

Toward Sustainable Biosensing: Green-Synthesized Gold Nanoparticles from *Ocimum Americanum* Enhance the Performance of NcCDH-Based Lactose Biosensor

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Abstract

The increasing demand for sustainable biosensing calls for alternative approaches that integrate green chemistry and nanotechnology. This study reports an eco-friendly and facile method for synthesizing gold nanoparticles (AuNPs) utilizing *Ocimum americanum* leaves extract as a bioreductant. Furthermore, we demonstrate their application to improve the performance of lactose biosensor employing cellobiose dehydrogenase from *Neurospora crassa* (NcCDH) as a promising approach for lactose detection in the dairy industry. Ultrasound-assisted extraction of dehydrated *Ocimum americanum* leaves for 3 min shows highest antioxidant activity ($IC_{50} = 83.53$) which was subsequently used as the bioreductant for the green synthesis of AuNPs. Characterization of AuNPs spectra, zeta potential and imaging results confirmed that the chemically synthesized AuNPs were monodisperse and stable while green synthesized AuNPs were smaller but polydisperse. Electrodeposition at varying concentration of chitosan showed highest current response at 0.1% chitosan, while highest immobilization efficiency ($40.51 \pm 3.56\%$) was achieved at 0.5% chitosan. Cyclic voltammetry characterization revealed 2 distinct electron transfer pathways of NcCDH via the FAD and heme domains. The incorporation of chemically synthesized AuNPs increased current responses by 327.77% (FAD) and 14.23% (heme), whereas Green-AuNPs yielded 549.74% (FAD) and 14.23% (heme) in comparison to the modified SPCEs without AuNPs. Chemical-AuNPs resulted in higher sensitivity, but Green-AuNPs provided lower detection limit to the biosensor analytical performance. Both chemically and green synthesized AuNPs-modified electrodes showed good stability up to 21 days, exceeding the control electrode which showed 2.5-times higher activity lost within 21 days. Our findings confirmed that AuNPs, particularly Green-AuNPs, significantly enhance the analytical performance of NcCDH-based lactose biosensor by facilitating electron transfer between the enzyme and electrode through both the FAD and heme domain.

Keywords: AuNPs, Biosynthesis, Electrochemical, Biosensor, *Neurospora crassa*, Cellobiose dehydrogenase, *Ocimum americanum*

Introduction

A biosensor is a selective analytical tool that involves communication between a bioreceptor and physical transducer to produce detectable signals. In its operation, the specific interaction between a target analyte and its bioreceptor induces physicochemical changes at the transducer surface, which are then converted into measurable signals [1]. Enzyme-based biosensors are particularly interesting among different types of biosensors because of their remarkable specificity at recognizing substrates. The interaction between enzyme and analyte creates detectable signals that are directly related to the concentration of analyte. Enzymatic biosensors commonly employ an amperometric approach, in which redox reactions produce electrical currents measured by an electrode. The combination of high selectivity of the enzymes and remarkable sensitivity of the electrochemical detection method makes this approach the most studied branch in biosensor research [2].

In food industries, enzymatic biosensors have been used to detect monosaccharides, such as glucose oxidase (GOx)-based biosensor, which has been used to detect glucose in fruit juice, nectar, honey, and wine [3]. Recent studies also validate that the biosensor shows results that are highly consistent with the conventional detection method, High Performance Liquid Chromatography (HPLC) [4]. However, disaccharide detection requires a more complex approach. Lactose, a glucose-galactose disaccharide, stands as one of the most critical disaccharides in the food industry and serves as a fundamental carbohydrate in milk and dairy products. Detection of maltose became crucial to maintain dairy product quality, especially in lactose-free milk. According to the Italian Ministry of Health, the threshold of lactose concentration to be labelled “lactose-free milk” is lower than 0.01% and has been adopted in a few other European countries [5]. The development of enzyme-based biosensors for lactose detection mostly involves cascade reactions, which combine hydrolysis by β -galactosidase followed by oxidation by glucose oxidase. However, these systems face several problems, such as the instability of the enzyme, cross-reactivity, and complexity in the immobilization process. In order to overcome these limitations, a single-enzyme reaction system employing cellobiose dehydrogenase (CDH) becomes a promising

alternative, and cellobiose dehydrogenase from *Neurospora crassa* (NcCDH) has been reported to be able to detect lactose [6].

Cellobiose dehydrogenase (CDH, EC 1.1.99.18) is a hemoflavoenzyme produced extracellularly by fungi such as *Neurospora crassa*, which plays a crucial role in lignocellulose degradation. This enzyme has a wide range of substrates it may work with, which include β -1,4-glycosidic bond linkages such as lactose, cellobiose, and glucose [7]. CDH consists of 2 functional domains structurally, a flavin-binding dehydrogenase domain (DH) that catalyzes substrate oxidation and a cytochrome domain (*cyt*) containing heme *b* that enables direct electron transfer (DET) to electrodes [8-10]. NcCDH present in different phylogenetic classes such as Class II variants (IIA and IIB), have been produced recombinantly in *Pichia pastoris* exhibiting high specific activity (21.2 U/mg) [6,11]. NcCDH have been proven to have a good sensitivity to lactose (K_m : 22 - 91 mM). It can detect lactose through 2 different electron transfer mechanisms, which are Direct Transfer Electron (DET) and Mediated Electron Transfer (MET) [12]. DET can occur via intramolecular electron transfer from $FADH_2$ (DH) to heme *b* (*cyt*) and be detected by the electrode. MET can be employed if heme *b* in *cyt* domain are inaccessible due to immobilization issues [13] or domain flexibility or movement [14]. Redox mediators such as 1,4-benzoquinone and 2,6-dichlorophenolindophenol (DCIP) are used as electron acceptors from $FADH_2$ to reduced mediators that can be detected by electrode [7]. Due to its unique characteristics and its ability to detect low substrate concentrations without requiring enzyme cascade reaction, NcCDH is an exceptionally suitable biocatalyst for developing efficient lactose biosensors.

One of the main challenges in developing enzymatic biosensors is the immobilization of enzymes on the electrode surface. Chitosan is widely used for enzyme immobilization via entrapment. However, conventional entrapment methods generally result in uneven enzyme distribution and uncontrolled film thickness [15]. Electrodeposition provides an alternative immobilization strategy to address this limitation. Chitosan is sensitive to pH ($pK_a \approx 6.3$), water-soluble, and present in cationic form at pH below the pK_a , which lets it interact with other negatively charged molecules via electrostatic forces. During electrodeposition at a

negative charge, chitosan is reduced, resulting in a localized increase in pH near the surface of the electrode. Chitosan becomes insoluble when the pH goes above the pKa, producing a thin and porous film that entraps the enzymes [16]. This strategy allows us to control the thickness and stability of the film, making it more porous because of the formation of H₂ gas when H⁺ is reduced [17].

