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ORIGINAL ARTICLE

Development of glucose oxidase-chitosan immobilized paper biosensor using screen-printed electrode for amperometric detection of Cr(VI) in water

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Abstract
Hexavalent chromium is a toxic heavy metal getting discharged into the environment and water bodies through various industrial processes. Conventional analysis methods call for expensive equipment and complicated sample pretreatment that made unsuitable for onsite detection. Paper is used as an enzyme immobilization platform because of its property to wick the liquid by capillary action; lightweight, cheap and can be easily patterned or cut according to the requirements for developing biosensor. In this study, enzyme immobilization of glucose oxidase (GOx) on filter paper were examined using three polysaccharides such as chitosan, sodium alginate and dextran for entrapment efficiency, activity and stability of the immobilized enzyme. Among the three, chitosan proved efficient for enzyme entrapment with about 90% efficiency at 0.3% (w/v) chitosan. The stability was checked after 1 week at 4 °C and room temperature, where the chitosan entrapped enzyme retained nearly 97% stability at 4 °C. Enzyme inhibition study of GOx and Cr(VI) was carried out using chronoamperometry shown uncompetitive type of inhibition. A paper-based electrochemical biosensor strip was developed by immobilizing GOx enzyme on filter paper using chitosan as an entrapping agent and associating it with a screen-printed carbon electrode for amperometric measurements. The linear range of detection was obtained as 0.05–1 ppm with the limit of detection as 0.05 ppm for Cr(VI), which is the standard permissible limit in potable water. The relative standard deviation (5.6%) indicates good reproducibility of the fabricated biosensor.

Keywords Paper biosensor · Screen-printed carbon electrode · Glucose Oxidase · Inhibition · Chronoamperometry · Hexavalent Chromium

Introduction

Hexavalent chromium is the most hazardous heavy metal discharged into the environment particularly from chromium mines and other industrial processes (Wilbur et al. 2012). It is extensively used in the steel industry for stainless steel manufacturing and also used for metal finishing, alloying, leather tanning, and textile dyes (Biswas et al. 2017). It can leach into groundwater and remain unchanged over a long period. Cr(VI) has great potential to cross biological barriers. It can enter the human body by oral route with a significant transfer that may lead to stomach disturbance, liver damage, kidney failure, and cancer (DesMarais and Coasta 2019). World Health Organisation (WHO) had categorized Cr(VI) as a carcinogen and as per standards, the concentration of chromium in drinking water should have less than 0.05 mg/L (WHO 1998; Banchhor et al. 2017). Chromium mines of Sukinda valley in Odisha state produce 98% of total chromium in India (Biswas et al. 2017). As an outcome, the water sample from the Damsala Nala of Sukinda mining area in the Odisha state of India was found with 5 mg/L of chromium and that of the soil sample collected from the same site contains 47 mg/L of chromium, which is a quite high amount (Nayak et al. 2020a). The ability to analyze the Cr(VI) concentrations is an important issue considering the

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environmental safeguard. Conventional analysis methods for Cr(VI) consist of spectrophotometry, flame atomic absorption spectrometry, and atomic fluorimeter spectrophotometry (Bansod et al. 2017). The most common method used is the spectrophotometric analysis by 1,5-diphenylcarbaide (DPC) colorimetric assay (Lace et al. 2019). These methods require complicated and expensive instruments as well as technical staff for operation, which will make them unsuitable for onsite and rapid detection.

Among various biosensors, recent attention has turned towards inhibition-based enzyme sensors to determine the concentrations of inhibitors in the assayed sample by measuring the inhibition degree of enzyme, sensor activity, and the lower detection limits (Amine et al. 2016). Glucose oxidase enzyme (GOx) (EC number 1.1.3.4) is an oxidoreductase that catalyzes the oxidation of glucose to hydrogen peroxide and D-glucono-*δ*-lactone which further converted to gluconic acid by spontaneous hydrolysis (Bankar et al. 2009). This enzyme is produced by certain species of fungi and insects. The pH optimum for glucose oxidase from *A. niger* is 5.5, while it has a broad activity range of pH 4–7. The glucose oxidase binds specifically to β-D-glucopyranose and does not act on α-D-glucose (Osorio-Gonzalez et al. 2019). It can oxidize all of the glucose in solution because the equilibrium between α and β anomers is driven towards the β side as it is consumed in the reaction. Owing to the low cost, high specific activity, and good stability glucose oxidase enzyme proved an ideal enzyme for inhibition-based biosensor development (Wilson and Turner 1992; Osorio-Gonzalez et al. 2019). Zeng et al. (2004) developed a glucose oxidase inhibition-based biosensor for the detection of trace Cr(VI). An electropolymerized aniline membrane has been prepared on a platinum electrode containing ferrocene as an electron transfer mediator, on which GOx is crosslinked by glutaraldehyde. Chen et al. (2011) examined the inhibitory effect of heavy metal ions on the bioactivity of the enzyme glucose oxidase (GOx) in solution, electrode surface adsorbed GOx and polymer entrapped GOx. The author reported that Ag^{+} showed the strongest inhibition effect among the HM ions examined, and the inhibitive assays of Ag^{+} based on GOx entrapped in poly-(L-noradrenalin) (PNA) give limits of detection (LOD) of 2 nM. Inhibition effects of Hg^{2+}, Cu^{2+}, and CO^{2+} are detectable only at 15 µM or higher concentrations.

Calvo-Perez et al. (2014) developed tyrosinase and GOx inhibition-based amperometric biosensor for selective detection of Cr(III) and Cr(VI). The bipotentiostat biosensor was fabricated by immobilizing the enzyme on a screen-printed carbon electrode using glutaraldehyde as a crosslinking agent. A bienzyme amperometric biosensor for simultaneous detection of H_2O_2, Cr(III), and Cr(VI) was developed by Liu et al. (2019). The enzymes were entrapment in poly(noradrenaline) matrix which is catalyzed by HRP on the platinum electrode surface. The chronoamperometric detection was based on GOx inhibition and shows 0.2 nM LOD for Cr(VI) sensing. da Silva et al. (2020) demonstrated a sensitive GOx inhibition-based biosensor for electrochemical determination of heavy metals like Hg^{2+}, Cd^{2+}, Pb^{2+}, and Cr^{6+} with a nanomolar limit of detection. The biosensor was fabricated by immobilizing GOx on poly (brilliant green) film on a multiwalled carbon nanotube modified glassy carbon electrode. Furthermore, Yang et al. (2019) used an electroactive bacteria *Shewanella oneidensis* as a new approach for mediator free heavy metals sensing in water with 2.48 ppm LOD for Cr(VI). Also, Prabhakaran et al. (2020) developed a voltammetric biosensor implanting a bacterial strain of *Shyngopyxis macrogoltabida* isolated from the chromium mining area. These bacteria possess enzymes that help them to reduce Cr(VI) generating an electrochemical signal. The main limitation of whole-cell bacteria-based biosensors is the need for nutrients and optimum conditions for the viability of the strain. Owing to the complicated, time-consuming, and expensive fabrication methods there is a need for the development of easy and cost-effective biosensor fabrication methods. Using paper as a platform for enzyme immobilization, the complexity and the cost of the fabrication can be reduced to a certain.

Paper-based biosensors integrate the paper as a substrate with a microfluidic advantage because of its property to wick the liquid by capillary action; it is lightweight, cheap and can be easily patterned or cut according to requirement (Liana et al. 2012; Juang et al. 2017). Paper offers a stable thin film of water that delivers the analyte to the surface of the electrodes. Electrodes can be printed on paper using special inks by screen printing which is a simple low-cost fabrication technique (Dungchai et al. 2011).

