under optimal working conditions of biosensor except that the lactose solution was replaced by processed milk.

Results and Discussion

Characterization of β-GALNPs and GODNPs

The average size of β-GALNPs and GODNPs as shown by TEM was 14 nm and 33 nm respectively (Fig.3 and Fig.4). FTIR showed transmittance at 3318cm^{-1} in native β-GAL (Fig.5a) and 3300 cm^{-1} in β-GALNPs (Fig.5b) which shows presence of free NH_2 groups. FTIR signals at 3318 and 3300 cm^{-1} can also be assigned for -OH and -COOH groups. The presence of amide bond in both β- GAL and β-GALNPs was indicated by transmittance at 1636 cm^{-1}. Transmittance at 1636 cm^{-1}in both GOD (Fig.6a) and GODNPs (Fig.6b) shows the presence of amide I bond. Native GOD and GODNPs also showed free NH_2 group at 3314 and 3285 respectively. FTIR spectra of native GOD and GODNPs had peak at 1100 cm^{-1} showing the presence of C-O bond.

Characterization of β-GALNPs /GODNPs/Au Electrode by SEM

Smooth surface shown by bare gold electrode (Fig.7a) and globular surface morphology shown by β-GALNPs/GODNPs/Au modified electrode (Fig.7b) confirmed the immobilization of both ENPs together onto Au electrode.

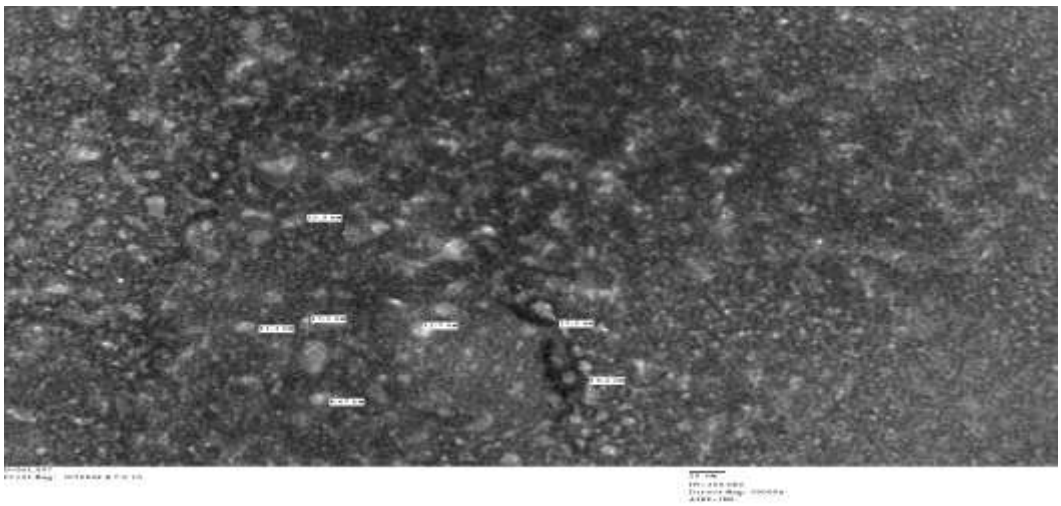


Fig. 3: Transmission electron microscopy (TEM) images of β-GALNPs (β-GALNPs=β-galactosidase nanoparticles)

