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Electrochemical Biosensor Based on Surfactant Doped Polypyrrole (PPy) Matrix for Lactose Determination

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ABSTRACT: Lactose biosensor based on surfactant doped polypyrrole (PPy) was developed. Galactose oxidase and β-galactosidase was coimmobilized in PPy matrix during electropolymerization process with the presence of sodium dodecylbenzene sulphonic acid as surfactant. Bi-enzyme entrapped PPy was characterized with Fourier transform infrared spectroscopy (FTIR), cyclic voltammetry (CV), and scanning electron microscopy (SEM). The response of the enzyme electrode was measured by CV in the range of -0.1 to 1.0 V versus Ag/AgCl which was due to the electrooxidation of enzymatically produced H_2O_2. The effect of lactose concentration was investigated. Response time of biosensor was found to be 8–10 s (the time required to obtain the maximum peak current) and upper limit of the linear working portions was found to be 1.22 mM lactose concentration with a detection limit of (2.6×10^{-6} M). The apparent Michaelis–Menten constant was found to be 0.117 mM lactose. The effects of interferents (ascorbic acid and uric acid) were determined. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40200.

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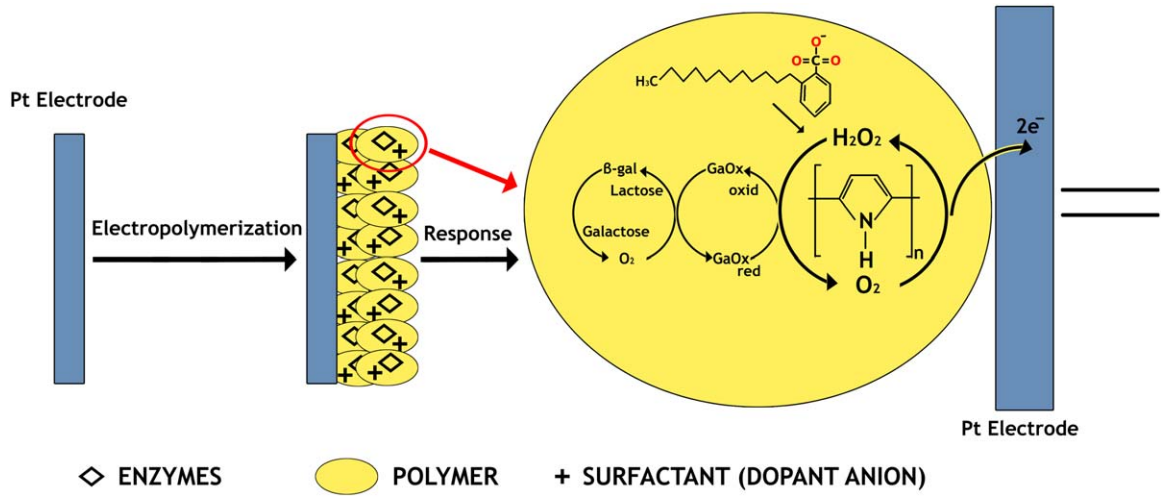
INTRODUCTION

Lactose determination is routinely carried out in milk and dairy products industry to insure powerful process and product control since lactose content is a basic indicator for evaluation of milk quality and detection of abnormal milk (with low lactose level) suffured from cows with mastitis.[1] Effective control of lactose levels is also important for fermented dairy products industry in order to control the fermentation processes. Lactose is hydrolyzed into its monosaccharides by β-galactosidase, which is bound to the mucosal membrane of the small intestine, and than these monosaccharides are absorbed from intestine. Low or absent intestinal lactase activity, called as lactose intolerance or lactase deficiency, does not allow metabolization of lactose into galactose and glucose.[2] Non-absorbed lactose passes into the colon where it causes an influx of water, causing diarrhea, and is fermented by intestinal micro-organisms, causing cramping and flatulence.[3] Therefore, it becomes essential to detect lactose precisely in milk and some milk products.

Various methods are used for the determination of lactose including titrimetry,[4] gravimetric analysis,[5] polarimetry, spectrophotometry, chromatography (gas, liquid and high-pressure liquid chromatography),[3] and infrared spectroscopy.[6] However, many of these methods are generally time-consuming, expensive, and labor-intensive for routine quality control applications.