The present study includes three approaches, first finding the potency and kinetics of inhibition for GOx and Cr(VI), second proposing an effective method for enyme entrapment on paper and third is the implementation of that method to develop a paper-based electrochemical biosensor. Enzyme immobilization study was carried out using three polymers such as sodium alginate, chitosan, and dextran on filter paper. The efficiency, as well as stability at a variable concentration of polymer, was studied. Enzyme GOx was immobilized on filter paper with chitosan entrapment cross-linked using glutaraldehyde. A paper-based biosensor strip was developed electrochemical detection of Cr(VI) by implanting the GOx immobilized paper onto the screen-printed carbon electrode (SPCE) and characterized using cyclic voltammetry and chronoamperometry.

Materials and methods

Materials

Glucose oxidase (GOx) (EC 1.1.3.4) and horseradish peroxidase (HRP) (EC 1.11.1.7) were purchased from Himedia. Sodium alginate and dextran were purchased from Sigma-Aldrich. Screen-printed carbon electrodes (SPCE) were procured from Palmsens (Netherlands). Enzyme stocks (1 µg/mL) were prepared in distilled water. Sodium acetate buffer 0.1 M, pH 4.6–5.6 was prepared using 0.1 M sodium acetate and 0.1 M acetic acid stock. Phosphate buffer (PB) 0.1 M was prepared by adding 1.2 ml of 1 M dibasic sodium phosphate and 8.8 ml of 1 M monobasic sodium phosphate making the final volume to 100 ml. β-D-glucose solution 10% (w/v) was prepared in distilled water. Hydrogen peroxide 0.3% (v/v) was prepared in distilled water. *O*-dianisidine solution 1% (w/v) was prepared in methanol freshly. Chitosan 1% (w/v) was prepared by dissolving chitosan in 0.5% acetic acid pH adjusted to 5 and kept on stirring for 1 h and further diluted to 0.5% using PB pH 6. Glutaraldehyde solution 3% (v/v) was prepared in distilled water and Whatman#1 filter paper was cut into 1 × 1 cm pieces. Cr(VI) stock solution of 1000 ppm concentration was prepared by dissolving 0.283 g of $K_2Cr_2O_7$ in 100 ml of distilled water.

Methods

GOx inhibition study by chronoamperometry

Inhibition kinetics of GOx was studied using chronoamperometry. A portable minipotentiostat Emstat3® (Palmsens) was used with the platinum working electrode, Ag/AgCl reference electrode, and platinum wire counter electrode. GOx at concentration 0.5 µg/mL was added to 10 mL of β-D-glucose solution prepared in 0.6 M PB pH 7 and current was measured at 0.7 V vs Ag/AgCl for 300 s (Fenton et al. 2008; Zeng et al. 2004). Equation (1) shows the calculation of the degree of inhibition. I_0 is the current signal in µA in the absence of Cr(VI) and I is the current response at a particular concentration of Cr(VI).

$$\text{Degree of inhibition } (\%) = \left(\frac{I_0 - I}{I_0}\right) \times 100. \quad (1)$$

Enzyme immobilization by polymer entrapment

The preparations in the range 0.1–0.5% (w/v) polymer gel mixtures of chitosan, sodium alginate, and dextran were compared for efficient enzyme immobilization. 25 µL of 8 µg/mL GOx concentration was added to the gel mixtures. For chitosan gel mixture glutaraldehyde was used as a crosslinking agent in the 1:1 chitosan-glutaraldehyde ratio. The mixture was stirred and 10 µL volume was spotted on the Whatman#1 filter paper square and allowed to dry for 60 min. The paper squares with enzyme spotted were washed six times (three times on both sides) with 100 µL of in wash buffer (0.1 M PB + 0.1% v/v Tween-20, pH 7) and allowed to dry for 60 min. 10 µL of HRP + β-D-glucose + *o*-dianisidine mix containing 20 µg/mL HRP, 0.1% *o*-dianisidine, and 0.1% β-D-glucose was added and the intensity of the colour developed was measured using ImageJ software (Cao et al. 2015). The experiments were performed in triplicates and the entrapment efficiency were calculated based on Eq. (2).

$$\text{Entrapment efficiency } \% = \frac{\mu\text{g of protein spotted on paper} - \mu\text{g of protein in wash buffer}}{\mu\text{g of protein spotted on paper}} \times 100. \quad (2)$$

Preparation of GOx paper squares and electrochemical characterization

GOx concentration of 1 mg/mL was prepared in 0.3% chitosan and about 10 µL of enzyme gel mixture was added to the 1 × 1 cm paper square and allowed to dry for 60 min. The GOx paper square was kept upside down onto SPCE and electrochemical characterization was done using cyclic voltammetry. 100 µL of 5 mM β-D-glucose solution was added on GOx-paper-SPCE and cyclic voltammetry was performed at potential range from − 2.5 to + 1.5 V and scan rate of 0.1 V/s. The response to β-D-glucose and inhibition by Cr (VI) was analyzed using chronoamperometry at 0.68 V for 100 s. A sample volume of 100 µL containing 5 mM β-D-glucose and a known concentration of Cr(VI) was spotted onto the GOx-paper-SPCE for chronoamperometric analysis. The β-D-glucose concentration was kept constant and the current response to the increasing Cr(VI) concentrations was recorded. The experiments were done in triplicates.

Results and discussion

GOx inhibition study

For inhibition-based biosensor, the study of inhibition kinetics is needed which helps in getting ideas about the susceptibility of the enzyme for inhibition by the inhibitor. In enzyme activity assay, GOx and HRP are used in a coupled reaction where GOx oxidizes β-D-glucose to give hydrogen peroxide which in turn oxidizes the chromogen in presence of HRP to give a colour that can be detected spectrophotometric method. Use of spectrophotometric method

for inhibition study cannot be reliable as two enzymes are involved at the same time and we cannot figure out the actual GOx inhibition kinetics because HRP may also get inhibited by Cr(VI) to a certain extent. Another method is using potassium iodide and starch to give purple color in the presence of hydrogen peroxide produce by catalytic oxidation of β-D-glucose (Nery and Kubota 2016). Since Cr(VI) is an oxidizing agent it reacts and helps in colour formation in the absence of hydrogen peroxide owing to which this method cannot be used for GOx inhibition study.

Chronoamperometry is an electrochemical method in which β-D-glucose oxidation can be measured by measuring the change in current with respect to time at a fixed voltage on the addition of the enzyme. According to the literature chronoamperometry for GOx is performed at 0.7 V which is selected as a fixed voltage for GOx inhibition study (Zeng et al. 2004; Fenton et al. 2009). A decrease in current was recorded with increasing Cr(VI) concentration from 0.5–4 ppm at 1 mM and 5 mM β-D-glucose concentrations. The plot of inverse of current versus Cr(VI) concentration shows a parallel pattern suggesting uncompetitive inhibition (Fig. 1a). Furthermore, Dixon's plot does not confirm the type of inhibition especially in case of mixed inhibition (Attar et al. 2014). To confirm the inhibition type Cornish-Bowden plot is plotted as a ratio of substrate concentration to current versus inhibitor concentration (Cornish-Bowden 1974). The Cornish-Bowden plot shows the intersection of the lines above the inhibitor line confirms the uncompetitive inhibition (Fig. 1a, b). The straight vertical line from the intersection on the inhibitor line gives a value of binding constant K_i as 2.88 ppm (Fig. 1b) which on molar conversion gives 55.4 µM. This K_i value in a lower micromolar range is

