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The effect of different immobilization strategies on the electrochemical performance of enzymatic carbonaceous electrodes developed using carbonized biomass sources

Şevki Furkan Küçükayar [a], Veli Şimşek [b], Mustafa Oguzhan Caglayan [c], Zafer Üstündağ [d], Samet Şahin [a,c,*]

[a] Bilecik Seyh Edebali University, Department of Energy Systems Engineering, 11100 Bilecik, Turkey
[b] Bilecik Seyh Edebali University, Faculty of Engineering, Department of Chemical Engineering, 11100 Bilecik, Turkey
[c] Bilecik Seyh Edebali University, Faculty of Engineering, Department of Bioengineering, 11100 Bilecik, Turkey
[d] Kütahya Dumlupınar University, Faculty of Arts and Sciences, Department of Chemistry, 43100 Kütahya, Turkey

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ABSTRACT

In this study, typha tassel (TT) and pussy willow (PW) as biomass sources were used to synthesize carbonaceous materials for enzymatic electrode modification. Carbonization was performed at high temperatures of up to 1000 °C under an inert atmosphere and the resulting carbonized TT (CTT) and PW (CPW) were dispersed in dimethylformamide. CTT and CPW were then characterized by SEM/EDX, MAPPING, FTIR, and XRD to confirm the carbonaceous structures. Four different immobilization strategies were used to demonstrate the use of CTT and CPW together with a glucose oxidizing enzyme (glucose oxidase, GOx), an electron transfer mediator (ferrocene, Fc), and a protective polymer coating (Chitosan, Chit). The effect of the Chit layer was first investigated on the performance of the prepared enzymatic electrodes and it was shown that Chit could help to preserve the GOx activity. The effect of the electron transfer mediator whether in a solution or co-immobilized with GOx was also investigated using a mixture of Fc and CTT or CPW and Fc-only redox active film approaches. The results indicate that when Fc co-immobilized with GOx, a better performance was achieved. The prepared electrodes showed promising results for glucose biosensing with a limit of detection and limit of quantification values of 0.97 mM and 3.2 mM, respectively, operating up to 10 mM glucose. This study presents a comprehensive investigation of different immobilization strategies of GOx on carbonaceous electrodes and provides insight into the possible use of such materials as biomass to bioelectronics approaches.

1. Introduction

Carbonaceous materials (CMs) have been widely used in literature for electrochemical applications due to their high conductivity and chemical stability [1]. Graphene derivatives such as graphene oxide (GO) and carbon nanotubes (CNTs) are among the most widely used CMs specifically in electrochemical biosensing applications [2]. However, such materials often suffer from complicated and high-cost synthesis procedures [3]. Although much effort has been made to improve the shortcomings of these materials, different types of CMs, especially those derived from lignocellulosic biomass, offer great potential for many applications. CMs derived from biomass are often produced using green chemistry approaches, therefore can be a green alternative to the most widely used CMs without compromising electrical conductivity and high surface area. Such materials can be used in various applications such as solar cells [4], water treatment [5], catalysis [6], batteries [7], supercapacitors [8], and electrochemical biosensing [9].

Electrochemical sensors can be considered the most widely used sensors the because of several advantages such as low-cost, easy detection, and high sensitivity [10]. They can also be employed in a wide range of applications from bench-top to wearable devices. Since the active electrode surface area and conductivity are two of the most important desired properties for electrochemical electrode design, several materials such as metals, metal-oxides [11], polymers [12], and carbon-based substances [13,14] have been used to develop active electrodes. The material type has become very important for

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* Corresponding author at: Bilecik Seyh Edebali University, Department of Energy Systems Engineering, 11100 Bilecik, Turkey.
E-mail address: samet.sahin@bilecik.edu.tr (S. Şahin).

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electrochemical systems to increase sensitivity, selectivity, and robustness. Therefore, the use of CMs in electrochemical sensing applications has attracted many researchers to even enhance the properties of the electrode designs [15–17].

There are several biochar-based electrochemical sensors using biomass feedstock as electrode material. Such electrodes can be used as an adsorbent [18], catalyst [19], or carrier [20] for many applications. The porous structure of the CMs with a large surface area makes them an ideal candidate for the adsorption of an inorganic or organic catalyst such as metal ions or enzymes, respectively. Recent studies show that CM-based electrodes can improve the stability and the catalytic activity of the enzymes and could be of potential use for the fabrication of electrochemical biosensors [21]. One of the first studies using immobilized enzymes on CM-modified electrodes was a tyrosinase enzyme biosensor for bisphenol A detection [22]. A mixture of tyrosinase enzyme and biochar nanoparticles was drop coated followed by a protective coating of Nafion polymer to prepare the enzymatic electrodes. Tyrosinase enzymes have been widely used in preparing CM-based electrochemical sensors using different immobilization strategies such as covalent, cross-linking, and precipitation [23]. On the other hand, there haven't been many studies using CM-based enzymatic electrodes for glucose-oxidizing enzymes such as glucose oxidase. Although preliminary studies showed that CM-based electrodes can be used for enzymatic electrochemical glucose oxidation [24], there hasn't been a comprehensive study in the literature.