Biosensors offer a cheap, quick, and reliable alternative for the determination of lactose. Several types of lactose biosensors have been reported in literature.[1,7–16]

Conducting polymers are a significant group of organic conductors because of their mechanical and electronic properties such as flexibility and facile processability. These properties have made them very useful for biological sensor applications.[17] Conducting polymers are promising candidates as a transducer of the enzyme electrodes because they are synthesized readily in the form of thin films by electrochemical polymerization of heterocyclic compounds such as pyrroles. Among the conducting polymers, polypyrrole (PPy) is one of the most studied polymers because of its high electrical conductivity, stability and interesting technological applications. Nevertheless, to the best of our knowledge, use of PPy in the preparation of a lactose biosensor is unavailable in the literature. PPy is an excellent choice as sensor materials because of its ease of structural modification, the high conductivity, and environmental stability. However, the synthesis of conducting polymers in the presence of ionic surfactants imparts several interesting properties such as self-organized structure and solubility.[18,19] As shown previously, adding



Scheme 1. Electropolymerization and response mechanism of the PPy-DBS enzyme electrode to lactose. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

surfactant in to the synthesis media affect the chemical, mechanical, morphological, and electronic properties of the PPy.[20–22] Omastova et al.[20,21] have recently shown that anionic surfactants are incorporated in PPy structure, while cationic and nonionic surfactants affect the properties of PPy only marginally.

The immobilization of enzymes on conducting can be achieved by various methods. Generally, the immobilization of enzymes is achieved either by *in situ* entrapment during the electrochemical polymerization in enzyme containing solution.[23] The electropolymerization of the monomers in an aqueous electrolyte provides the irreversible entrapment of enzyme in the resulting polymer matrix.[24]

In this context, the aim of this article is to develop a novel lactose biosensor using an anionic surfactant doped PPy as a transducer for the first time. For this purpose, dodecylbenzene sulphonate doped PPy, shown in Scheme 1, was used as a novel conducting support–material to manufacture β-Gal/GaOx-immobilized electrode. Conducting layer was synthesized electrochemically in the presence of surfactant and enzymes on to platinium disc electrode. After characterizing the conducting layer with cyclic voltammetry (CV), Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM) methods, modified electrode was tested for the lactose sensing. The response of the enzyme electrode was investigated in relation to the lactose amount. The effect of interferents on the response of modified electrode was also determined.

EXPERIMENTAL

Reagents

Pyrrole (Merck) was purified by distillation at reduced pressure before use. Sodium dodecylbenzenesulfonate (Fluka), Galactose oxidase (GaOX, EC 1.1.3.4, 179,000 units/g, type VII-S from *Aspergillus niger*, Sigma), and β-galactosidase (EC 3.2.1.23, $\geq$8000 units/g solid, Lactase from *Aspergillus oryzae*, Sigma) were used to activate the polymer modified Pt disc electrode though lactose. $NaH_2PO_4{\cdot}2H_2O$ (Riedel De Haen) and NaOH (Riedel De Haen) was used to prepare buffer soltion. Alumina polishing suspension agglomerate (0.05cr micron) (Baikowski) was used as electrode polisher. Double-distilled water was used for preparation of the buffer solution. All other compounds were analytical reagent grade.