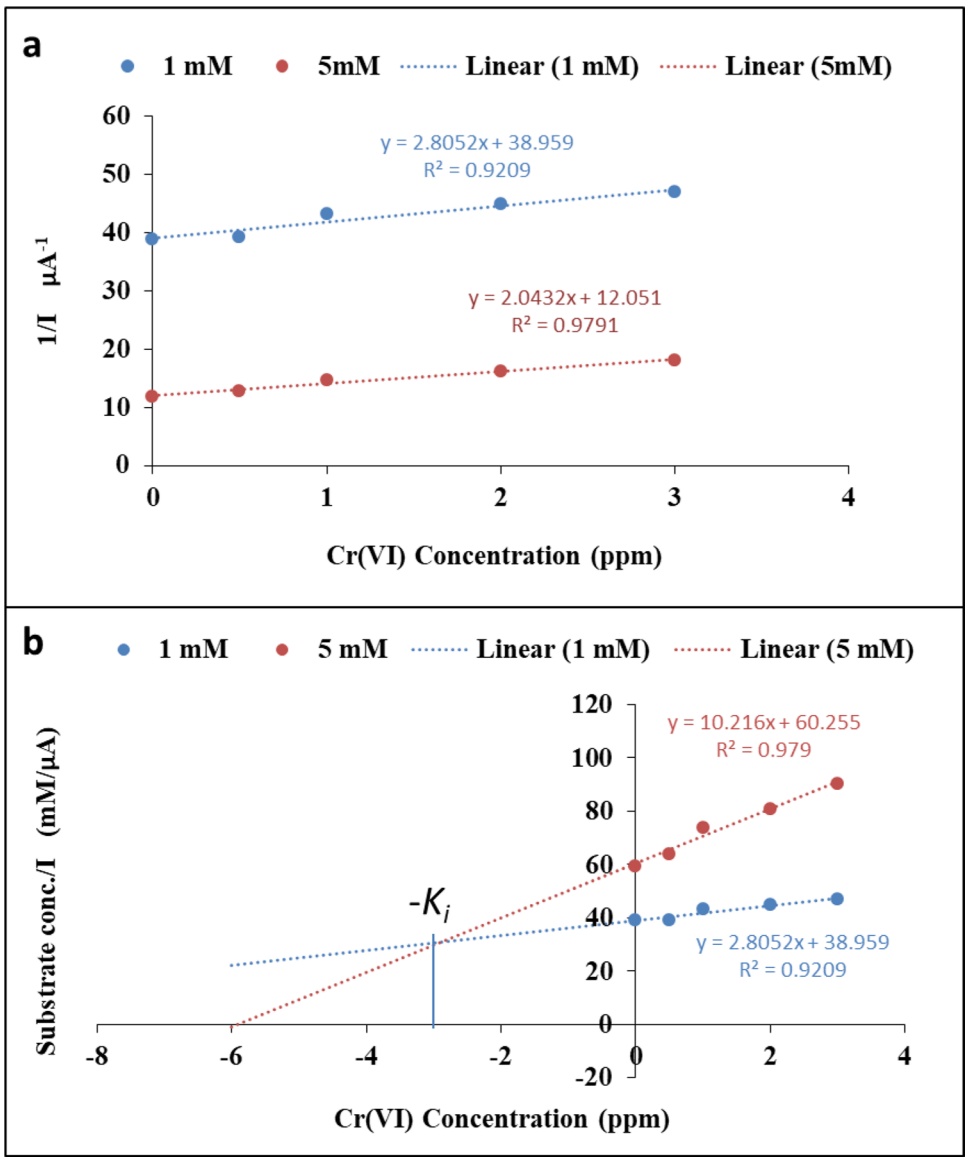

Fig. 1 GOx inhibition type analysis; **a** Dixon plot, **b** Cornish-Bowden plot confirms uncompetitive inhibition

acceptable for considering Cr(VI) as a good inhibitor. The binding constant K_i reflects the binding affinity of the inhibitor towards the enyme. Lesser the K_i value more is the binding potency of the inhibitor henceforth from the obtained results Cr (VI) proved as a potential inhibitor of GOx enyme.

Enzyme immobilization study

Polysaccharides like chitosan, dextran and alginate are natural biodegradable and biocompatible polymers that are usually used as a carrier matrices for enzyme immobilization (Bezerra et al. 2015). Glucose oxidase (GOx) was immobilized on Whatman#1 filter paper by simple polymeric entrapment methods using chitosan, sodium alginate, and dextran. For determining the immobilization efficiency, the paper squares with immobilized enzyme were washed with PB containing 0.1% Tween 20, pH 7. Tween 20 removed nonspecific bindings which eventually leach out unbound enzyme in wash buffer. The washed-out protein (GOx) content was measured by Bradford protein assay (Bradford 1976). The obtained protein content was subtracted from initial protein content giving the actual enzyme immobilized on paper (Eq. 2). A standard curve was prepared for Bradford assay using GOx concentrations from 10–100 µg/mL in PB pH 7 (Fig. 2a). In the case of chitosan immobilization with glutaraldehyde crosslinking, the immobilization efficiency is highest (near 100%) at 0.5% chitosan concentrations (Fig. 2b) as the protein content was found very less in the washed-out buffer. However, maximum colour intensity was observed at 0.3% chitosan concentration with 90% enyme immobiliation efficiency where the colour intensity is getting lower above 0.3% chitosan concentration (Fig. 3a, b). The possible reasons can be given as: (1) High glutaraldehyde concentration can cause enzyme inactivation by binding to its catalytic site. (2) Higher chitosan concentration may limit the enzyme exposure to the substrate affecting the substrate diffusivity.

Chitosan possesses free amino groups that bind to the aldehyde group of glutaraldehyde (Lei et al. 2003). Glutaraldehyde has two –CHO groups that can form crosslinking between chitosan molecules forming a mesh that entraps enzyme in it on crosslinking (Krajewska 2004; Beppu et al. 2007). Also, glutaraldehyde can bind to the amino group of lysine residues in protein crosslinking the protein to chitosan strengthening the immobilization (Kildeeva et al. 2009). Lysine residues present most outside the catalytic site which is beneficial for glutaraldehyde crosslinking however higher concentration of glutaraldehyde can react with enzyme active site decreasing the enzyme-specific activity (López-Gallego et al. 2013). From Figs. 2b and 3b, sodium alginate at 0.3%, and 0.4% concentrations show nearly the same colour intensities but highest immobilization efficiency at 0.5% concentration indicating the negative effect of higher concentration on the enzyme. Dextran shows almost same colour intensity at 0.3%, 0.4% concentrations and highest at 0.5% immobilization efficiency (Figs. 2b, 3b). Figure 3c shows colorimetric intensity comparisons of different polymeric entrapment methods with 0.3% polysaccharide concentration. Chitosan entrapment method was found as efficient with respect to enzyme immobilization efficiency as well as its activity.