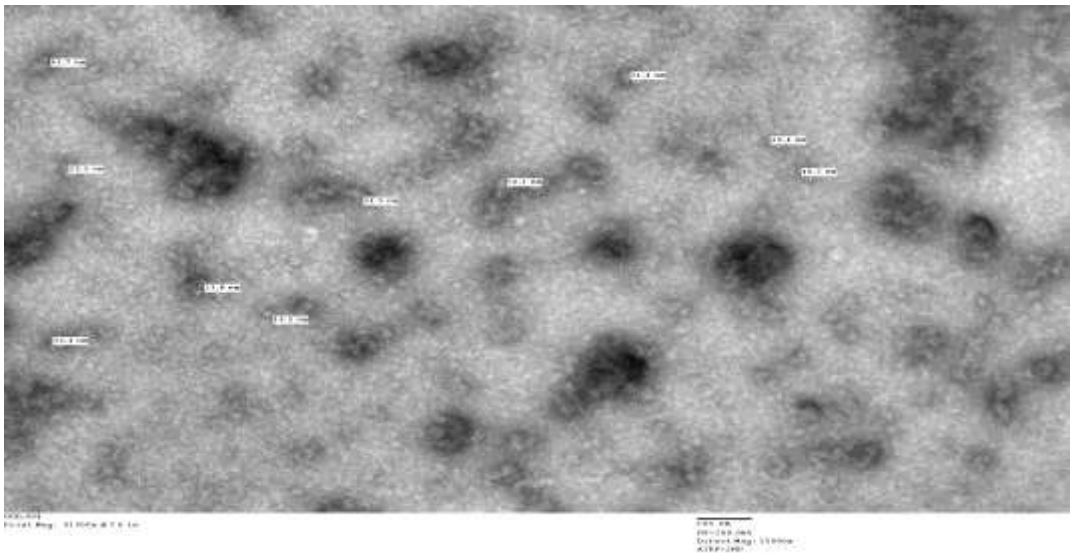


Fig. 4: Transmission electron microscopy (TEM) images of GODNPs (GODNPs = Glucose oxidase nanoparticles)

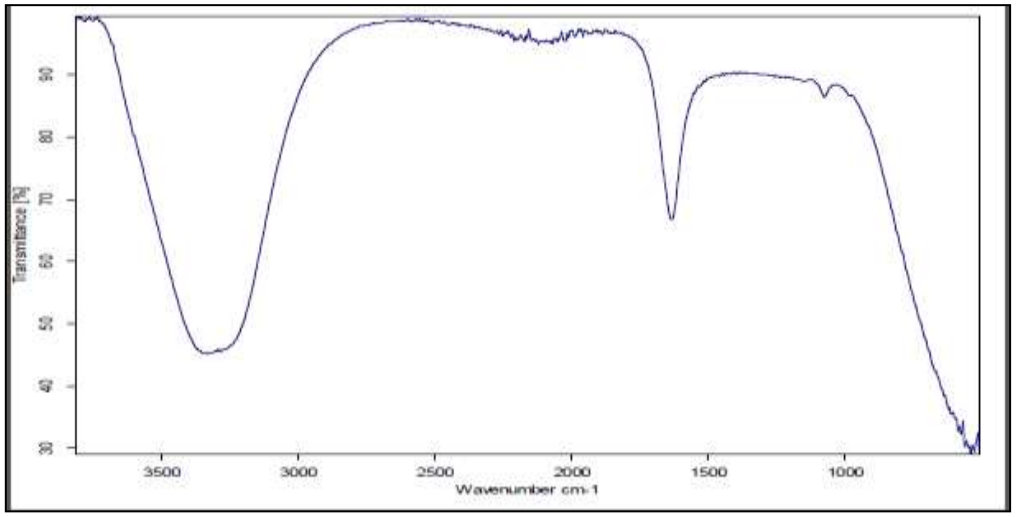


Fig. 5(a): Fourier Transform Infra-Red Spectra (FTIR) images of β-GAL (β-GAL=β-galactosidase)