Instrumental

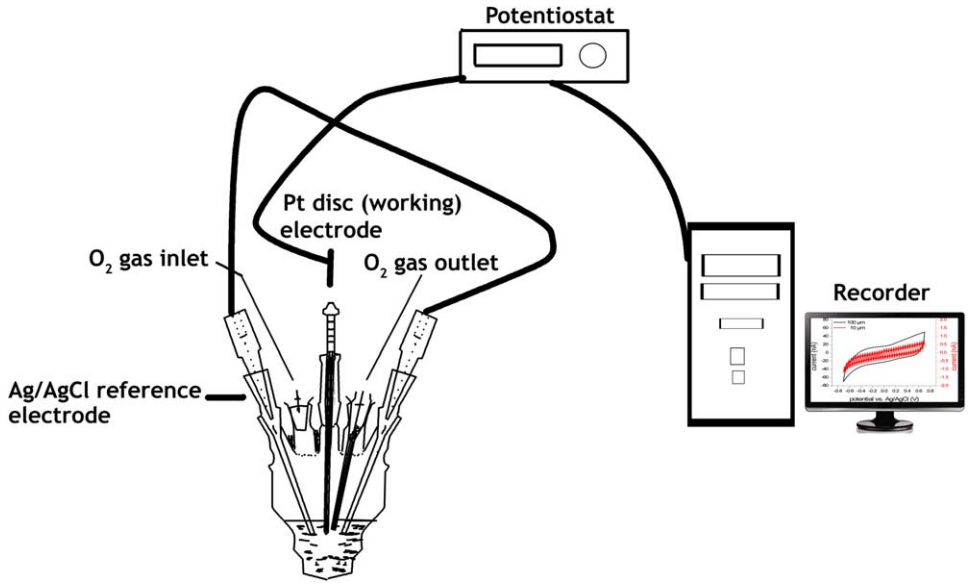
FTIR spectrum was obtained using FTIR spectrophotometer between 400 and 4000 cm^{-1} with a 4 cm^{-1} resolution on a Perkin Elmer model (Beaconsfield, Beuckinghamsshire, HP91QA, England). SEM was performed using Phillips scanning electron microscope (XL-30S FEG). Coimmobilization of enzymes and polymerization of pyrrole and biosensor measurements were conducted in the three-electrode cell equipped with a Gamry Model potentiostat (Gamry Instruments, US). PPy-DBS modified platinium disc electrode was used as working electrode, platinum wire and Ag/AgCl electrodes were used as counter and reference electrodes, respectively. Indium thin oxide (ITO) electrode was used for the deposition of polymer for the analysis of FTIR and SEM.

Electrode Fabrication

Agglomerate-free alumina polishing suspension was used as polisher for Pt disc working electrode. The working electrode was washed with phosphate buffer solution before all electrochemical measurements. The PPy film was electrochemically formed using pyrrole (0.5 M) in 0.05 M NaDBS, 0.1 M phosphate buffer, pH 7.4, containing 2.94 mg/mL GaOx (40 unit) and 2.0 mg β-galactosidase (20 unit). Chronoamperometry method was used to deposit enzyme immobilized PPy onto Pt disc electrode at 1.0 V during 30 min at room temperature. Than, polymer modified working electrode was washed with buffer solution and distilled water, respectively, to remove non-entrapped enzyme and remaining monomer. The prepared working electrode was stored at 4°C in buffer (pH 7.4) solution.

Lactose Estimation and Apparatus

A three-electrode cell has five separate inputs for the working, reference and auxiliary electrodes, and the gas entry and exit (Scheme 2). Oxygen gas is passed through the cell at a constant speed for 10 min to obtain measuring medium saturated with



Scheme 2. Electrochemical measurement system (modified from Gulce[49]). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

oxygen. During measurement, oxygen gas flow was continued over the solution to keep the constant oxygen concentration. The response of enzyme electrode to lactose was investigated by CV method based on electrooxidation of H_2O_2 produced enzymatically in potantial range of −0.1 to 1.0 V. Lactose solution was prepared at 0.02 M. The measurements were recorded after addition of known amounts of lactose to the buffer solution (pH 7.4) at 0.4 V, which is attributed to the denaturation potential for couple of enzymes (β-gal/GaO).[10]

RESULTS AND DISCUSSION

In this study, we aimed to develop a new lactose biosensor using surfactant doped conducting polymer. For this purpose, dodecylbenzensulphonic acid doped PPy, was synthesized in presense of galactose oxidase and β-galactosidase in pH 7.4 buffer solution. The black and stable polymeric film layer was obtained on Pt disc electrode by electrochemical process. The same procedure was applied to ITO coated glass electrode to get much more materials for FTIR and SEM studies of PPy and enzyme entrapped PPy.

CV, FTIR, and SEM techniques were used with the aim of characterization of the polymer. The convenience of usage of the characterized polymer as an enzyme electrode was also investigated. The changes in the response of the enzyme electrode through the substrate concentration and the effect of interferents were examined.

According to the below reactions, determination of the amount of lactose is based on a couple biochemical and an electrochemical reaction. The first two biochemical reactions are hydrolysis of lactose by β-galactosidase and catalytic oxidation of galactose by galactose oxidase, respectively [eqs. (2) and (3)]. And the last reaction is attributed to the oxidation of hydrogen peroxide, produced in the reaction as given eq. (3).[13]

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{-gal}} \text{Glucose} + \text{Galactose} \quad (1)$$

$$\text{Galactose} + O_2 \xrightarrow{\text{GaOx}} \text{D-Galacto}-\text{hexodialdose} + H_2O_2 \quad (2)$$

$$H_2O_2 \xrightarrow{\text{Pt}} O_2 + 2H^+ + 2e^- \quad (3)$$

H_2O_2 is polarized at +0.4 V vs. AgCl/Ag resulting in a voltammetric signal proportional to the lactose concentration.