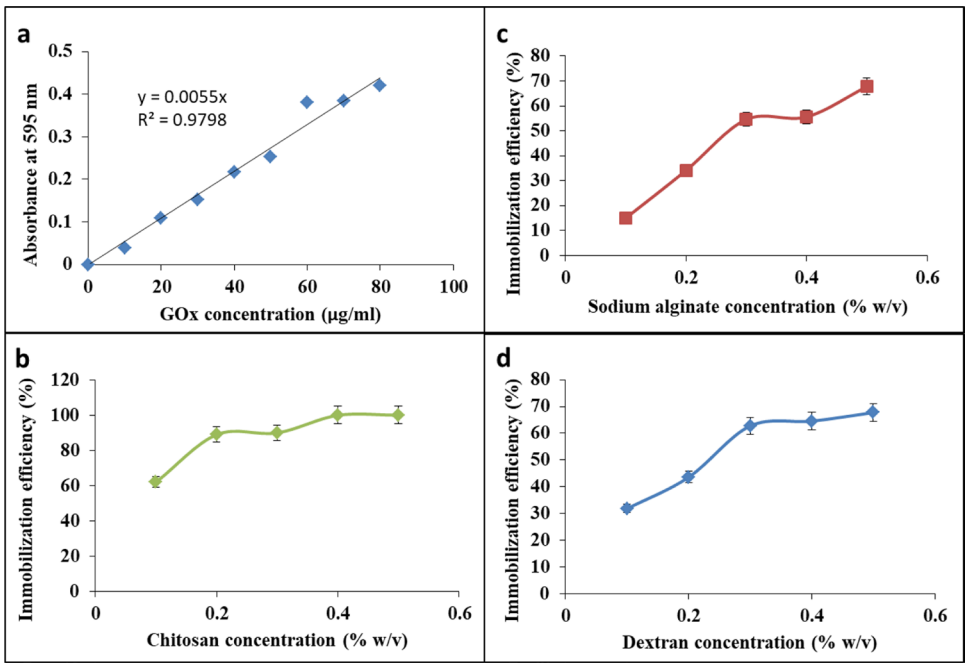

Fig. 2 **a** Standard curve for Bradford Test; enzyme immobilization efficiency using **b** chitosan, **c** sodium alginate, and **d** dextran

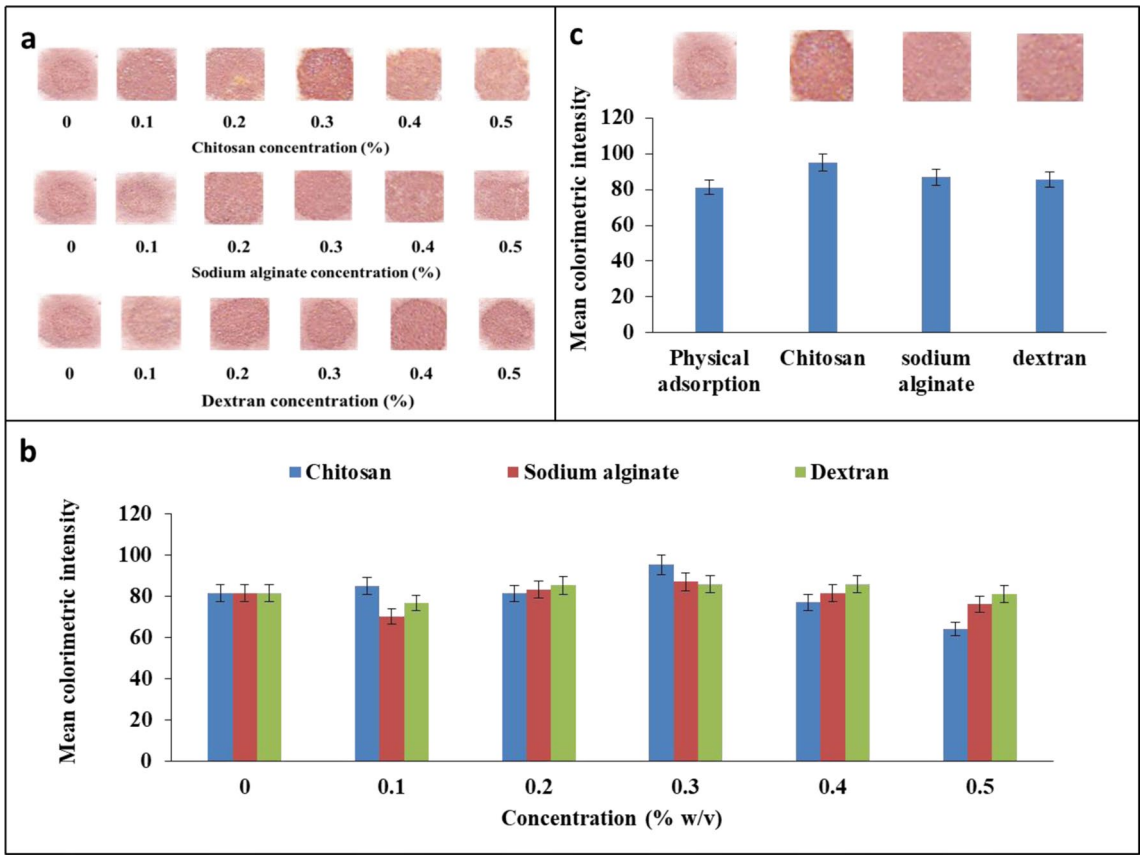

Fig. 3 **a** Colour developed on the addition of glucose + HRP + o-Dianisidine mix on the paper squares with immobilized GOx in different polymers; **b** colorimetric intensity comparison of images in (**a**); **c** colorimetric intensity comparison of 0.3% polymers with physical adsorption (0% polymer)

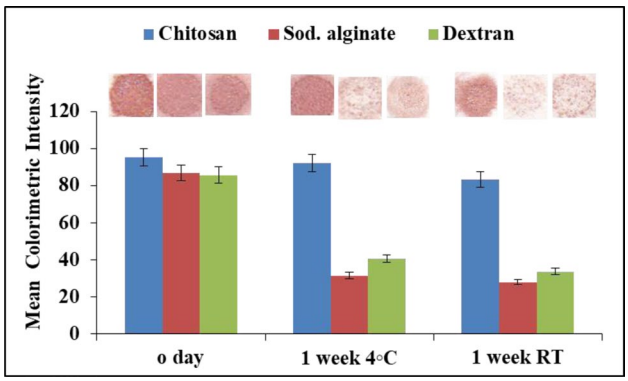

Fig. 4 Effect of time and temperature on enyme activity of GOx immobilied using chitosan, sodium alginate and dextran on paper

The stability of the chitosan entrapped enzyme was also found better with 97% of the initial enyme activity after 1 week at 4 °C (Fig. 4). Also, the enyme was slightly reduced at room temperature over a perod of a week sustaining 87% of the intial enyme activity. Compared to this, in case of other two polysaccharides more than 50% of the enyme activity was found to be decreased in a week even at 4 °C. A cross-linked polysaccharide like chitosan isolates enzyme from the surrounding and creates a microenvironment that helps in maintaining the conformation by proteting the active sites (Shuler and Kargi 2002). This may help in maintaining enyme catalytic activity over a period of time. Libertino et al. (2008) immobilized GOx in porous SiO_2 surface that showed stable activity even after 3 months when stored in phosphate buffer saline (PBS) at 4 °C. Enyme immobilization on porous silicon (PS) is well established method with numerous advantages like good stability over a wide range of pH and temperature, improved activity over the free form of enzyme, high recyclabilty, photoluminascent property with less interference by various factors (Letant et al. 2004; Lasmi et al. 2018; Rodrigues et al. 2019; Moretta et al. 2021). PS constitutes silicon nanocrystals which offer high surface area for enzyme immobilization (Song and Sailor 1999). Because of its semiconductor qualities as well as its compatibility with modern integrated circuit industry, PS proves a good support for enzyme immobilization (Moretta et al. 2021). Changes in the quantum confinement occur due to the formation of porous layer on silicon surface which is

a remarkable property for use in enzyme-based biosensing applications (Dhanekar and Jain 2013). The pore size can be tuned easily using different established etching methods such as strain, plasma dry and electrochemical etching (Fopase et al. 2020). However, these methods are complicated, time consuming and requires acid neutralization in case of electrochemical etching in the presence of hydrofluoric acid (Fopase et al. 2019). Also, enzyme immobilization on PS support necessarily requires linker molecules like (3-aminopropyl)triethoxysilane (APTES) and glutaraldehyde (Gunda et al. 2014; Fopase et al. 2019). Althogh PS offers many advantages over the chitosan entrapment, the immobilization method opted here is quite simple, quick, requires no linkers and cost effective compared to the PS fabrication and its use.