In this study, two different biomass sources, typha, and pussy willow (PW), were chosen to produce carbonaceous electrodes. Typha is aquatic (or semi-aquatic) and short-lived plat that is abundant around mostly lakes [25]. It can grow very rapidly disturbing the wetland and causing the elimination of species diversity while reducing the chances of other plants to survive [26]. On the other hand, pussy willow is also an abundant plant that eventually becomes waste biomass in nature. The potential utilization of these two abundant plants makes them very interesting candidates for biomass carbonization studies, especially based on their different structures. Therefore, the tassels of typha (TT) and PW flowers were carbonized to obtain conducting biomass-based materials for enzyme immobilization. Different immobilization strategies were then systematically investigated to find the best performance in electrochemical glucose oxidation using glucose oxidase (GOx). GOx is one of the most widely used industrial enzymes for the preparation of enzymatic electrodes, therefore, it was chosen as a model enzyme to demonstrate the performance of the carbonaceous electrodes for different immobilization strategies. Hence, a systematic study on the immobilization of GOx using TT and PW-modified electrodes is presented for the first time in the literature. A comparative investigation of different immobilization strategies of GOx on carbonaceous electrodes provides insight into the possible use of such materials as biomass to bioelectronics approaches.

2. Experimental

2.1. Materials

All chemicals were obtained at analytical grade from Sigma-Aldrich or Merck and used as received.

2.2. Synthesis and characterization of carbonaceous materials

To synthesize the carbonaceous electrode materials, 10 g of TT or PW was pyrolyzed in a tube furnace (Protherm, Turkey) with a heating ramp of 10 °C/min at 1000 °C under nitrogen for 1 h [27]. The carbonaceous materials were denoted as CTT and CPW for TT and PW, respectively. 10 mg/mL dispersions of CTT and CPW in dimethylformamide (DMF) were prepared after being ground and sieved post-synthesis. The dispersions were stored at room temperature when not in use and sonicated for 15 min before experiments.

CTT and CPW were characterized after carbonization using scanning electron microscopy (SEM, Zeiss SUPRA V 40), energy-dispersive X-ray spectroscopy (EDX), Fourier-transform infrared spectroscopy (FT-IR, Agilent Cary 360), and X-Ray diffraction analysis (XRD, Panalytical-Empyrean). The synthesized carbonaceous materials were also electrochemically characterized after being coated on electrodes using a potentiostat/galvanostat (Ivium Technologies, Netherlands).

2.3. Preparation and characterization of enzymatic electrodes

All electrochemical experiments were conducted using SPEs (Metrohm Dropsens, Switzerland) with a working electrode surface area of 0.059 cm^2. The working and counter electrodes were carbon and the reference electrode was made of silver (~74 mV vs SHE, data is obtained from the manufacturer). Screen-printed electrodes (SPEs) were first cleaned to remove impurities on the electrode surface using linear sweep voltammetry (LSV) between 0 and −2 V (Ag/Ag^+) at 20 mV/s scan rate in 0.1 KCl solution. The clean SPE was then drop-coated with either CTT or CPW using 10 mg/mL dispersions and dried, denoted as SPE/CTT and SPE/CPW, respectively. Four different enzyme immobilization strategy was employed to prepare enzymatic electrodes using glucose oxidase (GOx). The schematic representation of the enzyme immobilization strategies used in this study is shown in Fig. 1.

In this first strategy (Fig. 1**, purple**), 1 μL of GOx (1 mg/mL) was drop coated on either SPE/CTT or SPE/CPW and kept at + 4 °C for 24 h. The modified electrodes were then soaked in 0.1 M PBS (pH 7.4) for 15 mins followed by three consecutive washing steps to remove weakly bounded proteins before any test. The prepared electrodes were denoted as SPE/CTT/GOx and SPE/CPW/GOx. In the second strategy (Fig. 1**, yellow**), SPE/CTT/GOx and SPE/CPW/GOx electrodes were further coated with 2 μL of 1% (w/v) chitosan (Chit) to obtain a biocompatible and protective layer. The prepared electrodes were denoted as SPE/CTT/GOx/Chit and SPE/CPW/GOx/Chit. In the latter methods, ferrocene (Fc) was co-immobilized with the enzyme using two different approaches. The third strategy (Fig. 1**, lime**) includes the coating of 10 mg/mL CTT or CPW containing 50 mM Fc on SPEs, denoted as SPE/CTT-Fc and SPE/CPW-Fc. GOx and Chit were then modified the same as in the previous strategies to obtain SPE/CTT-Fc/GOx/Chit and SPE/CPW-FcGOx/Chit. Finally, Fc was applied as a separate layer after the carbonaceous material modification to get 1 mg/cm^2 Fc loading followed by the same GOx and Chit modifications as in the previous strategies to obtain SPE/CTT/Fc/GOx/Chit and SPE/CPW/FcGOx/Chit (Fig. 1**, pink**).