Characterization of PPy and Enzyme Entrapped PPy

Cyclic Voltammetry (CV) Studies. CV method was used to investigate the electrochemical behavior of PPy and enzyme entrapped PPy via the changing the scan rate. First, PPy doped with surfactant was deposited onto Pt disc electrode. Then, this modified electrode was removed from polymerization solution and washed with buffer solution (pH 7.4). After that, electrode was dipped into clean buffer solution (pH 7.4) without monomer and surfactant. The similar procedure was performed to the electrode modified with enzyme entrapped PPy-DBS. CV experiments were carried out in unstirred solutions at a scan rate of 50 mV s^{-1}. There is an irreversible oxidation peak corresponding to the enzyme entrapment to the PPy structure was observed at approximately 0.4 V for the enzyme entrapped PPy (Figure 1). This oxidation potential value belongs to galactose oxidase. This value is in agreement with the previous studies.[25,26]

The electrochemical performance of enzyme entrapped PPy modified Pt disc electrode was investigated by changing the scan rate. The measurements were taken into buffer solution (pH 7.4). CV studies show that the current values are increasing with the scan rate increase [Figure 2(a)]. In cyclic voltammetry, the Randles–Sevcik equation describes the effect of scan rate on the peak current, i_p, depends not only on the concentration and diffusional properties of the electroactive species but also on the scan rate.[43]

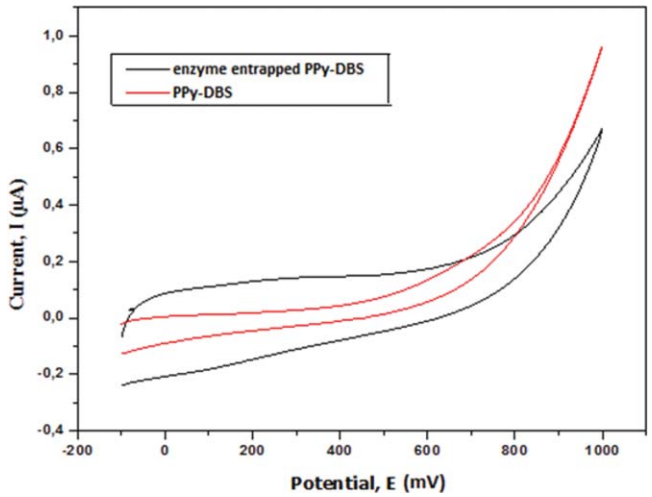


Figure 1. Comparison of the current–potential curves of PPy and enzyme entrapped PPy onto Pt disc electrode with 50 mV s^{-1} scan rate in buffer solution. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

If the solution is at room temperature:

$$i_p = kn^{3/2}AD^{1/2}Cv^{1/2} \quad (4)$$

where i_p is the current maximum in A; n, number of electrons transferred in the redox event; A, electrode area in cm^2; F, The Faraday Constant in C mol^{-1}; D, diffusion coefficient in cm^2/s; C, concentration in mol/cm^3; v, scan rate in V/s. According to the equation anodic current (i_p) is proportional to the square root of scan rate (v). In this case, a linear dependence of irreversible anodic peak intensity against with square root of scan rates was given in Figure 2(b). As indicated from the linearity of the plot, it can be seen obviously that the redox process has no diffusional obstacles and the electroactive polymer is well fastened on the working electrode surface.[27]

FTIR Results. Figure 3 shows the FTIR spectrum of electrodeposited PPy and enzyme entrapped PPy films upon Pt disc electrode recorded using KBr in the wavenumber range 400–4000 cm^{-1} and under transmission mode. It can be seen that the spectrum present characteristic and conspicuous bands corresponding to various inter-atomic and ring stretching vibrations. Especially the band at 1544 and 1542 cm^{-1} may be assingned to the C=C ring stretching of pyrrole whereas the C–N ring stretching peak of pyrrole ring be formed at 1459 and 1458 cm^{-1} in the spectrum of polymers. The band at around of 1285 cm^{-1} is the C–H or C–N in-plane deformation modes and the peak at vanisity of 1162 cm^{-1} is due to C–C stretching of pyrrole. These bands correspond to the characteristic bands for electrochemically synthesized PPys; it shows very good agreement with earlier reported work.[28] These FTIR data observed from spectrum of polymers justify the formation of PPy on the