Development of paper-based biosensor strip

The electrochemical detector used here is a SPCE, which is a miniature version of the conventional three electrode system. It is associated with a GOx entrapped Whatman filter paper by a simply placing in inverted manner onto the electrodes. Similar kind of work was done by Noiphung et al. (2013), where the author demonstrated that the paper-based microfluidic glucose biosensor by placing SPCE beneath the GOx immobilized filter paper. Such carbon electrodes can be directly printed on the filter paper along with hydrophobic and hydrophilic patterns (Yang et al. 2017). Screen printing, inkjet printing, wax printing, photolithography, Polydimethylsiloxane (PDMS) printing are some established methods commonly used for electrode printing and pattern development (Zang et al. 2006; Liu et al. 2016; Cinti et al. 2017; Amatatongchai et al. 2019). Hughes et al. (2018) demonstrated screen printing of glucose sensitive electrode using GOx mixed carbon ink. Dungchai et al. (2009) used photolithography for printing electrodes and creating hydrophilic pattern on the paper with immobilized enzymes. The hydrophobic and hydrophilic patterns developed on the hydrophilic surface like paper serve as channels to control and navigate the fluid flow to the reaction zone or electrode surface.

In the current study, the developed biosensing system uses a GOx immobilized paper with the area of 1 cm^2 simply covers all the three screen-printed electrodes (working, reference and counter). A drop of water sample is sufficient to carry out the electrochemical analysis as the paper has a property to hold the water. The functionality of the electrode system was analysed using cyclic voltammetry analysis which shows oxidation and reduction occuring at the electode surface (Fig. 5a). This simple one step fabrication method eliminates the need of channeling and hydrophilic patterning. However electrodes printing on paper will significantly decrease the fabrication cost as paper is a very cheap support material and also it is suitable for use and through application.

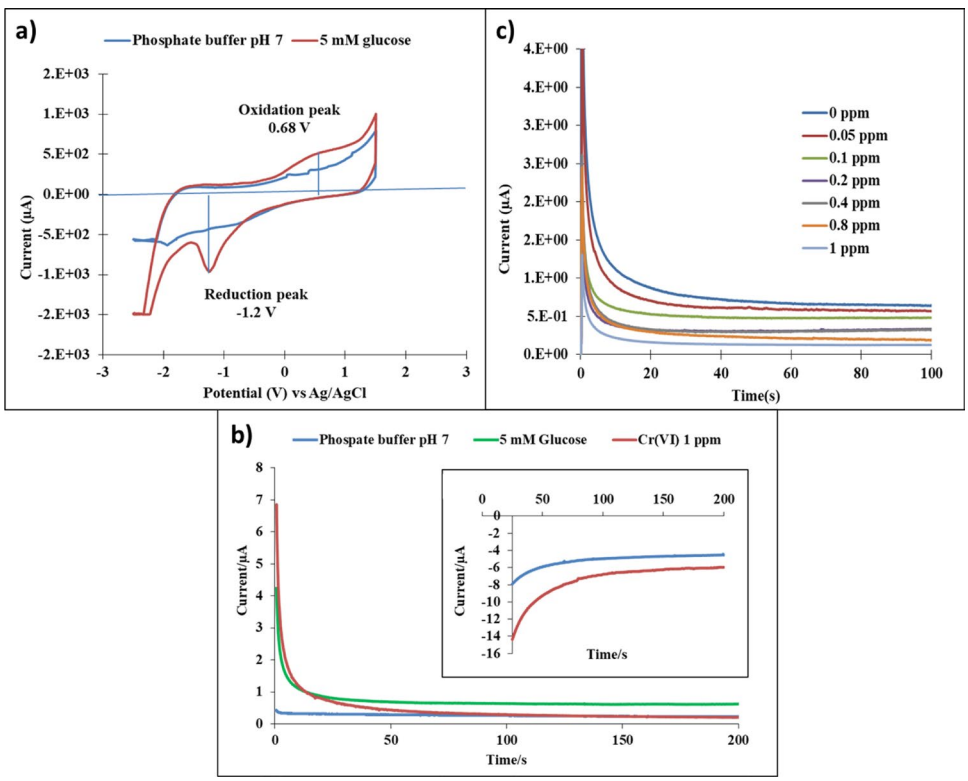

Fig. 5 **a** Cyclic voltametric analysis of screen-printed electrode with paper immobilized glucose oxidase; **b** chronoamperometry at 0.68 V and at − 1.2 V and **c** chronoamperometry using glucose oxidase-paper-screen-printed electrode at increasing Cr(VI) concentrations

Electrochemical characterization

Enzyme immobilized paper square was placed on the sensing area of the screen-printed electrode and the working of the electrode was characterized by cyclic voltammetry by spotting 100 µL of 5 mM β-D-glucose solution in phosphate buffer pH 7. From Fig. 5a, the oxidation and reduction peak was obtained at 0.68 V and − 1.2 V, respectively. During the GOx-mediated β-D-glucose oxidation reaction in the presence of oxygen, the oxidation peak was formed as shown in Eqs. (3) and (4).

$$\beta\text{-D-glucose} + O_2 \xrightarrow{GOx} \text{Gluconolactone} + H_2O_2 \xrightarrow{H_2O} \text{Gluconic}, \quad (3)$$

$$H_2O_2 \xrightarrow{\text{Electrode}} 2H^+ + O_2 + 2e^-.$$

The chronoamperometry was performed at − 1.2 V and 0.68 V to analyze the current response to Cr(VI) in the absence of β-D-glucose. Although the oxidation curve is not sharp, at 0.68 V the current response to 1 ppm Cr (VI) is almost equal to that of phosphate buffer (PB) which shows no current change on Cr(VI) addition (Fig. 5a, b). Conversely at -1.2 V on the addition of Cr(VI), the current rises above the current response of PB which can interfere with the current response of β-D-glucose oxidation by GOx (Fig. 5b). The biosensor response should be selective to β-D-glucose oxidation as inhibition of GOx by Cr(VI) lowers the current response due to a reduction in the moles of β-D-glucose oxidized. Thus chronoamperometry was performed at positive voltage 0.68 V for the generation of calibration curve shown in Fig. 5c. For glucose biosensing, H_2O_2 detection occurs at around + 0.6 V for the conventional electrode versus Ag/AgCl reference electrode (Karyakin et al. 1995). Sekar et al. (2014) used Prussian Blue as a mediator lowering the operating potential to − 0.3 V for GOx paper disc-based SPCE glucose biosensor. This biosensor was developed to detect glucose in blood samples, with increased selectivity towards H_2O_2 as at higher positive operating potential the oxidizing compounds like ascorbic acid present in the blood cause interference. Lowering of the operating potential does not require necessarily for the analysis in water samples. In the present work, the biosensor showed a considerable sensitivity of 143.83 µA/mM at + 0.68 V without the use of any mediator. Zeng et al. (2004) reported anodic peak and highest sensitivity at around + 0.7 V for GOx immobilized platinum electrode. Ayenimo and Adeloju (2016) also reported the operating potential of + 0.7 V for GOx entrapped in polypyrrol modified platinum electrode at pH 7.