In this study, we incorporated gold nanoparticles (AuNPs) into the chitosan matrix to solve the limitations that arise from performing entrapment methods to immobilize the enzymes, such as low sensitivity due to the limited substrate diffusion. Adding AuNPs to the system will greatly improve the electrical conductivity and speed up the electron transfer in the detection system. Thus, combining chitosan biocompatibility with AuNPs' catalytic properties may help to improve the performance of biosensors while mitigating the problems caused by diffusion [18]. Synthesis of AuNPs has been carried out using physical, chemical, and green methods, which allow control over particle size, shape, and surface chemistry of the AuNPs. Chemical reduction is the most common method used for synthesizing AuNPs, which uses gold salts, such as HAuCl₄ in aqueous solution, reduced by sodium citrate or ascorbic acid. Variation of reducing agent concentration is commonly used to tune the size of the nanoparticles. The chemical reductant used in this method allows more control of particle size but unfortunately raises environmental and safety concerns. The biological approach, or green method is carried out by utilizing plant extracts, microorganisms, or enzymes. It is considered an alternative, cleaner method in comparison to the chemical method [19]. Plant source extracts, such as those from basil leaf (*Ocimum americanum*), have potential for green synthesis of AuNPs due to their phytochemical compounds (flavonoids, phenolics, terpenoids, and polyphenols) [20-24]. To date, *Ocimum americanum* has also been reported effective in synthesizing AgNPs (8 - 55 nm), MnONPs (13.4 nm), and ZnONPs (20.07 ± 0.34 nm) [25-27]. This study aimed to evaluate the potential of incorporating green-synthesized AuNPs in a CDH-based lactose biosensor onto screen-printed carbon (SPCEs) through electrodeposited-chitosan entrapment in comparison with the chemically synthesized AuNPs. This application of an eco-friendly, plant-mediated

synthesis of gold nanoparticles (AuNPs) using *Ocimum americanum* leaf extract as a bioreductant, to the best of our knowledge, has not been previously carried out for electrochemical biosensor application. This study is the first investigation utilizing *O. americanum*-derived Green-AuNPs as a nanomaterial mediator in an enzyme-based electrochemical biosensor employing cellobiose dehydrogenase (NcCDH) for lactose detection. The findings of this study may offer insights on the development of sustainable biosensing integrating eco-friendly nanomaterials with advanced biocatalytic systems, with potential for food industry application.

Materials and methods

Fresh *Ocimum americanum* leaves were obtained from a local farmer in Malang Regency, East Java, Indonesia. Cellobiose dehydrogenase from *Neurospora crassa* (NcCDH) was kindly provided by Laboratory of Food Biotechnology, BOKU University, Austria. Screen Printed Carbon Electrodes (SPCEs, c110) with a geometrical surface area of 0.1256 cm². The SPCEs consist of working electrode: Carbon, auxiliary electrode: Carbon and reference electrode: silver, were purchased from Metrohm, Switzerland. SPCEs were washed using ultrapure water, followed by sonicating in ethanol for 5 min prior to further modification. Medium molecular weight of chitosan, acetic acid (CH₃COOH), HAuCl₄, sodium citrate (Na₃C₆H₅O₇), K₂HPO₄, KH₂PO₄, Potassium chloride (KCl), lactose monohydrate, 2,6-Dichlorophenolindophenol (in 10% ethanol), sodium fluoride (NaF), Coomassie Brilliant Blue G-250, ethanol (95%), phosphoric acid (85%), and nitrogen gas were all purchased from Merck in their highest purity form available, and all solutions were prepared with ultrapure water (Millipore, Germany).

Preparation of *Ocimum americanum* extract

The sample of fresh basil (*Ocimum americanum*) leaves was cleaned by washing them with distilled water, and then the moisture was removed by wiping the leaves with paper towel. Three different batches of variations were prepared to assess which preparation treatment was best for sample extraction. For the first batch, fresh leaves were dehydrated at 40 °C for 8 h and subsequently ground into fine powder using a blender. The leaf powder was then ready to be extracted. For the second batch, the fresh leaves were crushed using a

chopper prior to extraction. The following extraction steps for each of sample were using the ultrasound-assisted extraction (UAE) method for different time durations (1, 3, 5, 10 min) by adding water at a 1:20 (w:v) ratio to the sample, followed by filtration through Whatman No. 42 filter paper. The extract produced was then centrifuged (3 min, 5,000 rpm) in order to remove impurities, followed by sonicating for 15 min. Finally, the third batch was prepared by adding 15% of maltodextrin to the extract and freeze-drying the mixture for 17 h. All the extracts (liquid and powder) were ultimately kept at 4 °C before further analysis.

Determination of antioxidant activity of *Ocimum americanum* extract

Determination of antioxidant activity of the sample extract was conducted using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method with minor modification [28,29]. Different extract concentrations of 6.25, 12.5, 25, 50, and 100 ppm were prepared, followed by mixing each of the concentrations with 150 µL of 0.1 mM DPPH using a vortex. All the mixtures were then incubated for 30 min under dark conditions at room temperature. Measurements were performed using 96-well microplate reader, and absorbances were observed at 517 nm. The antioxidant activity of each sample was assessed using the IC₅₀ value, which indicates the concentration needed to inhibit 50% DPPH radical activity. The lower the IC₅₀ value, the higher the scavenging activity of the sample.

Synthesis of chemically and green AuNPs

Gold nanoparticles (AuNPs) were both green and chemically synthesized following the modified Turkevich synthesis method as reported [30]. Briefly, 6.3 mL of MilliQ water (MQW) was mixed with 0.028 mL of 0.5 mM of HAuCl₄ followed by heating the mixture to 90 °C while stirring at 200 rpm. For the chemical synthesis, 0.17 mL of 1% sodium citrate was added as a reductant under a continuous stirring condition for 2 min, followed by the addition of 0.28 mL of HAuCl₄. For the green synthesis, 0.75 mL of *Ocimum americanum* leaf extract, was used as the reducing agent under the same condition and equivalent volume. In both methods, heating was maintained for 30 min until the color of the colloidal solution changed from yellow to cherry red, which indicates the final formation of nanoparticles with the expected size. The synthesized

AuNPs were purified by mixing 200 µL of the colloidal solution with 800 µL MQW, followed by 9000×g for 15 min by centrifugation. The resulting pellet was resuspended in 1 mL MQW, centrifuged at 5000×g for 10 min, and finally diluted in 100 µL MQW, which was stored at 4 °C in aluminum-foil covered vials.