2.4. Electrochemical characterizations

All electrochemical experiments were performed at 23 ± 2 °C using four independently prepared electrodes (N = 4). In the first two enzyme immobilization strategies, the electrodes were tested in 0.1 M PBS (pH 7.4) solution containing 1 mM Fc carboxylic acid (FcCOOH) and only 0.1 M PBS (pH 7.4) solution was used for the latter two strategies.

3. Results and discussion

3.1. Characterization of CTT and CPW

After the carbonization process, CTT and CPW were characterized using SEM-EDX, FTIR, and MAPPING. Fig. 2 **(a) and (b)** show the SEM-EDX and MAPPING analysis images of CTT and CPW, respectively. SEM images of CTT (Fig. 2 **(a)**) confirm the carbonaceous fibers forming a polycrystalline carbon structure (along with XRD results) with graphite-like connections similar to the literature [24]. Mostly carbonized 3-D hollow cylindrical structures of CPW were observed without interconnected graphite-like structures contrary to CTT. The MAPPING images of CTT and CPW show the carbon content of both carbonaceous materials in Fig. 2 **(a) and (b)** confirming the successful carbonization of the biomasses. Furthermore, EDX analysis results and the selected areas

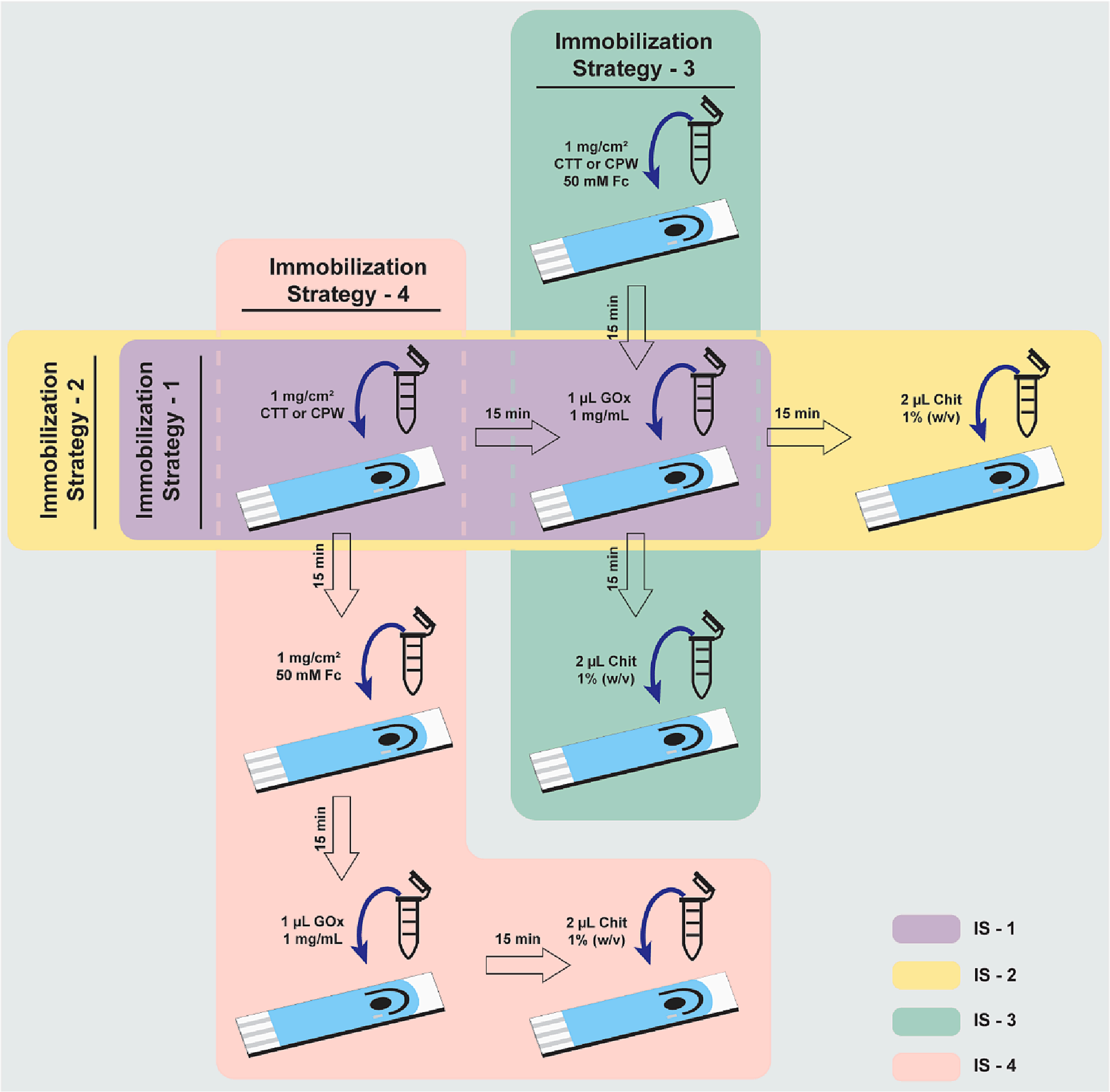

Fig. 1. Schematic representation of the enzyme immobilization strategies used in this study.

for the Mapping analysis for C and O elements were also shown in **Fig. S1-S2**.

Fig. 2 **(c)** shows the FTIR spectra of CTT and CPW before and after carbonization to illustrate the carbonaceous structure of the synthesized materials. The major IR spectrum bands that appeared at around 3300, 1300, and 1000 cm^{-1} for TT (Fig. 2 **(c)**) and PW (Fig. 2 **(c)**) show characteristics of a cellulosic structure. Specifically, the peaks around 3300 cm^{-1} can be assigned to hydroxyl group stretching, and the band at around 900–1000 cm^{-1} could indicate characteristics of β-glycosidic linkage between glucose units and -C-O- groups in the cellulose chain backbone [28]. The FTIR spectra of CTT and CPW, on the other hand, show characteristic resemblance with carbonaceous materials such as graphite or graphene [29]. The peaks between 1650 and 1700 cm^{-1} correspond to the aliphatic $C{=}O$ stretching. The peak between 1500 and 1600 cm^{-1} corresponds to the aromatic ring deformation. The weak band around 3000–3100 cm^{-1} is assigned to the stretching vibration of aromatic –CH [30]. The peak around 3300 cm^{-1} for RTT and RPW was shifted to around 3800 cm^{-1} due to the removal of bound water after the carbonization process.

XRD analysis was also performed to obtain information about the structure and chemical composition of CTT and CPW (Fig. 2 **(d)**). The broad diffraction patterns in the high angle range between $2\theta = 18$-28° were associated with the amorphous carbon structure [31,32]. The amorph and crystal structures between $2\ \theta = 27^\circ$, 40-45°, and 50-60° indicate characteristic graphitic structures [33]. Briefly, the first peak (0 0 2) indicates the formation of carbon layers [34], and characteristic carbon diffraction reflections of (1 0 0), (1 0 1), and (0 0 4) were also observed between $2\ \theta = 40$–55° [35]. As a result, SEM-EDX, FTIR, and XRD analysis showed that CTT and CPW were shown to have a carbonaceous structure after the carbonization process.

3.2. Electrochemical characterization of the CTT and CPW-modified electrodes

After the carbonization process, CTT and CPW were mixed with DMF, ethanol, and water for a range of concentrations to obtain the best results for homogenous coating on SPEs. The best results were obtained by dispersing 10 mg CTT or CPW in 1 mL DMF after sonicating for 3 h. After the initial preparation, the dispersions were sonicated for 15 mins before any electrode modification. First, different material loadings on