Calibration curve for biosensor

The chronoamperometric study was done for analyzing the current change with respect to time at variable analyte concentrations. The chronoamperometry was performed using phosphate buffer pH 7 since most of the GOx-based biosensor development studies have been done at neutral pH pH (Ghica et al. 2013; Ayenimo and Adeloju 2016; da Silva et al. 2020). Also, the pH of most of the conventional water resources is in the neutral range. Calculation of relative standard deviation (RSD) is important to find out the percentage of deviation relative to other replicates. The RSD calculated from three replicates is 5.6% at a fixed β-D-glucose concentration of 5 mM with a standard deviation of 0.02–0.06 which indicates a good reproducibility. Calvo-Perez et al. (2014) also obtained RSD value of 5.5% from five replicates of GOx-based SPCE. Variations among the replicates occur as manual dissension of enzyme might give handling error. I_{50} value was calculated as 0.5 ppm from the equation obtained by plotting the degree of inhibition calculated as per Eq. (1) versus Cr(VI) concentration (Fig. 6a). The I_{50} value of indicates the functionality of the inhibitor more specifically than its binding affinity to the enyme. It shows 50% of enyme inhibition occurs at this Cr(VI) concentration. A regression line for current change with respect to Cr(VI) concentration is illustrated in Fig. 6b with the following Eq. (5), which shows the current change (ΔI) is directly proportional to the Cr(VI) concentration (C).

$$\Delta I = 0.3573C + 0.1066 \quad (5)$$

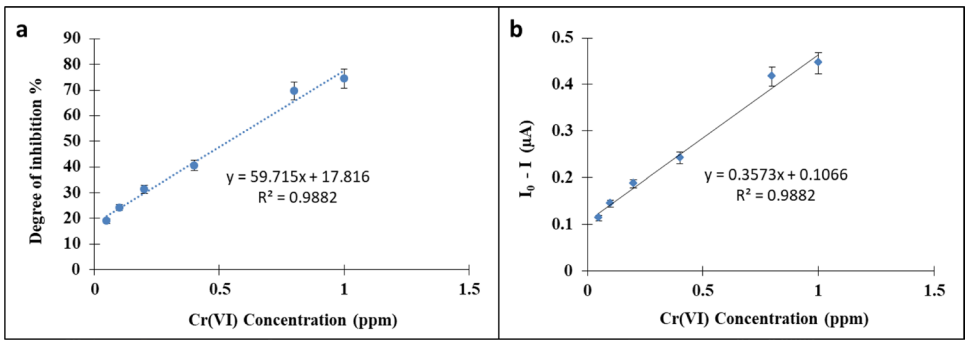

Fig. 6 Regression line for **a** degree of inhibition and **b** current change with respect to Cr(VI) concentration

The limit of detection as well as the limit of quantitation was achieved as 0.05 ppm (Fig. 6b). The linear range obtained is 0.05–1 ppm with a regression factor of 0.988. Zeng et al. (2004) demonstrated the development of amperometric biosensor with GOx deposited in electropolymerized polyaniline membrane on the platinum electrode for determination of trace Cr (VI) in water. The LOD obtained as 0.49 µg/L which is significantly low quantity. Although it provides low LOD the limitation of such type of biosensors is a reuse of the enzyme, which can lose its catalytic activity over time due to repetitive use. Also, the biosensor works at acidic pH 2 which may prove limitation for onsite detection as at neutral pH it limits the flow of electrons to the sensing electrode. Liu et al. (2019) presented the fabrication of GOx and HRP mix-based amperometric biosensor for simultaneous detection of Cr(VI) and Cd(II). The authors reported LOD of 0.2 nM in the linear range of 0.5–6 nM for Cr(VI). The lower LOD and higher sensitivity might be due to the use of HRP which acts as a mediator for electron transfer to the platinum electrode. According to Attar et al. (2014), HRP is also getting inhibited by Cr(VI) with a K_i value of 0.78 µM. Considering this, the work presented by Liu et al. (2019) does not explain the effect of HRP inhibition factor as GOx and HRP are present in a mixed form which makes it complicated, thus questioning the reliability of the biosensor. In the present work, a mediator free SPCE-based disposable biosensor strip was developed using a single enzyme GOx. This can minimize the fabrication cost as well as the error caused due to inactivation of the immobilized enzyme in repetitive use. Also, the platinum electrode is quite costly as compared to the screen-printed electrodes; the screen-printed electrodes can be fabricated by printing electrodes on paper using carbon conductive ink that can reduce the cost, as the paper is a cheap and easily available material (Liana et al. 2012).

Interference by other heavy metals

Aqueous medium contains a wide number of heavy metal ions other than Cr(VI) which can interfere with the biosensor functionality. Possible heavy metal ions like Cr(III), Cu(I), Ni(II), Pb(II) show interference whereas Cd(II) and Hg(II) show higher interference at lower concentrations (Table 1). The degree of inhibition was calculated at 0.5 ppm concentration of the above heavy metals and compared to the Cr(VI) concentration giving the same degree of inhibition. Higher inhibition was recorded due to the presence of some common metal ions in the order Pb(II) < Cd(II) < Hg(II). Heavy metals like Cr(VI), Pb(II), Cd(II), and Hg(II) are called biotoxic metal ions and are collectively detected by GOx inhibition-based electrochemical biosensor (da Silva et al. 2020). Comparing to the literature demonstrating GOx-based biosensor Hg(II) and Ag(I) shows inhibition at lower concentrations (Zeng et al. 2004). Furthermore, as demonstrated by Ghica et al. (2013), Cd(II), Co(II), and Ni(II) show inhibition to GOx-based biosensor at I_{10} value of 2.4, 2.1, and 3.3 µM, respectively. Nayak et al. (2020b) also reported the influence of other heavy metals by HRP for the Cr(VI) detection in water samples. da Silva et al. (2020) also reported the influence of heavy metals like Ag(I), K(I), Na(I), Ni(II), Zn(II), Cu(II), CO(II) and Fe(III) on GOx-based amperometric biosensor as low as 10% at high concentration.

Conclusion

Paper platforms-based biosensor is an emerging approach of biosensor development that helps in easy, rapid, and reliable onsite detection by utilizing paper as a cheap and inert material for the efficient immobilization of biorecognition agents. This study demonstrated the simple polymer entrapment method using 0.3% (w/v) chitosan as an efficient method (about 90%) for glucose oxidase (GOx) immobilization on paper, which aids in enzyme stability for 1 week at 4 °C and room temperature, prevents enzyme leaching by retaining 97% stability at 4 °C. Cr(VI) shows uncompetitive mode of inhibition with *Ki* value of 2.88 ppm (55.4 µM) which states that the enzyme can be implemented for the construction of inhibition-based biosensors. The developed biosensor exhibited the linear range detection in 0.05–1 ppm with the limit of detection of 0.05 ppm for Cr(VI), which is

Table 1 Effect of heavy metal ions other than Cr(VI) on biosensor functionality

Heavy metal ion	Concentration (ppm)	Degree of inhibition (%)	Cr(VI) concentration giving same degree of inhibition (ppm)
Cr(III)	0.5	29.41	0.13
Cu(II)	0.5	30.38	0.15
Ni(II)	0.5	32.35	0.18
Pb(II)	0.5	34.92	0.23
Cd(II)	0.5	49.47	0.49
Hg(II)	0.5	74.39	0.92

the standard permissible limit in potable water. Heavy metal ions like Hg(II), Cd(II), and Pb(II) can be proved as potential interference to the biosensor functioning, which could be overcomed by chelating reactions. Relative standard deviation (5.6%) indicates good reproducibility of the fabricated biosensor with maximum 0.06 of standard deviation. Furthermore, validation of the technique using real-time samples is required to be performed for analyzing variation in comparison with the conventional method.