Characterization of chemically and green-AuNPs

The formation of AuNPs was confirmed through observation of their absorbance spectra using UV-Vis spectrophotometry in the 400 - 800 nm range. Spectra data was collected using a UV-Vis spectrophotometer (Shimadzu UV-1280, Kyoto, Japan). The zeta potentials were characterized using a Particle Size Analyzer (HORIBA NanoPartica SZ-100) to obtain surface charges information. The nanoparticle formation and size were characterized using Transmission Electron Spectrophotometry (F200X G2 (S)TEM, ThermoFischer Scientific, Massachusetts, US). For Fourier-transform infrared spectroscopy (FT-IR) analysis, the AuNPs were centrifuged to separate the solid AuNPs from the aqueous solvent, and the excess water was subsequently dried in an oven at 40 °C for 48 h. FT-IR (SENSOR II Spectrometer, Bruker Optics, Billerica, US) spectra analysis was conducted in the range of 4,500 to 500 cm⁻¹ with resolution of 4 cm.

Electrode modification and biosensor fabrication

Screen-printed carbon electrode modification was conducted through chitosan entrapment via electrodeposition. All solutions were sonicated for 30 min, followed by purging with nitrogen gas for 15 min prior to measurements. In order to evaluate the effect of chitosan concentration on the immobilization of NcCDH onto the electrode, varied concentrations of chitosan solution (0% - 0.5%) were applied to modify the electrode. As much as 10 µL of chemical-AuNPs, 10 µL of different concentrations of chitosan solution (0% - 0.5%) and 10 µL NcCDH enzyme solution (10 U/mg) were mixed together. About 30 µL of the mixture was carefully drop-casted onto the SPCE surface, followed by electrodeposition, performed by applying a constant potential of -1 V for 50 s to ensure the formation of a stable layer. The modified SPCE was then rinsed with

phosphate buffer (pH 6) and nitrogen-dried before storage at 4 °C as illustrated in **Figure 1**. The best

concentration of chitosan was further used for fabrication of biosensors incorporating green-AuNPs.

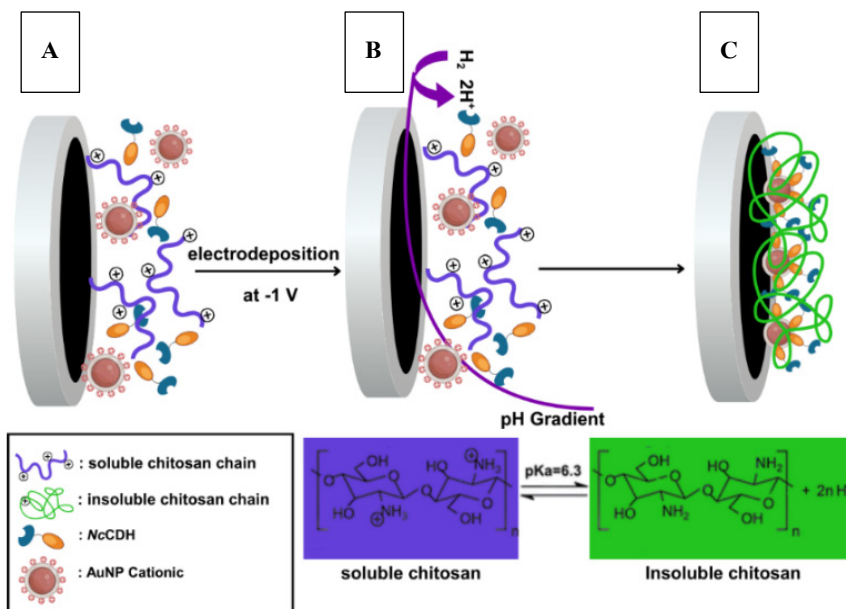


Figure 1 Fabrication of modified-SPCE. Entrapment of *NcCDH*/Green-AuNPs on the SPCE surface (A) was achieved using chitosan, transitioning from soluble (B) to insoluble (C) form via pH-gradient method.

The percentage of the total immobilized enzyme on the SPCE was calculated as % Immobilization Yield as follows:

$$\% \text{ Immobilization Yield} = [(C_0 - C_1)/C_0] \times 100\% \quad (1)$$

where: C_0 = specific activity of free-enzyme (U/mg)
 C_1 = specific activity of enzyme lysate in the soaking solution (U/mg)

Lactose detection performance

All electrochemical measurements were performed using a portable potentiostat Galvanoplot GX-102 (Solar Biyoteknoloji, Kordon Kule, Turkey) controlled with GalvanoPlot SUITE software. Unless stated otherwise, all measurements were performed in 100 mM phosphate buffer at pH 6.0 containing 100 mM of KCl. All solutions were purged with nitrogen for 15 min prior to measurements. Three different modified SPCEs (SPCE/*NcCDH*/Chitosan, SPCE/*NcCDH*/Chemical-AuNPs/Chitosan, and SPCE/*NcCDH*/Green-AuNPs/Chitosan) were electrochemically characterized using Cyclic Voltammetry (CV) and Differential Pulse Voltammetry

(DPV). The CV measurements were performed at a scan rate of 10 mV/s across a potential window of -750 to $+750$ V to establish baseline current profiles. Subsequent CV analysis with 100 mM lactose solution enabled identification of the oxidation with the net response calculated by subtracting the background currents. DPV measurement was performed at 200 to 700 mV. Calibration curves were created from 3 independent measurements for each point of the curve using varied concentrations of 0 - 100 mM lactose. The calculation of the limit of detection (LOD) was carried out using SigmaPlot 12 (Systat, Chicago, IL, USA) and calculated as the minimum concentration of analyte that can be reliably identified, resulting in a current response greater than the background value plus 3σ . Error bars represent the standard deviation of independent measurements obtained from 3 electrodes.

Results and discussion

Extraction of *Ocimum americanum* leaves

Ocimum americanum leaves contain a spectrum of phytochemicals including phenolics, flavonoids, terpenoids, alkaloids, tannins, saponins, amino acids, and carbohydrates [21,31]. These classes contribute

functional groups (e.g., -OH, -C=O, -C=C-, amide, amino, carbonyl-containing moieties) capable of reducing Au^{3+} to Au^0 and simultaneously stabilizing nascent AuNPs via surface binding. The scavenging activity of hydroxyl radical is directly correlated with the antioxidant activity of the phytochemical [32]. In this study, antioxidant activity evaluation was performed by assessing the IC_{50} value of different preparation methods (**Figure 2(A)**) and the duration of the extraction using ultrasound-assisted extraction (UAE) method (**Figure 2(B)**). The antioxidant activity was measured to evaluate the *Ocimum americanum* leaves' ability to synthesize nanoparticles. The IC_{50} value of the extract prepared by dehydrating the leaves has shown the lowest value (163.69 ppm) in comparison to other treatment methods. This value is considered as a moderate activity category [33]. Thus, practically, *Ocimum americanum* leaves extract is potential reducing agent for the biosynthesis of the gold

nanoparticles. The antioxidant properties of the sample depend on the extraction process. Applying drying methods prior to extraction helps to inactivate the enzymes and inhibit the oxidative reaction, thus maintaining the bioactive compounds contained in the sample. Several researchers reported that different drying methods may affect the total phenolic contents, flavonoids, and antioxidant activities of plant extracts [34-36].