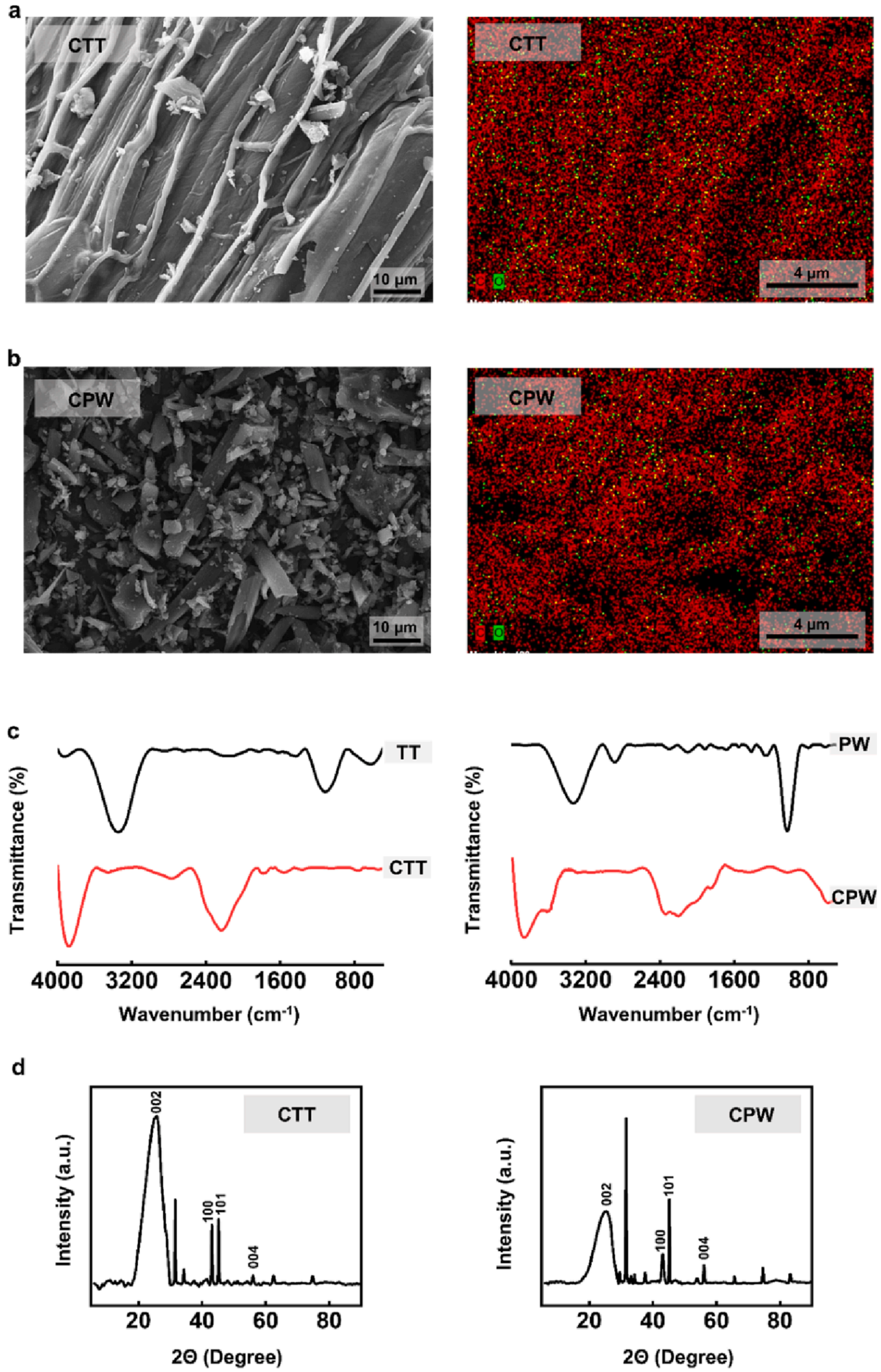

Fig. 2. (a) and (b) SEM-EDX MAPPING images, (c) FTIR spectra, and (d) XRD analysis of CTT and CPW.

SPEs were tested to optimize the best conditions for coating and electrochemical performance.

Fig. 3 **(a) and (b)** show the voltammograms at 50 mV/s at different materials loadings on SPEs tested in 1 mM FcCOOH for CTT (SPE/CTT) and CPW (SPE/CPW), respectively. A quasi-reversible chemical and electrochemical behavior were observed with a peak separation of ca. 80 mV and a peak current ratio (i_{pa}/i_{pc}) of ca. 2 for bare SPEs (0 mg/cm^2 loading). On the other hand, electrochemical reversibility was increased upon different material loadings reaching peak separation values of 60 mV and 65 mV for 1 mg/cm^2 loading of CTT and CPW, respectively [36]. The optimum material loading was also found to be 1 mg/cm^2 based on the electrochemical response, reproducibility of the response (relative standard deviation (RSD) % of 3.8 and 2.7 for CTT and CPW, respectively), and the physical constraints of the coating process on the electrode surface (Fig. 3 **(c) and (d))**. Although higher electrochemical currents were achieved for both modified electrodes, the RSD % increased due to the physical difficulties of the coating process causing material loss. The modified electrodes with the optimized material loading were also tested for their response to enzymatic reactions.