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Author contributions AD: conception and design, data curation, writing- original draft preparation, final approval of the article; SJ: supervision, methodology, writing- reviewing and editing; BP: conceptualization, supervision, writing- reviewing and editing.

Declarations

Conflict of interest The authors declare no conflict of interest to disclose.

References

Amatatongchai M, Sitanurak J, Sroysee W, Sodanat S, Chairam S, Jarujamrus P, Nakapricha D, Lieberzeit PA (2019) Highly sensitive and selective electrochemical paper-based device using a graphite screen-printed electrode modified with molecularly imprinted polymers coated Fe3O4@ Au@ SiO_2 for serotonin determination. Anal Chim Acta 1077:255–265. https://doi.org/10.1016/j.aca.2019.05.047

Amine A, Arduini F, Moscone D, Palleschi G (2016) Recent advances in biosensors based on enzyme inhibition. Biosens Bioelectron 76:180–194. https://doi.org/10.1016/j.bios.2015.07.010

Attar A, Ghicaa ME, Amine A, Brett CM (2014) Poly (neutral red) based hydrogen peroxide biosensor for chromium determination by inhibition measurements. J Hazard Mater 279:348–355. https://doi.org/10.1016/j.jhazmat.2014.07.019

Ayenimo JG, Adeloju SB (2016) Rapid amperometric detection of trace metals by inhibition of an ultrathin polypyrrole-based glucose biosensor. Talanta 148:502–510. https://doi.org/10.1016/j.talanta.2015.11.024

Banchhor A, Pandey M, Pandey PK (2017) A review of hexavalent chromium contamination in India. Res J Chem Sci 7:39–44

Bankar SB, Bule MV, Singhal RS, Ananthanarayan L (2009) Glucose oxidase-an overview. Biotechnol Adv 27:489–501. https://doi.org/10.1016/j.biotechadv.2009.04.003

Bansod B, Kumar T, Thaur R, Rana S, Sing I (2017) A review on various electrochemical techniques for heavy metal ions detection with different sensing platforms. Biosens Bioelectron 94:443–455. https://doi.org/10.1016/j.bios.2017.03.031

Beppu MM, Vieira RS, Aimoli CG, Santana CC (2007) Crosslinking of chitosan membranes using glutaraldehyde: Effect on ion permeability and water absorption. J Membr Sci 301:126–130. https://doi.org/10.1016/j.memsci.2007.06.015

Bezerra CS, de Farias Lemos CMG, de Sousa M, Gonçalves LRB (2015) Enzyme immobilization onto renewable polymeric matrixes: past, present, and future trends. J Appl Polym Sci. https://doi.org/10.1002/app.42125

Biswas P, Karn AK, Balasubramanian P, Kale PG (2017) Biosensors for detection of dissolved chromium in potable water: a review. Biosens Bioelectron 94:589–604. https://doi.org/10.1016/j.bios.2017.03.043

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Calvo-Perez A, Domínguez-Renedo O, Alonso-Lomillo M, Arcos-Martínez M (2014) Speciation of chromium using chronoamperometric biosensors based on screen-printed electrodes. Anal Chim Acta 833:15–21. https://doi.org/10.1016/j.aca.2014.05.006

Cao R, Guan L, Li M, Tian J, Shen W (2015) A zero-step functionalization on paper-based biosensing platform for covalent biomolecule immobilization. Sens Biosens Res 6:13–18. https://doi.org/10.1016/j.sbsr.2015.09.002

Chen C, Xie Q, Wang L, Qin C, Xie F, Yao S, Chen J (2011) Experimental platform to study heavy metal ion-enzyme interactions and amperometric inhibitive assay of Ag^+ based on solution state and immobilized glucose oxidase. Anal Chem 83:2660–2666. https://doi.org/10.1021/ac1031435

Cinti S, Mazzaracchio V, Cacciotti I, Moscone D, Arduini F (2017) Carbon black-modified electrodes screen-printed onto paper towel, waxed paper and parafilm M®. Sensors 17:2267. https://doi.org/10.3390/s17102267

Cornish-Bowden A (1974) A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. Biochem J 137:143. https://doi.org/10.1042/bj1370143

da Silva W, Ghica ME, Brett CM (2020) Biotoxic trace metal ion detection by enzymatic inhibition of a glucose biosensor based on a poly (brilliant green)-deep eutectic solvent/carbon nanotube modified electrode. Talanta 208:120427. https://doi.org/10.1016/j.talanta.2019.120427

DesMarais TL, Costa M (2019) Mechanism of chromium induced toxicity. Curr Opin Toxicol 14:1–7. https://doi.org/10.1016/j.cotox.2019.05.003

Dhanekar S, Jain S (2013) Porous silicon biosensor: current status. Biosens Bioelectron 41:54–64. https://doi.org/10.1016/j.bios.2012.09.045

Dungchai W, Chailapakul O, Henry CS (2009) Electrochemical detection for paper-based microfluidics. Anal Chem 81:5821–5826. https://doi.org/10.1021/ac9007573

Dungchai W, Chailapakul O, Henry CS (2011) A low-cost, simple, and rapid fabrication method for paper-based microfluidics using wax screen-printing. Analyst 136:77–82. https://doi.org/10.1039/C0AN00406E

Fenton K, Apblett C, Atanassov P (2008) Rapid electrochemical assay of glucose oxidase activity within electrophoresis gels. In: ECS meeting abstracts 7:202–202. https://iopscience.iop.org/article/https://doi.org/10.1149/MA2009-01/41/1425/meta

Fopase R, Nayak S, Mohanta M, Kale P, Balasubranian P (2019) Inhibition assays of free and immobilized urease for detecting hexavalent chromium in water samples. 3 Biotech 9:1–12. https://doi.org/10.1007/s13205-019-1661-4

Fopase R, Paramasivam S, Kale P, Balasubramanian P (2020) Strategies, challenges and opportunities of enzyme immobilization on porous silicon for biosensing applications. J Environ Chem Eng. https://doi.org/10.1016/j.jece.2020.104266

Ghica EM, Carvalho RC, Amine A, Brett CM (2013) Glucose oxidase enzyme inhibition sensor for heavy metals at carbon film electrodes