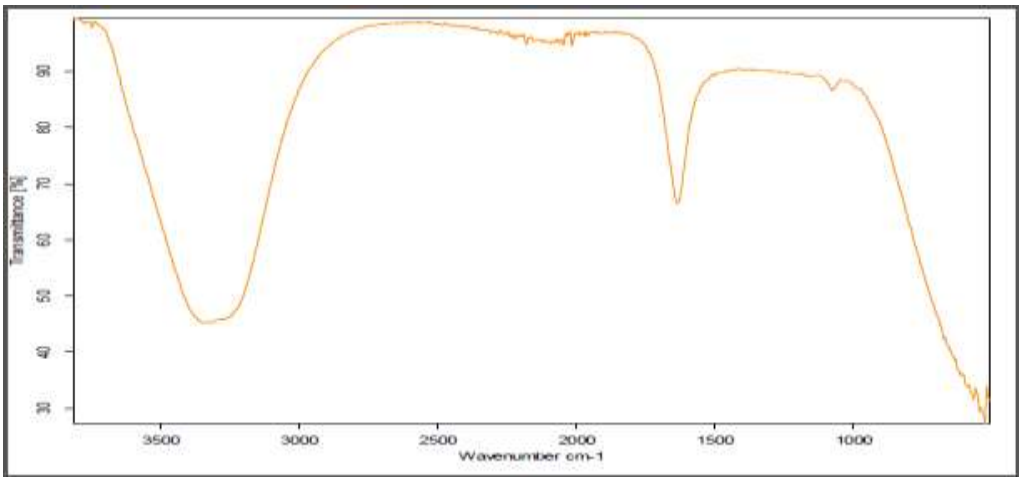


Fig. 5 (b): Fourier Transform Infra-Red Spectra (FTIR) images of β-GALNPs (β-GALNPs = β-galactosidase nanoparticles)

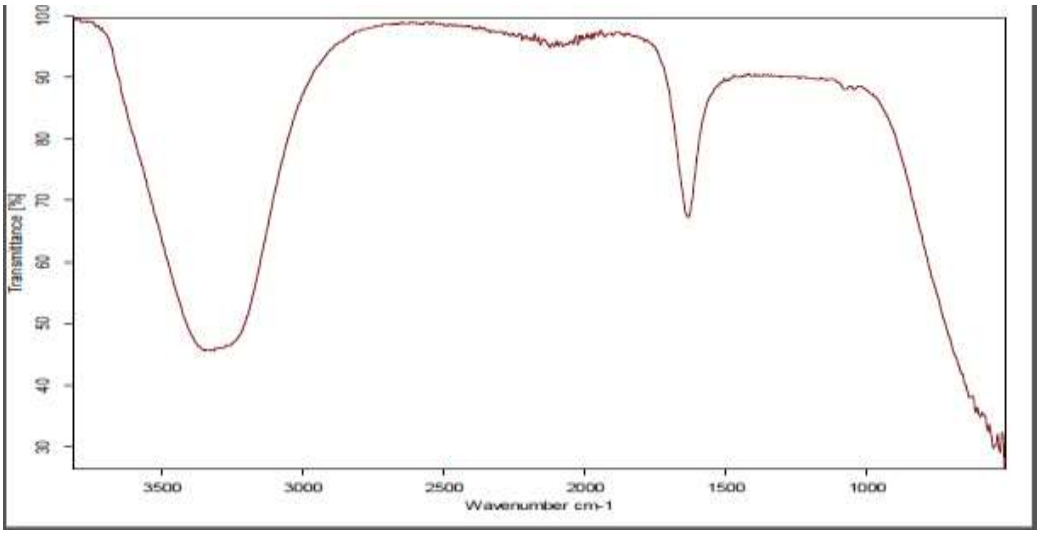


Fig. 6 (a): Fourier Transform Infra-Red Spectra (FTIR) images of GOD (GOD= Glucose oxidase)