Fig. 3 **(e) and (f)** show the CV tests of the GOx coated (1 μL of 1 mg/mL) modified electrodes (SPE/CTT/GOx and SPE/CPW/Gox, respectively) tested in 1 mM FcCOOH containing different glucose

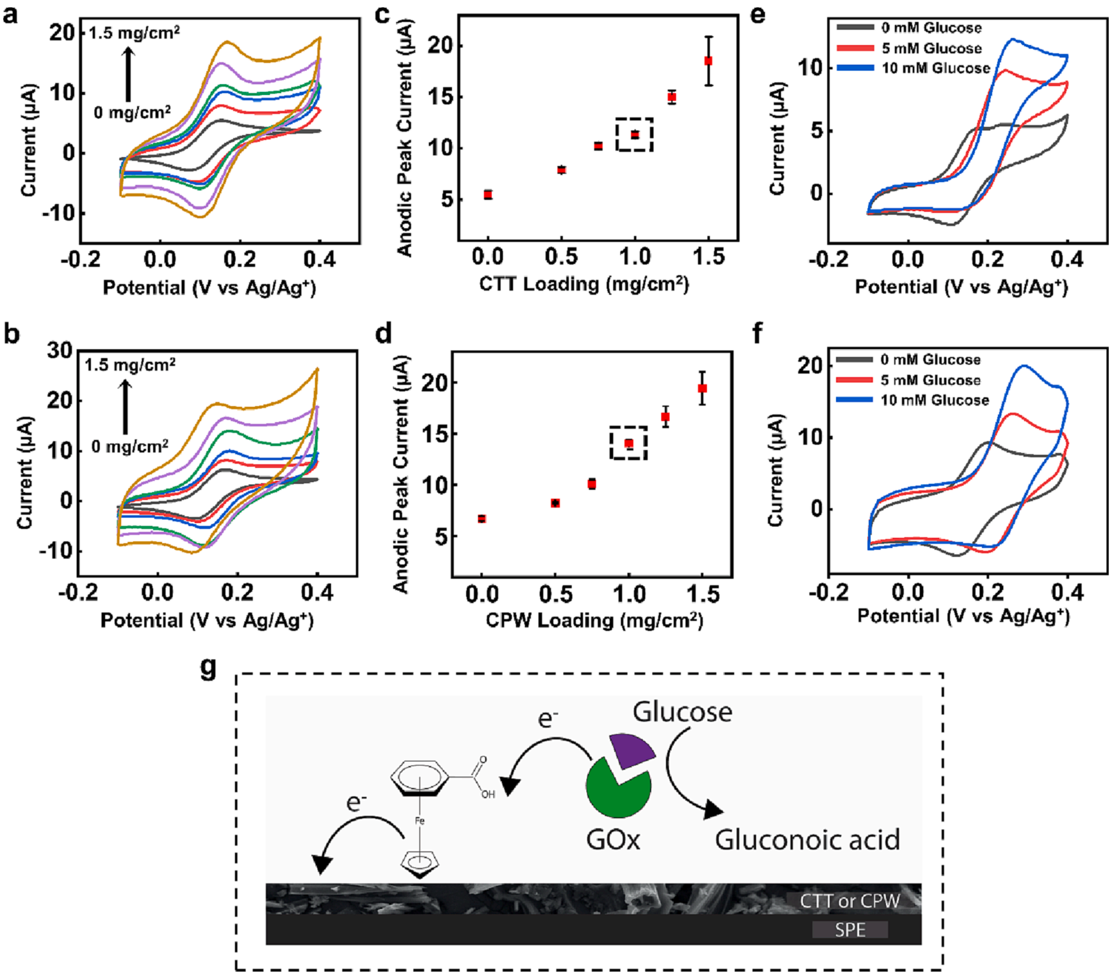

Fig. 3. **(a)** and **(b)** the voltammograms at 50 mV/s at different materials loadings on SPEs tested in 1 mM FcCOOH **(c)** and **(d)** anodic peak currents for different material loading, **(e)** and **(f)** CV tests of the GOx coated (1 μL of 1 mg/mL) modified electrodes (SPE/CTT/GOx and SPE/CPW/GOx, respectively) tested in 1 mM FcCOOH containing different glucose concentrations and **(g)** the schematics of the electron transfer route for the electrochemical enzymatic glucose oxidation reaction.

concentrations. For both modified electrodes with GOx, increasing oxidation and decreasing reduction peaks suggest a fast electrochemical reaction took place, and a proportional increase in the current was observed upon the addition of different glucose concentrations. Therefore, modifications of CTT and SPW might have enhanced the electrochemical performance of the bare SPEs. Enzyme immobilization was also successfully achieved and electrochemical glucose oxidation was performed in the presence of an electron transfer mediator. Fig. 3 **(g)** shows the schematics of the possible electron transfer route for the electrochemical enzymatic glucose oxidation reaction from the solution to the modified electrode surface.

3.3. Electrochemical performance investigation of different enzyme immobilization methods

In this study, four different electrode configurations were investigated for the electrochemical enzymatic glucose oxidation reaction. Initially, SPE/CTT and SPE/CPW electrodes were investigated using GOx enzyme immobilized on the electrode surface in FcCOOH solution as an electron transfer mediator. In enzyme immobilization processes, protective coatings with high protein affinity, mechanical stability, analyte permeability, reproducibility, and easy preparation are often preferred [37]. Therefore, Chit was chosen due to its suitable properties for enzyme immobilization and its biocompatibility.