Extraction using the UAE probe in various time durations showed that 3-min extraction performed the highest antioxidant activity ($\text{IC}_{50} = 83.53$), while prolonged extraction up to 10 min showed a decrease in antioxidant activity. This led to the degradation of the phytochemical compound as an effect of excessive exposure to ultrasonic waves [37,38]. Thus, the extract prepared by extracting dry *Ocimum americanum* leaves using UAE probe for 3 min will be used as a bioreductant for further Green-AuNPs synthesis.

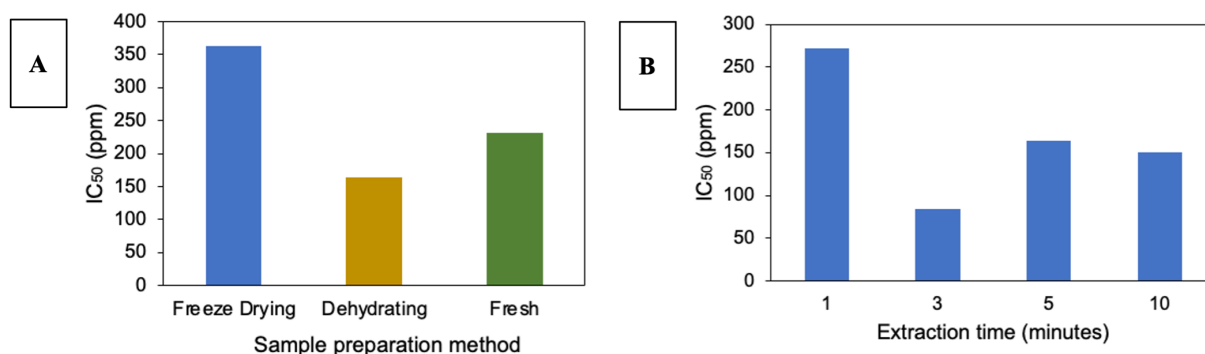


Figure 2 Effect of different preparation conditions of *Ocimum americanum* leaves extract on the IC_{50} value. Three different leaves' preparation methods (A) and 4 different extraction times using ultrasound-assisted extraction (UAE) method (B) were compared.

Characterization of chemically and green-synthesized AuNPs

TEM analysis results showed spherical particles with average sizes of 15.49 ± 2.880 nm for Chemical-AuNPs (**Figures 3(A)** and **3(C)**) and 12.05 ± 4.725 nm for Green-AuNPs (**Figures 3(B)** and **3(D)**). The synthesis of AuNPs using chemical and green reducing agents showed distinct reaction times of 3 and 2.5 min, respectively, as evidenced by color changes from yellow to cherry red (Chemical-AuNPs, **Figure 3(E)**) and yellow to blue (Green-AuNPs, **Figure 3(F)**). The UV-Vis spectra results showed a single peak at 521 nm for Chemical-AuNPs, while Green-AuNPs exhibited

multiple peaks at 558, 535, and 640 nm, as shown in **Figure 3(G)**. Zeta potential values of -60.7 mV (Chemical-AuNPs) and -45.4 mV (Green-AuNPs) confirmed good colloidal stability (zeta potential $> \pm 30$ mV) in which negative potential values come from the reductor residual as a stabilizing agent [39].

The FT-IR spectrum of AuNPs (**Figure 3(H)**) chemically synthesized using sodium citrate showed typical absorption bands of carboxylate ($-\text{COO}^-$) groups with asymmetric stretching (ν_{as}) at $1,516\text{ cm}^{-1}$ and symmetric stretching (ν_{s}) at $1,421\text{ cm}^{-1}$. These bands indicate a citrate adsorption through carboxylic groups, which is shown by ν_{as} and ν_{s} vibration that appear

around $1,500 - 1,630 \text{ cm}^{-1}$ and $1,305 - 1,415 \text{ cm}^{-1}$, respectively. Typically, citrate will adsorb through oxygen atoms creating a bidentate binding mode, where both oxygen atoms of the carboxylate group coordinate with the gold surface. This confirms that citrate acts as a capping agent through the interaction of its carboxylate groups with Au. A previous study reported that citrate adsorption on gold occurred through both oxygen atoms of the carboxylate group in an $\eta^2\text{-COO}$ bridging configuration, with the C–C axis oriented perpendicular to the metal surface [40]. However, in the present study, the presence of both ν_{as} and ν_{s} bands suggests citrate interaction with Au but does not allow the adsorption configuration to be determined with certainty.

A similar adsorption pattern is observed in AuNPs synthesized with *Ocimum americanum* extract, where the ν_{as} band appears at $1,517 \text{ cm}^{-1}$ and the ν_{s} band at $1,422 \text{ cm}^{-1}$ (Figure 3(H)). This suggests that organic

compounds in the *Ocimum americanum* extract, particularly those containing carboxylate or organic acid groups, act similarly to citrate in stabilizing the gold nanoparticles. Therefore, both citrate and natural compounds from *Ocimum americanum* function as capping agents via –COO^- interactions with the Au surface. Chemical-AuNPs displayed monodisperse characteristics dominated by spherical particles, as indicated by the cherry-red colloidal color and single UV-Vis peak [41]. These AuNPs also exhibited higher stability. In contrast, Green-AuNPs showed polydispersity indicated by multiple UV-Vis peaks. Although Green-AuNPs had lower stability compared to Chemical-AuNPs, they maintained adequate colloidal stability. TEM size distribution analysis (Figures 3(A) and 3(B)) revealed that Chemical-AuNPs were larger in size but more uniformly distributed than Green-AuNPs. These size differences are expected to influence electron transfer efficiency in biosensor applications.