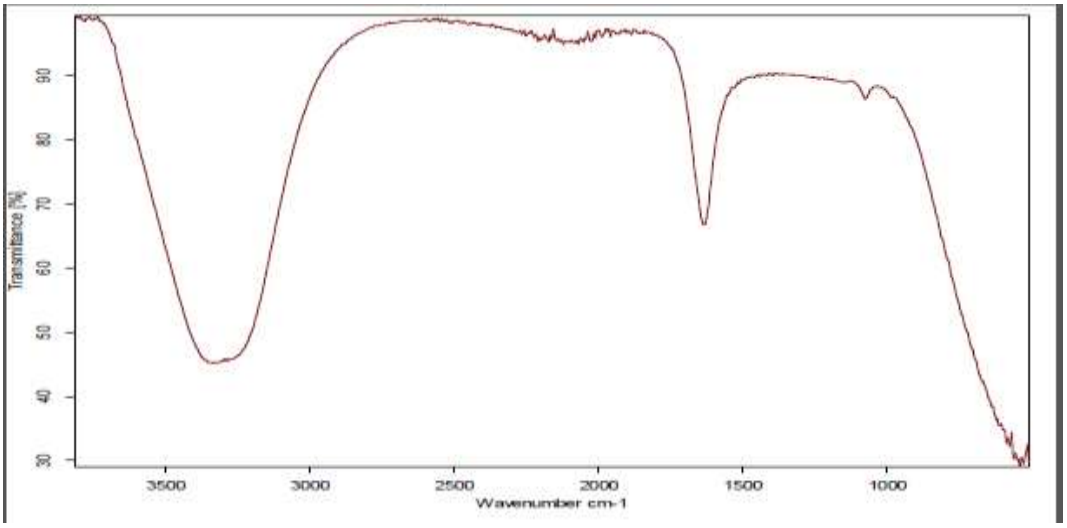


Fig. 6(b): Fourier Transform Infra-Red Spectra (FTIR) images of GODNPs (GODNPs = Glucose oxidase nanoparticles)

Fig. 7(a): Scanning electron microscopy (SEM) images of bare electrode

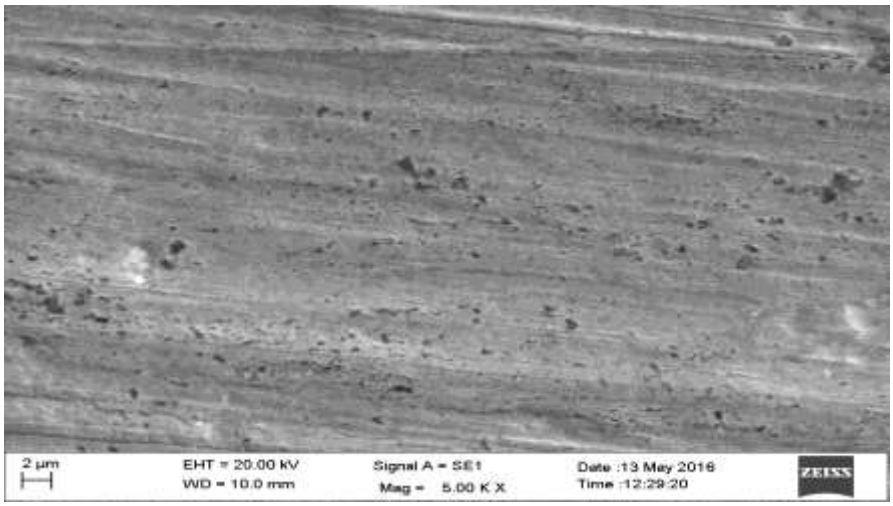


Fig.7(b): Scanning electron microscopy (SEM) images of immobilized β-GALNPs+GODNPs electrode (β-galactosidase nanoparticles; GODNPs = Glucose oxidase nanoparticles)

Effect of pH and Temperature on Lactose Biosensor

Amperometric measurements investigated effect of pH and incubation temperature on β-GALNPs/GODNPs/Au modified electrode in 1mg/mL lactose. Fig. 8 shows lactose biosensor showed optimum at pH 6.5, which is similar to (Sharma *et al*., 2007; portaccio and Lepore, 2017), but lower than (Sharma *et al*., 2004) and higher than (Ammam and Franscar, 2010). The optimum temperature of biosensor was 25^0C (Fig.9), which is lower than earlier reports (Sharma *et al*., 2007; Sharma *et al*., 2004; Gogtug *et al*., 2005; Ammam and Fransacr, 2010; Jasti *et al*., 2014; Yakovleva *et al*., 2012). Therefore, all experiments were carried out at pH 6.5 and 25^0C. The biosensor showed fast response within 5s, which is lower than earlier reports (Ammam and Fransacr, 2010; Soldatkin *et al*., 2013). There was a linearity between biosensor's current response and lactose concentration upto 10 mg/mL, after which it showed hyperbolic relationship (Fig.10a and 10b), which is better than previous reports (Sharma *et al*., 2007; Sharma *et al*., 2004; Jasti *et al*., 2014).