Fig. S3 (a to f) shows the schematics of the electron transfer mechanism and electrochemical characterizations using CA for the first two immobilization strategies. Similar electroanalytical performance was obtained for the different immobilization strategies with a small drop in sensitivity when Chit was used. This type of behavior may be a result of mass transfer limitations from the Chit film, where film thickness limits the transport rate of glucose proportionally [38,39]. Furthermore, different molecular weights of Chit can also result in a similar response for glucose permeability [39]. Especially when low-molecular-weight Chit was used, as it was in this study, it was shown that better glucose transport can be obtained due to swelling of the Chit [40]. It can be concluded that the sensitivity decrease for Chit-modified electrodes could be related to the decreased glucose transport rate.

The effect of the electron transfer mediator was also investigated using two additional immobilization strategies. In the third strategy, CTT or CPW was applied to the SPE surface as one composite material. To achieve this, a composite dispersion was obtained using 50 mM Fc and 10 mg/mL of the CTT or CPW. The prepared composite was coated on SPEs to yield 1 mg/cm^2 coating amount denoted as SPE/CTT-Fc/GOx/Chit and SPE/CPW-Fc/GOx/Chit. Then, the final strategy was implemented using 50 mM Fc (1 mg/cm^2) as a separate redox active layer following the carbon material layer denoted as SPE/CTT/Fc/GOx/Chit and SPE/CPW/Fc/GOx/Chit. In these latter modifications, closer electron transfer distance was aimed by using the Fc either embedded inside the conductive material or as a redox-active layer.