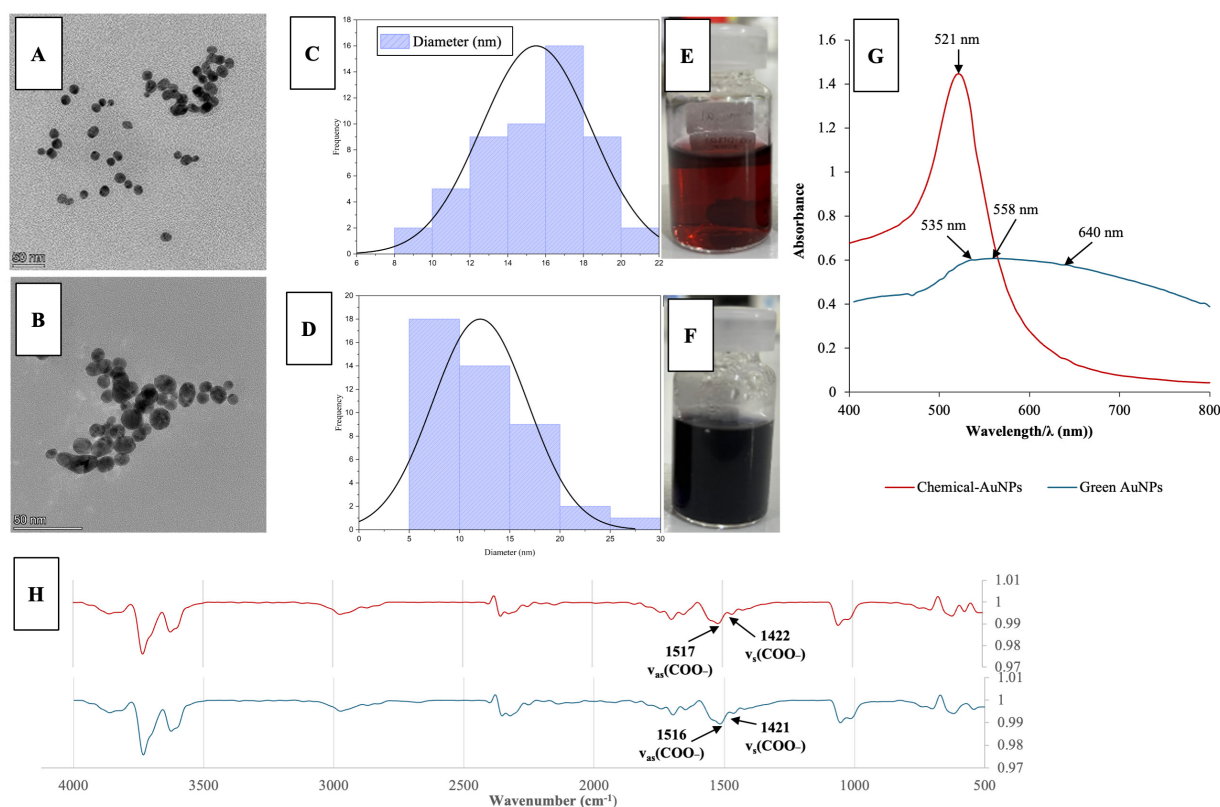


Figure 3 TEM Image Chemical-AuNPs (A) and TEM Image Green-AuNPs (B), Particle Size Distribution of Chemical-AuNPs (C), Particle Size Distribution of Green-AuNPs, Chemical AuNPs Colloid Color (E) Green AuNPs Colloid Color (F), UV-Spectra Curve of Chemical-AuNPs (red line) and Green-AuNPs (blue line) (G) FT-IR of Chemical AuNPs (red) and Green AuNPs (blue) (H).

Effect of chitosan concentration on immobilization of *Nc*CDH/AuNPs onto SPCE

Figure 4(A) shows that 0.5% (v/v) chitosan exhibited the highest immobilization yield of $40.51 \pm 3.56\%$, while 0.1% (v/v) chitosan gave the lowest ($19.42 \pm 2.96\%$). Mechanistically, the improvement at 0.2% was attributed to the formation of a stable, porous chitosan film optimal for enzyme entrapment. The decline at 0.3% resulted from increased viscosity disrupting film stability [42] and finally the rise at 0.4% - 0.5% correlated with denser matrix formation. Higher

concentrations of chitosan increased the number of positively charged $-\text{NH}_3^+$ groups, allowing electrostatic interaction with the negatively charged enzymes/AuNPs [12]. This was supported by ionotropic crosslinking between chitosan amine groups and the electroactive species [16]. The pH gradient properties of chitosan also facilitate on making the porous hydrogel such that entrapment may occur efficiently. Nevertheless, high concentration may slow down the diffusion of substrate, therefore lowering the activity of the enzymes [42].

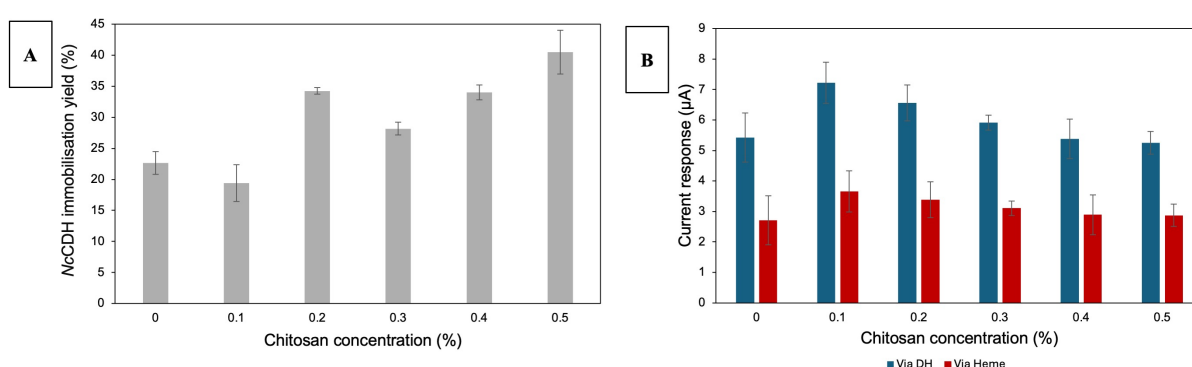


Figure 4 Effect of Chitosan on *Nc*CDH Immobilization Yield (A) and Electrochemical Response (B). The data represent average values from 3 different measurements, and the standard deviation is indicated by error bar.

Electron transfer efficiency was evaluated using cyclic voltammetry (CV) with 100 mM lactose as the substrate and 1 mM DCIP as the redox mediator at a scan rate of 10 mV/s between potentials of -750 and 750 mV | Ag vs AgCl (sat. KCl). The net current response was calculated by subtracting the peak current of the blank solution from that of the lactose-containing solution. The modified biosensor showed electron transfer behaviors through multiple peaks in the CV result, establishing 2 electron transfer pathways via flavin adenine dinucleotide (FAD) in the DH domain and heme *b* in *cyt* domain. The highest current response was observed at 0.1% chitosan ($7.22 \pm 0.67 \mu\text{A}$), followed by a significant decrease at higher concentrations: $6.56 \pm 0.59 \mu\text{A}$ (0.2%), $5.91 \pm 0.24 \mu\text{A}$ (0.3%), $5.38 \pm 0.65 \mu\text{A}$ (0.4%) and highest decrease was noted at 0.5% chitosan ($5.25 \pm 0.37 \mu\text{A}$) via FAD as shown in **Figure 4(B)**.