Evaluation of Biosensor

Present lactose biosensor showed low detection limit i.e. 1 mg/mL, which is lower than earlier reports (Jasti *et al*., 2014) but similar to ref. (Ammam and Fransacr, 2010) and higher than ref. (Goktug *et al*., 2015; Ammam and Fransacr, 2010; Campos *et al*., 2014; Marrakchi *et al*., 2008). The average recoveries of added lactose (5 and 10 mg/mL) in milk (n=5) was 94.73±0.5% and 9 6.4±0.9% for the present electrode. To check accuracy of biosensor, lactose containing five milk samples were taken in one run (within batch) and after storage at -20ºC for one week (between batches).The coefficient of variation (CVs) within and between batch were <3% and <4% respectively (Table 1), suggesting the consistency and repeatability of present biosensor.

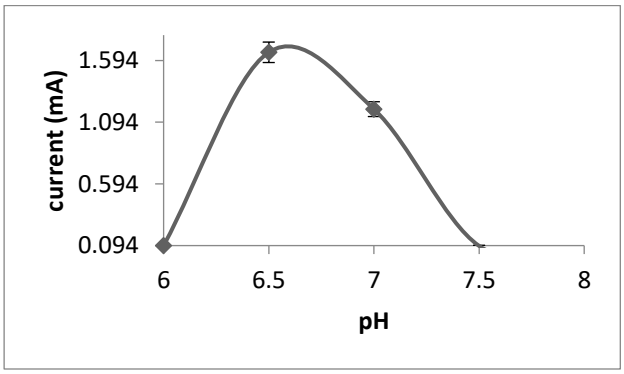


Fig. 8: Effect of pH on the current response of β-GALNPs+GODNPs on modified Au electrode. Standard assay conditions were used for biosensor except for the pH which was changed from 6.0 to 8.0 at an interval of 0.2. Sodium succinate buffer at pH 5 and 5.5 and sodium phosphate buffer between pH 6.0 to 8.0 were used at a final concentration of 0.05 M (β-galactosidase nanoparticles; GODNPs = Glucose oxidase nanoparticles; AuE gold electrode)

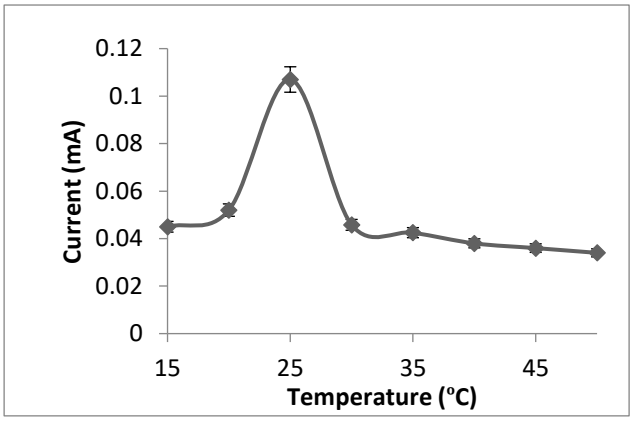
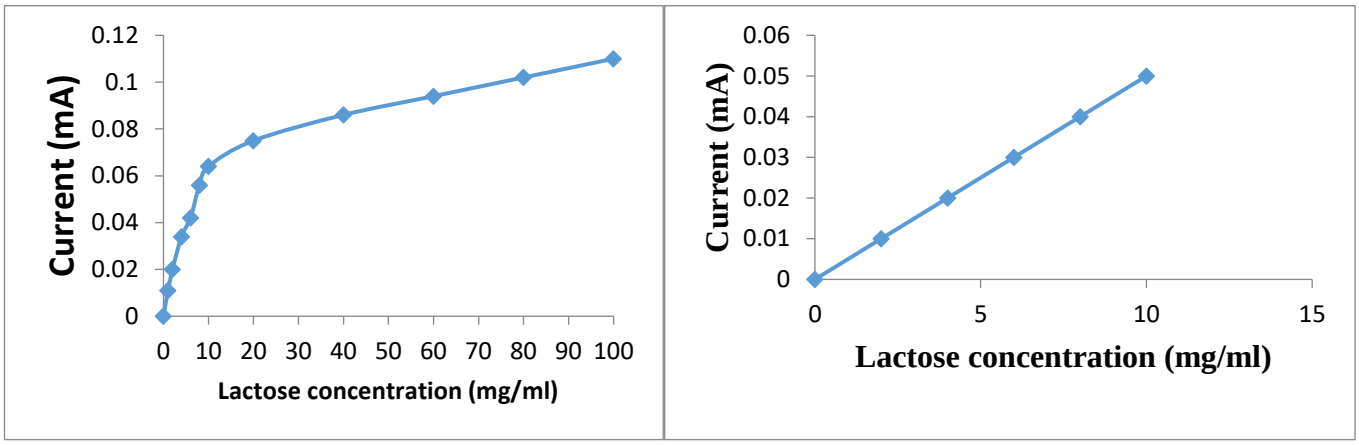