Fig. 4 **(a) and (b)** show the schematic of the electrochemical test

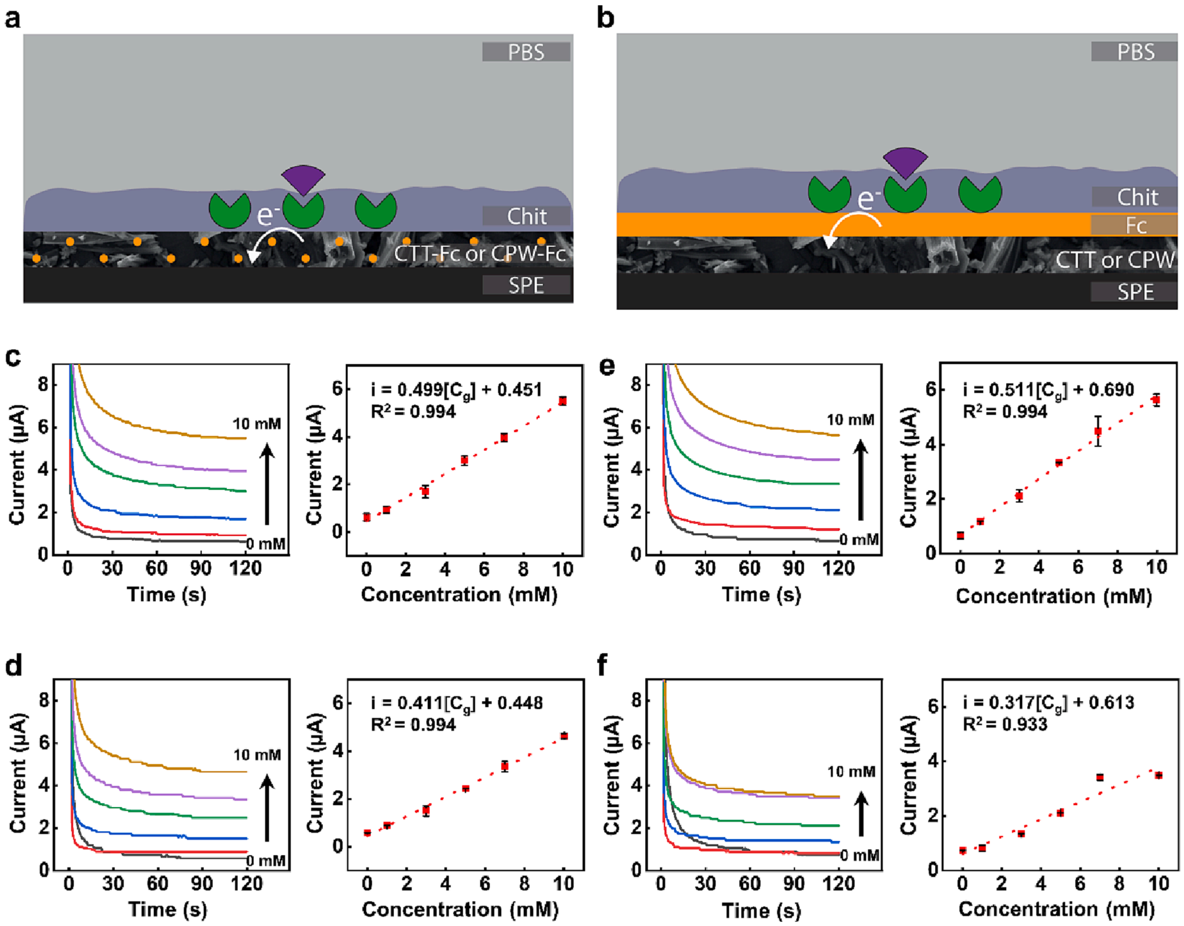

Fig. 4. **(a)** and **(b)** the schematic of the electrochemical test mechanism using the third and fourth immobilization strategies, **(c)** and **(d)** CA responses of CTT and CPW-modified electrodes for 120 s using the third immobilization strategy and respective calibration curves, **(e)** and **(f)** CA responses of CTT and CPW-modified electrodes for 120 s using the fourth immobilization strategy and respective calibration curves tested at 0.3 V (vs Ag/Ag$^+$) with increasing glucose concentrations up to 10 mM.

mechanism for SPE/CTT/Fc/GOx/Chit and SPE/CPW/Fc/GOx/Chit electrodes. These configurations were tested in PBS solution since the electron transfer mediator was modified on the electrode surface. Fig. 4 **(c), (d), (e), and (f)** show the CA responses recorded for 120 s and respective calibration curves in the presence of different glucose concentrations. A linear response to glucose for both methods was obtained at 0.3 V (vs Ag/Ag$^+$) with increasing glucose concentrations up to 10 mM. Both methods showed similar performance in terms of sensitivity and accuracy. However, SPE/CPW-Fc/GOx/Chit showed a notable performance decrease compared to other electrode configurations. This might be due to the non-homogenous interactions between Fc film and CPW causing unstable performance.