This trend is consistent with the findings of [42], who reported that thicker chitosan layers hinder substrate diffusion and thus reduce sensor performance. The decline in DH domain performance at higher

chitosan concentrations can be explained through several mechanisms. First, as revealed by [17], increased solution viscosity and the formation of thicker chitosan layers at higher concentrations create substrate diffusion barriers and prolong electron transfer distances. Second, the characteristics of entrapment immobilization methods tend to produce uneven enzyme distribution and orientation. This contributes to prolonged electron transfer distances and reduced electron transfer effectiveness [43].

In contrast, the heme domain exhibited a more stable response profile, with the highest peak value of $7.08 \pm 0.82 \mu\text{A}$ at a 0.1% concentration and limited fluctuations within the range of $6.50 - 6.63 \mu\text{A}$ for concentrations of 0.2% - 0.5%. The heme domain shows enhanced electron transfer efficiency and stability at higher polymer concentrations [44]. This stability is primarily attributed to strong electrostatic ionotropic crosslinking interactions between the NH_3^+ groups in chitosan and the negatively charged heme domain [16].

The difference between electron transfer performance via FAD and heme *b* highlights *Nc*CDH's

electrochemical properties and how its performance is influenced by chitosan concentration. Aligned with CV result, DH domain is more sensitive to changes in the matrix environment compared to the *cyt* domain, which is more robust due to electrostatic interaction between the heme domain and the chitosan matrix [12]. The superior biosensor performance at 0.1% chitosan is mainly attributed to the formation of a thinner chitosan layer, which minimizes substrate diffusion barriers while maintaining efficient electron transfer across both domains at the electrode. Higher concentration of chitosan generally increases network density, reduce pore size and lengthen diffusion pathways for substrates to access the enzyme. This produces slow diffusion response and lower the apparent reaction rates [45]. The selected concentration of chitosan was based on finding a balance between how well it immobilizes *NcCDH* and how well it transfers electrons. Higher chitosan concentration (0.5%) led to better immobilization efficiency, but they also made the polymer layer thicker, which can make it harder for electrons to move because of higher diffusion resistance and conductivity. Therefore, the 0.1% chitosan concentration was selected for subsequent experiments.

AuNPs effect on *NcCDH*/chitosan electrode electrochemistry

Cyclic voltammetry (CV) was conducted to evaluate the effect of gold nanoparticle (AuNP) incorporation on the electrochemical properties of immobilized *NcCDH* in a chitosan matrix. Three types of SPCEs were fabricated: *NcCDH*/Chitosan (Control), *NcCDH*/Chemical-AuNPs/Chitosan, and *NcCDH*/Green-AuNPs/Chitosan. The immobilized enzyme percentages on the SPCEs were 30.06%, 35.36%, and 34.94%, respectively. *NcCDH*/Chemical-AuNPs/Chitosan showed the highest percentage due to its monodisperse nature and higher zeta potential compared to *NcCDH*/Green-AuNPs/Chitosan, though both AuNPs-modified electrodes exhibited higher values than the control without AuNPs. The uniform distribution and surface charge of AuNPs enabled more homogeneous dispersion on the electrode surface, providing a larger and more optimal contact area for *NcCDH* enzyme binding [18].

Electrochemical properties evaluation using CV revealed multiple peaks indicating electron transfer occurs on both *NcCDH* domains on different electron transfer pathways. Each electrode produced different buffer values due to DCIP mediator accumulation, so currents were normalized to compare peak currents in the CV curves. The results demonstrated that AuNPs significantly influenced the current response in both domains. **Figure 5** presents a bar chart showing the oxidation peak currents for each variation.

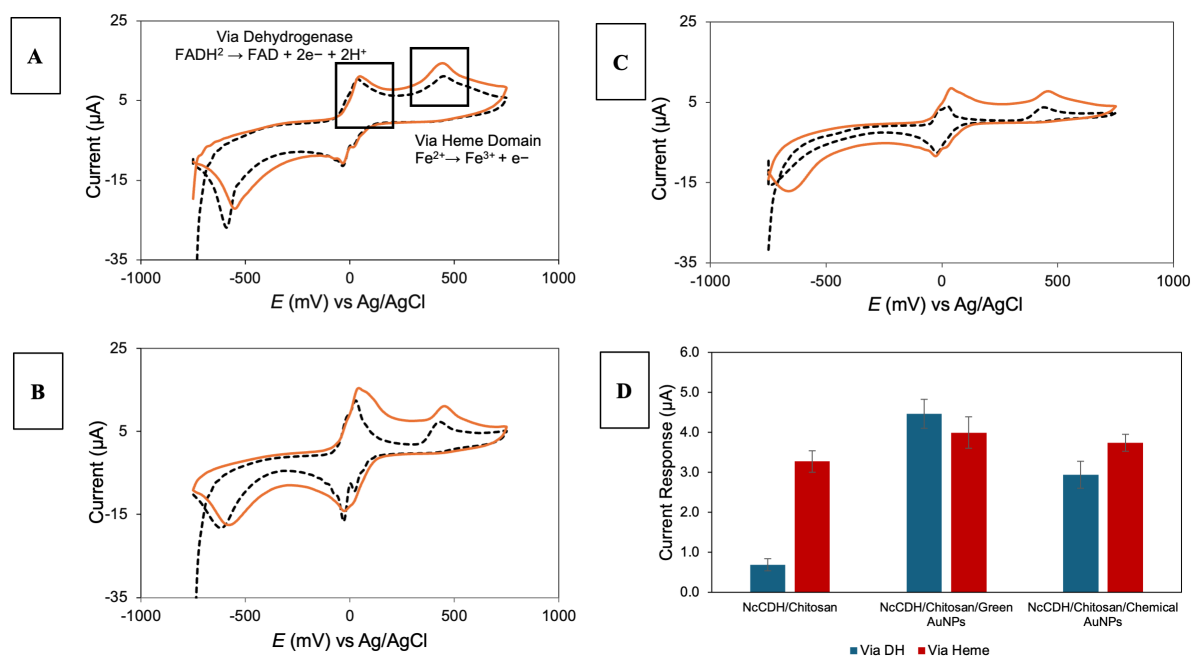


Figure 5 Cyclic voltammogram of *NcCDH*/Chitosan (A), *NcCDH*/Green-AuNPs/ Chitosan (B), *NcCDH*/Chemical-AuNPs/Chitosan (C) in the absence of (dashed black) and in the presence of (orange) 100 mM lactose, peak current response values for *NcCDH*/Chitosan modified electrodes with and without AuNPs (D). The data represent average values from 3 different measurements, and the standard deviation is indicated by error bar.