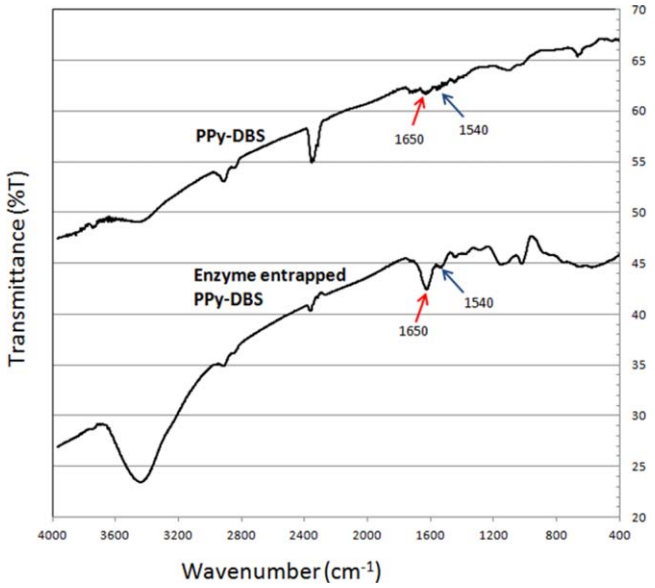


Figure 3. FTIR spectrum of PPy-DBS and enzyme entrapped PPy-DBS synthesized on to Pt electrodes in 0.1 M NaH_2PO_4 including 2.94 mg/mL galactose oxidase and 2 mg/mL β-galactosidase. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

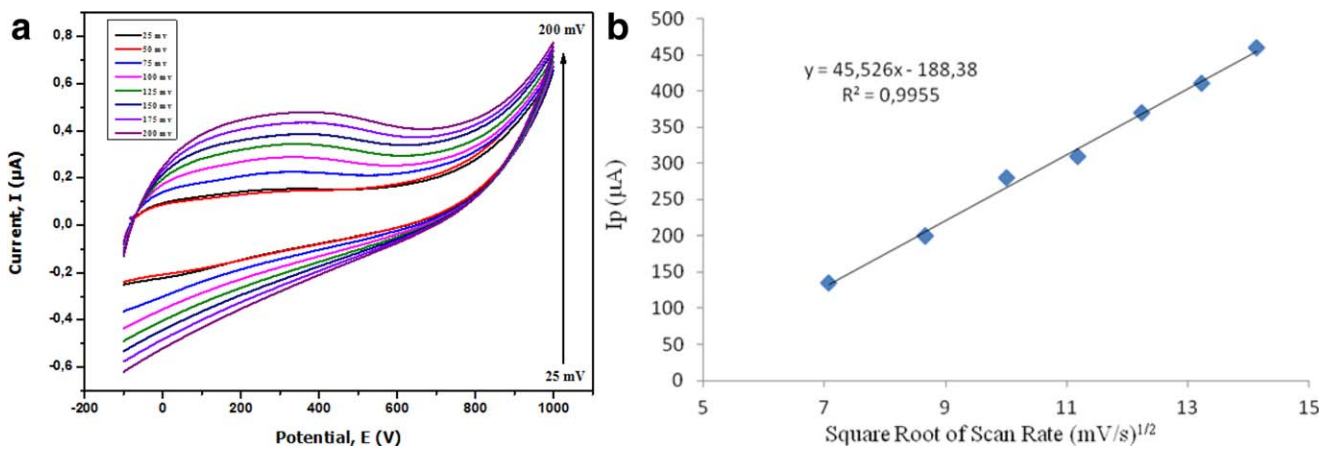


Figure 2. The current–potential plots of enzyme entrapped PPy film on Pt disc electrode with different scan rates between 25 mV s^{-1} and 200 mV s^{-1} (a). Plot of anodic current of the enzyme entrapped PPy in 0.1 M NaH_2PO_4, pH 7.4, vs. square root of scan rate (b). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