Fig. 9: Effect of temperature on current response of β-GALNPs+GODNPs on modified Au electrode. Standard assay conditions were used for biosensor except for the incubation temperature which was varied from 10 to 50ºC at an interval of 10 (β-galactosidase nanoparticles; GODNPs = Glucose oxidase nanoparticles; AuEgold electrode)



a **b**

Fig. 10: Effect of lactose concentration on the current response of the fabricated lactose biosensor based on GALNPs+GODNPs/Au electrode. **(a)** Standard assay conditions were used for biosensor except the lactose which was varied from 0 to 100 mg/ml at an interval of 10 **(b)** Standard curve of lactose by GALNPs+GODNPs/Au electrode. Standard assay conditions were used for biosensor except the lactose which was varied from 0 to 10.0 mg/ml. (β-galactosidase nanoparticles; GODNPs = Glucose oxidase nanoparticles; AuE =gold electrode)

Table 1: Within and between batches assay co-efficient of variation (CVs) for determination of lactose in milk by biosensor employing β-GalNPs+GOxNPs/Au electrode.

Total number of milk Samples (n=5)	**Mean milk Lactose(mg/ml)**	**%CV**
Within batch (5)		
38		
38.8		
39.6	39.48	2.8%
40.2		
40.8		
Between batch (5)		
36		
36.7		
37.2	37.44	3.2%
38.3		
39		

GalNPs=Galactosidase nanoparticles; GOxNPs=Glucose oxidase nanoparticles

Lactose Determination in Milk Samples

The concentration of lactose in processed milk was extrapolated from standard curve for lactose between biosensor response in current (mA) and lactose concentration (Figure 10a). The biosensor measured lactose concentration in milk of woman, buffalo, Indian cow, American cow and goat and found to be 73 mg/mL, 48 mg/mL, 43 mg/mL, 47 mg/mL and 41mg/mL respectively, which is comparable to previous reports (Stoica *et al.*, 2006).

Reusability and Storage of β-GALNPs+GODNPs/Au Electrode

After every single measurement, the enzyme electrode was washed in reaction buffer 3-4 times. The electrode was used for 120 times with only 50% of loss in its initial activity, due covalent co immobilization of ENPs onto to Au wire. After 50 % loss of its activity, the enzyme electrode was discarded and a new enzyme electrode was used. The electrode surface was not regenerated. It was stored dry at 4ºC.

Conclusion

Based on direct co-immobilization of mixture of enzyme nanopartcles (β-GALNPs and GODNPs) onto Au electrode, an improved lactose biosensor was developed, which showed better analytic performance in terms of linearity (1 mg/mL to 10 mg/mL), response time (5s), reusability (120 times) and stability (3 months) compared to earlier reported biosensors. This has also simplified the fabrication of lactose biosensor. The nanoparticles of other enzymes could also be prepared in this manner and used in fabrication of many more biosensors with improved analytic performance in future.

Authors' Contribution

Chandra Shekhar Pundir and Ranjana Jaiwal designed the work plan. Data collection, analysis and manuscript writing are done by Jyoti Ahlawat. Manuscript was checked by Chandra Shekhar Pundir and Ranjana Jaiwal. Final form of the manuscript is approved by all authors.

Conflict of Interest

The authors declare that there is no conflict of interest with the present publication.

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