modified with cobalt or copper hexacyanoferrate. Sens Actuators B 178:270–278. https://doi.org/10.1016/j.snb.2012.12.113
Gunda NSK, Singh M, Norman L, Kaur K, Mitra SK (2014) Optimization and characterization of biomolecule immobilization on silicon substrates using (3-aminopropyl) triethoxysilane (APTES) and glutaraldehyde linker. Appl Surf Sci 305:522–530. https://doi.org/10.1016/j.apsusc.2014.03.130
Hughes G, Pemberton RM, Nicholas P, Hart JP (2018) Fabrication of miniaturised screen-printed glucose biosensors, using a water-based ink, and the evaluation of their electrochemical behaviour. Electroanalysis 30:1616–1620. https://doi.org/10.1002/elan.201800104
Juang YJ, Li WS, Chen PS (2017) Fabrication of microfluidic paper-based analytical devices by filtration-assisted screen printing. J Taiwan Inst Chem Eng 80:71–75. https://doi.org/10.1016/j.jtice.2017.08.007
Karyakin AA, Gitelmatcher OV, Karyakina EE (1995) Prussian blue-based first generation biosensor: a sensitive amperometric electrode for glucose. Anal Chem 67:2419–2423. https://doi.org/10.1021/ac00110a016
Kildeeva NR, Perminov PA, Vladimirov LV, Novikov VV, Mikhailov SN (2009) About mechanism of chitosan cross-linking with glutaraldehyde. Russ J Bioorg Chem 35:360–369. https://doi.org/10.1134/S106816200903011X
Krajewska B (2004) Application of chitin-and chitosan-based materials for enzyme immobilizations: a review. Enzyme Microb Technol 35:126–139. https://doi.org/10.1016/j.enzmictec.2003.12.013
Lace A, Ryan D, Bowkett MM, Cleary J (2019) Chromium monitoring in water by colorimetry using optimied 1,5-diphenycarbazide method. Int J Environ Res Public Health 16:1803. https://doi.org/10.3390/ijerph16101803
Lasmi K, Derder H, Kermad A, Sam S, Boukhalfa-Abib H, Belhousse S, Tighilt FZ, Hamdani K, Gabouze N (2018) Tyrosinase immobilization on functionalized porous silicon surface for optical monitoring of pyrocatechol. Appl Surf Sci 446:3–9. https://doi.org/10.1016/j.apsusc.2018.03.037
Lei CX, Hu SQ, Shen GL, Yu RQ (2003) Immobilization of horseradish peroxidase to a nano-Au monolayer modified chitosan-entrapped carbon paste electrode for the detection of hydrogen peroxide. Talanta 59:981–988. https://doi.org/10.1016/S0039-9140(02)00641-0
Letant SE, Hart BR, Kane SR, Hadi MZ, Shields SJ, Reynolds JG (2004) Enzyme immobilization on porous silicon surfaces. Adv Mater 16:689–693
Liana DD, Raguse B, Gooding JJ, Chow E (2012) Recent advances in paper-based sensors. Sensors 12:11505–11526. https://doi.org/10.3390/s120911505
Libertino S, Aiello V, Scandurra A, Renis M, Sinatra F (2008) Immobilization of the enzyme glucose oxidase on both bulk and porous SiO_2 surfaces. Sensors 8:5637–5648. https://doi.org/10.3390/s8095637
Liu S, Su W, Ding X (2016) A review on microfluidic paper-based analytical devices for glucose detection. Sensors 16:2086. https://doi.org/10.3390/s16122086
Liu L, Chen C, Chen C, Kang X, Zhang H, Tao Y, Xie Q, Yao S (2019) Poly (noradrenalin) based bi-enzyme biosensor for ultrasensitive multianalyte determination. Talanta 194:343–349. https://doi.org/10.1016/j.talanta.2018.10.055
Lopez-Gallego F, Guisán JM, Betancor L (2013) Glutaraldehyde-mediated protein immobilization. Immobilization of enzymes and cells. Springer, Totowa, pp 33–41. https://doi.org/10.1007/978-1-62703-550-7_3
Moretta R, De Stefano L, Terracciano M, Rea I (2021) Porous silicon optical devices: recent advances in biosensing applications. Sensors 21:1336. https://doi.org/10.3390/s21041336
Nayak S, Rangabhashiyam S, Balasubramanian P, Kale P (2020a) A review of chromite mining in Sukinda Valley of India: impact and potential remediation measures. Int J Phytorem. https://doi.org/10.1080/15226514.2020.1717432
Nayak S, Kale P, Balasubramanian P (2020b) Inhibition assays of horseradish peroxidase by hexavalent chromium and other heavy metals. Int J Environ Anal Chem. https://doi.org/10.1080/03067319.2020.1776864
Nery EW, Kubota LT (2016) Evaluation of enzyme immobilization methods for paper-based devices-a glucose oxidase study. J Pharm Biomed Anal 117:551–559. https://doi.org/10.1016/j.jpba.2015.08.041
Noiphung J, Songjaroen T, Dungchai W, Henry CS, Chailapakul O, Laiwattanapaisal W (2013) Electrochemical detection of glucose from whole blood using paper-based microfluidic devices. Anal Chim Acta 788:39–45. https://doi.org/10.1016/j.aca.2013.06.021
Osorio-Gonzalez CS, Hegde K, Brar SK, Delgado-Cano B, Gomez-Falcon N, Avalos-Ramirez A (2019) Advancesin protein/enzyme-based biosensors for detection of metal contaminants in the environment. Tools, techniques and protocols for monitoring environmental contaminants. Elsevier, Amsterdam, pp 245–261. https://doi.org/10.1016/B978-0-12-814679-8.00012-1
Prabhakaran DC, Ramamurthy PC, Sivry Y, Subramian S (2020) Electrochemical detection of Cr(VI) and Cr(III) ions present in aqueous solutions using bio-modified carbon paste electrode: a voltammetric study. Int J Environ Anal Chem. https://doi.org/10.1016/j.jpba.2015.08.041
Rodrigues RC, Virgen-Ortiz JJ, Dos Santos JC, Berenguer-Murcia A, Alcantara AR, Barbosa O, Ortiz C, Fernandez-Lafuente R (2019) Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions. Biotechnol Adv 37:746–770. https://doi.org/10.1016/j.biotechadv.2019.04.003
Sekar NC, Shaegh SAM, Ng SH, Ge L, Tan SN (2014) A paper-based amperometric glucose biosensor developed with Prussian Blue-modified screen-printed electrodes. Sens Actuators B Chem 204:414–420. https://doi.org/10.1016/j.snb.2014.07.103
Shuler ML, Kargi F (2002) Bioprocess Engineering: Basic Concepts, 2nd edn. Prentice-Hall of India pvt ltd, New Delhi, pp 79–91
Song JH, Sailor MJ (1999) Chemical modification of crystalline porous silicon surfaces. Comments Inorg Chem 21:69–84. https://doi.org/10.1080/02603599908020416
WHO (1998) Guidelines for drinking-water quality. Heal Criteria Other Support Inf 2(1930128):1–283
Wilbur SB, Abdin H, Fay M, Yu D, Tencza B, Ingerman L,Klotzbach J, James S (2012) Toxicological profile for chromium. US Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry. https://pubmed.ncbi.nlm.nih.gov/24049864
Wilson R, Turner APF (1992) Glucose oxidase: an ideal enzyme. Biosens Bioelectr 7:165–185. https://doi.org/10.1016/0956-5663(92)87013-F
Yang Y, Noviana E, Nguyen MP, Geiss BJ, Dandy DS, Henry CS (2017) Based microfluidic devices: emerging themes and applications. Anal Chem 89:71–91. https://doi.org/10.1021/acs.analchem.6b04581
Yang Y, Fang Z, Yu YY, Wang YZ, Naraginti S, Yong YC (2019) A mediator-free whole-cell electrochemical biosensing system for sensitive assessment of heavy metal toxicity in water. Water Sci Technol 79:1071–1080. https://doi.org/10.2166/wst.2019.101
Zeng GM, Tang L, Shen GL, Huang GH, Niu CG (2004) Determination of trace chromium (VI) by an inhibition-based enzyme biosensor incorporating an electropolymerized aniline membrane and ferrocene as electron transfer mediator. Int J Environ Anal Chem 84:761–774. https://doi.org/10.1080/03067310410001730619
Zhang Q, Xu JJ, Chen HY (2006) Glucose microfluidic biosensors based on immobilizing glucose oxidase in poly (dimethylsiloxane) electrophoretic microchips. J Chromatogr A 1135:122–126. https://doi.org/10.1016/j.chroma.2006.09.052