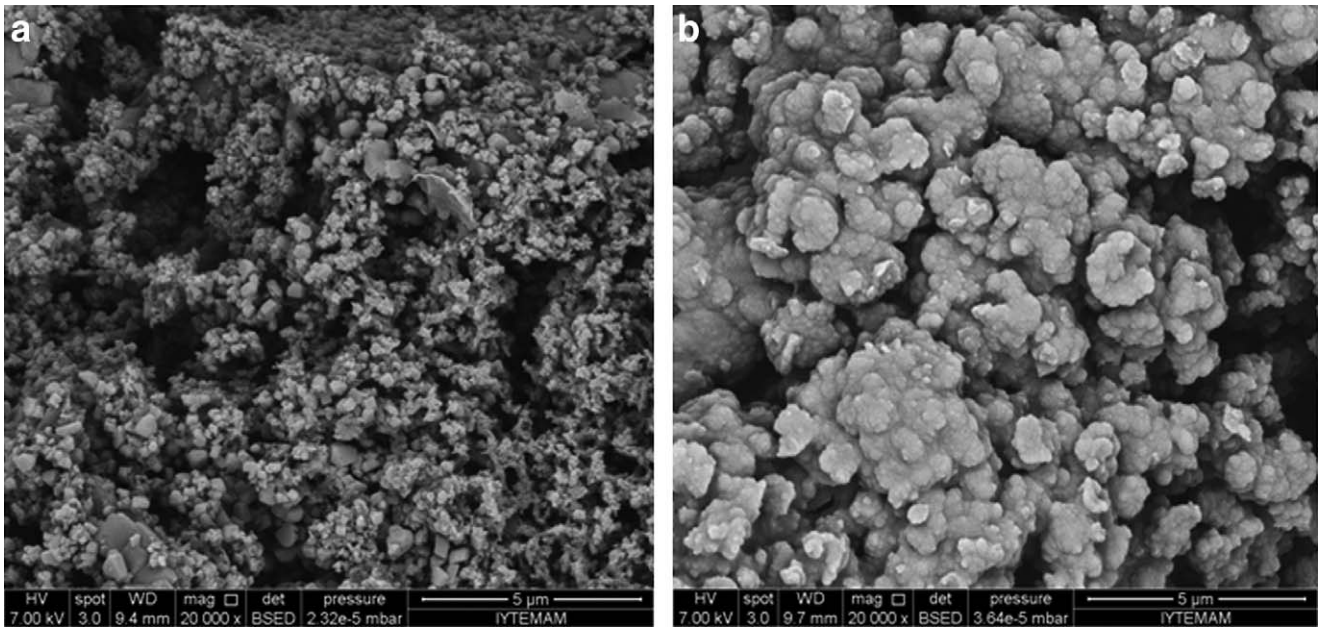


Figure 4. SEM images of (a) PPy and (b) enzyme entrapped PPy (PPy synthesized onto Pt electrode in 0.1 M NaH_2PO_4 including 2.94 mg/mL galactose oxidase and 2 mg/mL β-galactosidase).

Pt disc electrode. Spectral results support the polymerization of PPy. There is a triple peak at the range of 1400–1700 cm^{-1} in the spectrum. The bands observed in this area are assingned to the C=C ring stretching of pyrrole and responsible for a large extension of the conjugated system in the polypyrrole chain.[29] However, the peaks appearing at around 1650 and 1540 cm^{-1} can be attributed to the amide I band and amide II band, respectively, of the amide group of β-gal and GaOx.[30,31,44] These are the distinct bands of protein structure, demonstrating that the enzymes, β-gal, and GaOx are entrapped in the PPy structure. Besides, in the spectra of enzyme entrapped PPy, the intensities of these said bands were increased as a result of enzyme entrapment. Such types of spectra were also reported earlier by Petty.[45] However, the isoelectric points of β-gal and GaOx are pH 4.61 and pH 3.70, respectively. So, during the electrochemical procedure, the net charges of enzymes are negative in pH 7.4 buffer solution. In this case, enzymes with negative charges are more favorable to move to anode and entrapped into the PPy network.[46] All these results confirm the entrapment of β-gal and GaOx enzymes into PPy film.