The control electrode (*NcCDH*/Chitosan) generated currents of 0.69 and 3.28 μA (**Figures 5(A)** and **5(D)**), while the Green-AuNPs electrode achieved 4.46 and 3.99 μA (**Figures 5(B)** and **5(D)**), and Chemical-AuNPs electrode generated the highest result of 2.94 and 3.74 μA (**Figures 5(C)** and **5(D)**). Compared to the control, the SPCE/*NcCDH*/Chemical-AuNPs/Chitosan exhibited current increases of 327.77% and 14.23% via FAD and *cyt*, respectively. Meanwhile, the SPCE *NcCDH*/Green-AuNPs/Chitosan showed increases of 549.74% and 14.23%. These results confirm that AuNPs enhance electron transfer efficiency between the enzyme and electrode surface. In the MET system, AuNPs function as electron bridges, which reduce electron hopping distance. AuNPs also increase electrode active surface area and enhanced conductivity though higher conductive molecule density. These properties validate AuNPs' effectiveness in the MET system [12].

Although both AuNP types improved currents compared to the control, performance differences were observed between synthesis methods. Green-AuNPs yielded higher peak current responses than Chemical-AuNPs. When plant-based reductants are used to make

Green-AuNPs, the particles have phytochemical surface functions that help redox enzymes transfer electrons to the electrode more quickly. This makes the electrochemical response and overall biosensor performance better than chemically synthesized AuNPs. The better performance is also due to variations in physicochemical features, especially the smaller particle size, better distribution and better surface stability of Green-AuNPs [18,46]. For Green-AuNPs, phytochemical compounds work as both reducing and capping agents. This makes the nanoparticles smaller but more polydisperse. This smaller size can enlarge the active surface area and shortens the distance that electron have to travel between the redox centers of *NcCDH* (FAD and heme domains), therefore accelerates electron transfer.

Analytical performance of lactose biosensor

To assess the analytical performance of the fabricated *NcCDH*/AuNPs/Chitosan biosensor, a various range of lactose concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mM) were applied as depicted in **Figure 6**. DPV was performed to evaluate the dynamic concentration range as well as the

sensitivity and limit of detection (LoD) of the biosensor (**Figures 6(A)** and **6(C)**). The calibration curve was then prepared by plotting the current density (y) vs lactose concentrations (x) (**Figures 6(B)** and **6(D)**). The calibration curve of both biosensors showed the same dynamic range of 10 - 90 mM with correlation equations of $y = 2.6689x - 32.066$ ($R^2 = 0.9914$) for the biosensor incorporated with chemically synthesized AuNPs and $y = 2.464x - 22.511$ ($R^2 = 0.9957$) for the biosensor using green-AuNPs. The lactose sensitivity of *NcCDH*/Chemical-AuNPs/Chitosan ($2.669 \mu\text{A mM}^{-1} \text{cm}^{-2}$) was higher than that of *NcCDH*/Green-AuNPs/Chitosan ($2.464 \mu\text{A mM}^{-1} \text{cm}^{-2}$). The LoD of *NcCDH*/Green-AuNPs/Chitosan was found to be lower (8.921 mM) than *NcCDH*/Chemical-AuNPs/Chitosan

(12.015 mM). These detection limits were considered higher than that of biosensors published in other studies, as represented in **Table 1**. The higher sensitivity observed in the biosensor incorporating Chemical-AuNPs can be due to their more uniform size distribution which facilitate more efficient and consistent enzyme immobilization and electron transfer. On the other hand, Green-AuNPs are smaller which may improve the electron transport, but their phytochemical-derived surface coatings may partially hinder electron transfer pathways. However, the same surface bio-functionalities can act as mediators or help enzymes orient themselves in a way that lowers interfacial resistance and raises the effective electroactive surface area [47,48].

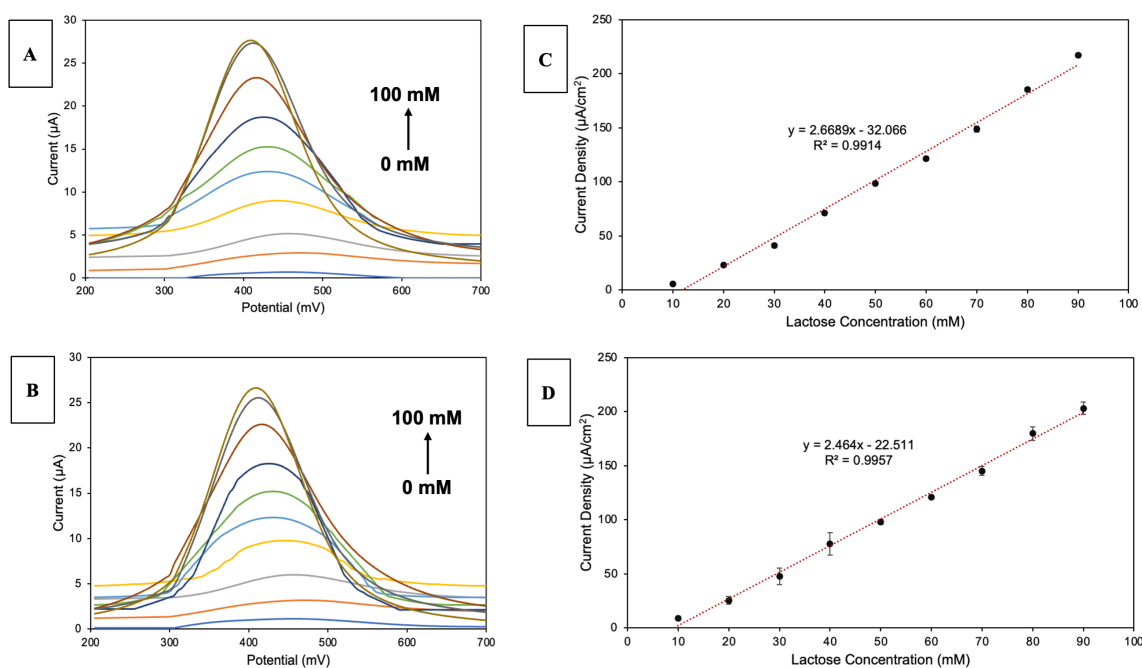


Figure 6 The DPV response and calibration curve of *NcCDH*/Chemical-AuNPs/Chitosan (A, C) and *NcCDH*/Green-AuNPs/Chitosan biosensor (B, D) towards 0 - 100 mM lactose in phosphate buffer (100 mM, pH 6.0 containing 100 mM of KCl). The data represent average values from 3 different biosensors measurements, and the standard deviation is indicated by error bar.

Table 1 Comparison of the analytical performance of different electrochemical enzyme-based biosensors for detection of lactose.