SEM Results. SEM studies of PPy and enzyme entrapped PPy were shown in Figure 4(a,b), respectively. This analysis tecniques were performed to confirm the entrapment of β-gal and GaOx enzymes into PPy frames. PPy has a granular and tightly stacked structure [Figure 4(a)]. These granules are very small and uniform, whereas the enzyme entrapped PPy shows very distinctive structure according to PPy [Figure 4(b)]. Enzyme entrapped PPy displays a cauliflower like structure and the diameter of granules are bigger than PPy.[44] SEM of PPy containing entrapped enzymes showing globular structure of proteins.[10] PPy film shows a spongy structure with a particle diameter of ~200 nm [Figure 4(a)]. This structure provides a large surface area and space for enzyme loading in entrapment procedure. Generally, the size of an enzyme particle is in the range of 10–100 nm.[47] The pore size of the polymer is suitable for the immobilization of the enzyme.[48] Thus, enzyme molecules can be immobilized into the PPy pinholes easily. After embedding the enzymes into polymer structure, the pores of PPy matrix attenuated considerably, and the enzyme loaded bright parts were observed clearly on the polymer film [Figure 4(b)]. As a result of this perspective, SEM results are supporting the entrapment of β-gal and GaOx enzymes into PPy film.

Lactose Sensing. Dodecylbenzene sulphonate doped PPy containing β-gal and GaOx enzymes were deposited onto Pt disc electrode in order to develop lactose biosensor. Figure 5 shows

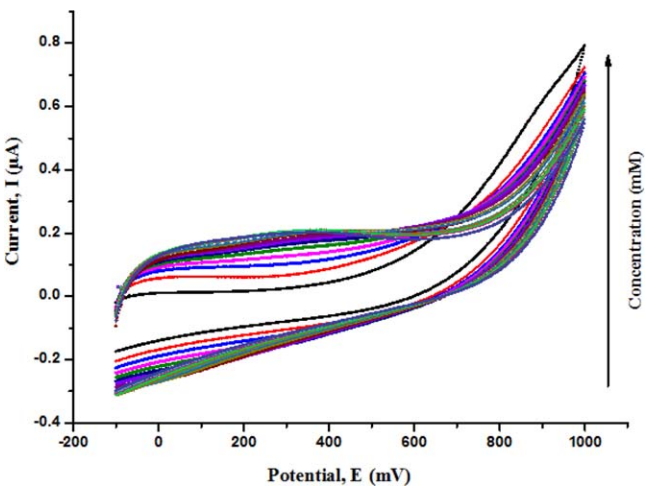


Figure 5. Typical cyclic voltammetric response (−0.1 to 1.0 V) of β-gal/GaOx immobilized film of DBS doped polypyrrole to lactose in 0.1 M NaH_2PO_4 (pH 7.4). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

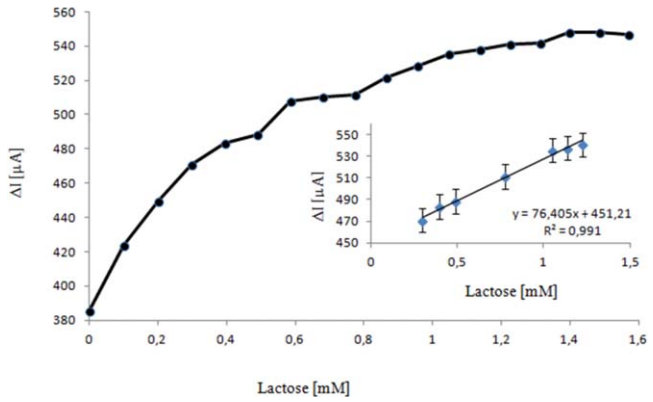


Figure 6. Effect of lactose concentration on current and calibration curve (inset graph) for the cyclic voltammetric response of the enzyme modified electrode in the range of −0.1 to 1.0 V applied potential, in the concentration range 0.3–1.22 mM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the response of the enzyme electrode to lactose. The variation of current values of working electrode against the potential values scanning between 0.1 and 1.0 V versus Ag/AgCl were measured to determine the increasing of current values at 0.4 V caused by electrooxidation of H_2O_2 propagated enzymatically. After having cyclic voltammogram of blank solution, known amounts of lactose solution were added and the currents at 0.4 V for each added concentration of lactose were recorded. Current values inceased with the each addition of lactose. These current values were used to form the calibration plots. Response time of enzyme electrode developed in this study is shorter than lots of studies in literature.[8,12,32] The response time of the developed lactose enzyme electrode is in agreement with the ones published in literatures.[13,14,33]