System	Electrochemical technique	Potential of detection (mV)	LoD (mM)	Linear range (mM)	Real sample	Ref.
SPCEN <i>NcCDH</i> /Green-AuNPs/Chitosan	Voltammetry	200 - 700	8.921	10 - 90	Not applicable	This study
MWCNT-RuO ₂ /Gox-β-Gal	Chronoamperometry	400	0.036	0.5 - 3.0	Semi-skim milk	[49]
TvCDH/AuNPs/graphite	Amperometry	250	0.35	10 - 300	Not applicable	[48]

System	Electrochemical technique	Potential of detection (mV)	LoD (mM)	Linear range (mM)	Real sample	Ref.
PsCDH NH ₂ -PD/SWCNT-GC	Amperometry	200	0.5	1 - 150	Not applicable	[50]
Pt/Chit/GOx β -gal/Chit/GA	Constant Potential Coulometry	650	1.29	2.47 - 40	whey permeates	[51]
GOx- β -Gal@AuNPs-g-C ₃ N ₄ -MnO ₂ -TiO ₂ /ITO	PEC amperometry	-400	0.023	0.008 - 2.5	Lactose free milk	[52]
CtCDH/AuNPs/BPDT/AuE	FIA amperometry	250	0.3	0.5 - 40	2% low fat milk, light cream, whipping cream, 80% lactose reduced milk	[18]

Storage stability

The storage stability of the electrode was evaluated using the same modified electrode over 21 consecutive days. When the modified electrode was not used, the electrode was stored at 4 °C under constant humidity in a closed container. The control SPCE without AuNPs showed a current response decrease by about 10% after 2 weeks of storage compared to initial current response and finally declined to 25% by the third week of storage, while *Nc*CDH/Green-AuNPs/Chitosan dropped to only 5.5% after 2 weeks and finally dropped to 10% by day 21.

*Nc*CDH/Chemical-AuNPs/Chitosan also showed comparable storage stability of 9.5% decrease by 21 days of storage. Through this study, we assumed that the AuNPs in the modified electrode were found to be strongly embedded in the *Nc*CDH/Chitosan matrix, assisting the electron transfer. These findings indicate that AuNPs not only enhance electrochemical activity but also maintain the stability and performance of the biosensor.

Possible sensing mechanism

To better illustrate the sensing mechanism of the proposed biosensor, a schematic representation is provided in **Figure 7**. The sensing mechanism relies on the catalytic function of cellobiose dehydrogenase (*Nc*CDH), which oxidizes lactose at the FAD site within the dehydrogenase (DH) domain, resulting the formation of lactobionate and the reduction of FAD to FADH₂. In the cytochrome domain, the electrons are then moved to *heme b* through internal electron transfer (IET). From this point on, electrons are sent to the electrode either directly (DET) or by a mediated (MET) pathway using 2,6-dichlorophenolindophenol (DCIP). DCIP works as an electron shuttle in the mediated system which takes electrons from the reduced enzyme and is re-oxidized at the electrode surface. This creates a quantifiable current signal that is directly proportional to the lactose concentration.

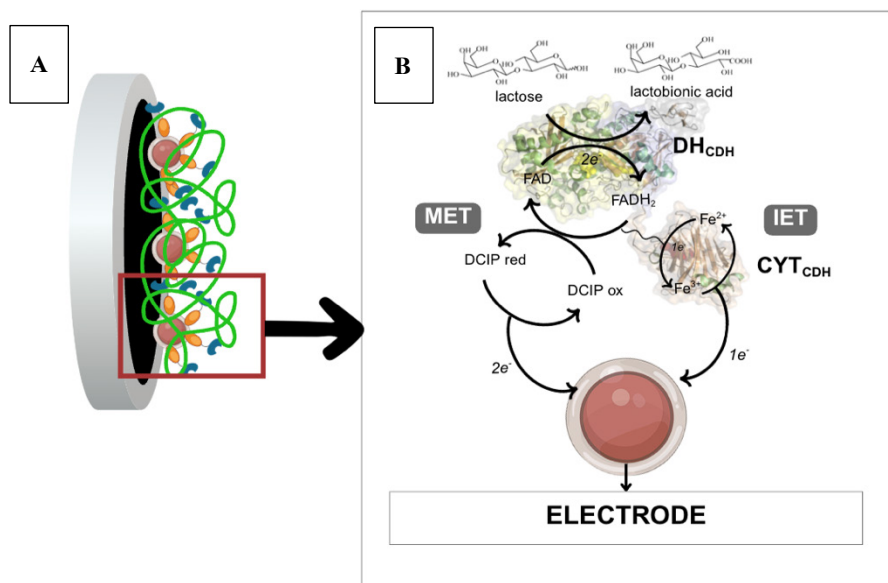


Figure 7 Schematic illustration of SPCE/*NcCDH*/Green-AuNPs/Chitosan biosensor sensing mechanism toward lactose. Immobilization of *NcCDH*/Green-AuNPs/Chitosan onto the SPCE (A) and possible electron transfer mechanism from the oxidation of lactose as an analyte to the SPCE surface (B).

Conclusions

This study demonstrated that both chemically and green-synthesized AuNPs can effectively enhance the electrochemical performance of *NcCDH*-chitosan modified electrodes for lactose detection. While Chemical-AuNPs exhibits mono-dispersity and uniform distribution size, Green-AuNPs yields a higher current response despite its polydispersity and lower negative charge. These different properties are because of the smaller size of the Green-AuNPs particle, therefore increasing the electron transfer and conductivity. The natural active compound in *Ocimum americanum* extract utilized for green synthesis may enhance electron transfer efficiency, offsetting the challenges associated with particle uniformity. In addition to this analytical performance study, more research into the structure and property relationships of AuNPs-based system is needed to better understand how the shape, surface chemistry, and dispersion of nanoparticles affect electron transfer mechanisms and long-term stability. Regarding the application in dairy products such as milk, the biosensor is incapable of detecting the lactose content for monitoring the lactose-free milk due to its high detection limit but is recommended to be applied in daily rapid assays for checking the lactose content in milk. Nevertheless, the results show that plant-based

AuNPs could be an alternative replacement for chemically synthesized nanoparticles in biosensor applications. This is in line with green nanotechnology principles and does not affect the performance.

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Declaration of Generative AI in Scientific Writing

The preparation of this manuscript did not use any generative AI tools. No content or data interpretation

was executed by generative AI. All authors are responsible for the content and conclusion of this work.

CRedit Author Statement

Sudarma Dita Wijayanti: Conceptualization, Supervision, Writing - review&editing, Project administration; Funding acquisition. **Josephine Laurentia Liem:** Investigation; Methodology; Formal Analysis; Data Curation, Writing-original draft and editing. **Haya Nurhidayah:** Investigation. **Wirya Sarwana:** Data curation; Formal Analysis, Writing - review&editing. **Yulizah Hanifah:** Data curation; Formal Analysis. **Fenty Nurtyastuti Eka Pertiwi:** Writing - review. **Nur Ida Panca Nugrahini:** Writing - review, Supervision. **Wisnu Arfian Anditya Sudjarwo:** Data Curation, Formal Analysis. All authors read and approved the final manuscript.

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