Figure 6 indicates the corelation between the reaction rate of the β-gal/GaOx immobilized film of DBS doped PPy with the lactose concentration in 0.1 M NaH_2PO_4. As can be seen from Figure 6, linear working range of the enzyme electrode is 0.3–1.22 mM lactose concentration. Detection limit of enzyme electrode was achieved at 2×10^{-6} M for lactose. The relation between the reciprocal of the response current (i^{-1}) and the reciprocal of lactose concentration (C^{-1}) was obtained

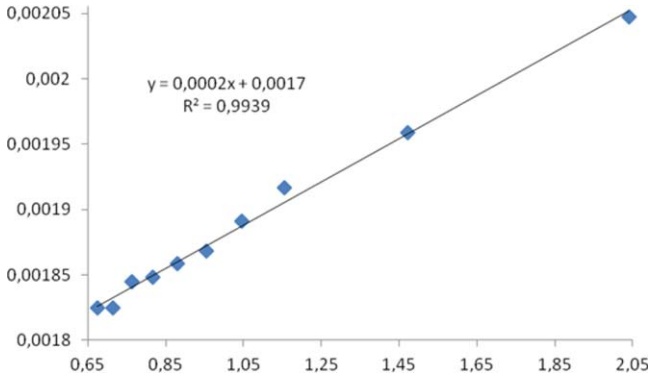


Figure 7. Lineweaver–Burk plot of β-gal/GaOx immobilized film of DBS doped PPy. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

according to the Lineweaver–Burk form of the Michaelis–Menten equation[34] (Figure 7). The Michaelis Menten constant is important to characterize the enzyme electrode due to the character of the aforementioned constant (KM). In Michaelis Menten assumption, transformation rate of substrate from products depends on the degradation rate of enzyme–substrate complex. When, the value of KM constant decreases, the affinity between the enzyme and the substrate is getting strengthened according to Michaelis Menten equation.[35] In this study, the apparent Michaelis–Menten constant (KM_{app} = 0.117 mM) for lactose was computed using by slope and intercept values of the Lineweaver–Burk equation. This value is lower than the value found in the literature for the free enzyme system (2.30 mM),[36] and may be due to stronger affinity between enzyme and electrode, and also encouragement of PPy-DBS electronically. KM values of both entrapped and free enzyme systems show that there is no diffusional limitations and denaturating character in the entrapment procedure of enzymes.[37]

The maximum reaction rate (V_{max}) was also estimated as 163 μA. Based on these data, designed enzyme electrode for the determination of lactose is appropriate than those given in the literature.[36,38–40]

Effect of Interferents. Interferents such as ascorbic acid (2.21 mg/dL)[41] and uric acid (1.5 mg/dL)[42] can affect the response of lactose biosensor. The effect of these interferents on current response was measured using 0.2 M lactose solution. The error in the response current was within 5% indicating that there is no significant effect of the interferent on the lactose sensor based on surfactant doped PPy film.

CONCLUSIONS

In the current study, enzyme electrode was developed using β-galactosidase and galactose oxidase immobilized dodecylbenzene sulphonate doped PPy for the determination of lactose. This work provides a fast, cheap, and sensitive option to determine lactose. Dodecylbenzene sulphonate doped PPy indicated enhanced response against lactose according to literature. Formation of PPy-DBS was carried out successfully on Pt disc electrode at constant potential (0.1 V) for a definite time in the presence of enzymes. KM values of both entrapped (0.117 mM) and free enzyme (2.30 mM)[36] systems show that there is no diffusional limitations and denaturating character in the entrapment procedure of enzymes. Linear working range of the enzyme electrode is up to 1.22 mM lactose concentration. Enzyme electrode has a fast response time (8–10 s), and a reasonable and stable polymer layer for the transporting electrodes. The detection limit of enzyme electrode was determined as 2.6×10^{-6} M. Furthermore, no significant effect of the interferents (ascorbic acid and uric acid) was observed at their physiological concentrations on the response of electrode. The sensor has an easy and simple manufacturing procedure and low cost that could facilitate the swimmingly detection of lactose in several samples. There is more work to be done to improve the properties of this enzyme electrode for the lactose estimation in milk and will be done in our future endeavors.

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