Fig. 5 **(a)** shows, on the other hand, that the average sensitivity of the CTT electrode modified with Chit was significantly improved for ca 80% compared to the case where Fc was used in the solution. This indicates that Fc, either embedded in CTT or as a separate film, showed a more efficient electron transfer than when used in the solution. This might be related to eliminating possible mass transport limitations in the solution.

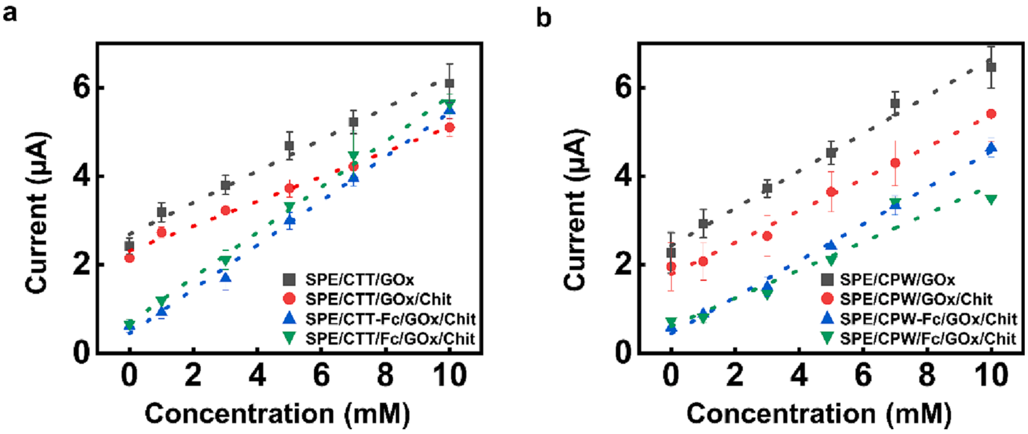

Fig. 5. Comparison of the calibration curves of different immobilization strategies using **(a)** CTT and **(b)** CPW.

On the other hand, a similar but lower increase of ca 15% was observed for the CPW electrode in the case of Fc used as embedded in the CPW Fig. 5 **(b)**. Like previous investigations when Fc was used as a film on top of the CPW layer the sensitivity of the electrode was not improved, yet negatively affected. However, in all the cases where Fc was not in solution, the baseline response (0 mM glucose) was significantly reduced due to better communication of the mediator with the electrode.

The electroanalytical performance of the SPE/CTT-Fc/GOx/Chit showed really promising performance for biosensing applications, specifically glucose in this special configuration. The glucose biosensor using this strategy can detect glucose concentrations between 0 and 10 mM with a limit of detection (LOD) and limit of quantification (LOQ) values of 0.97 mM and 3.2 mM, respectively (LOD: 3.3SD/Slope and LOQ = 10SD/Slope [41]).

4. Conclusion

In this study, a comprehensive investigation of the different enzyme immobilization strategies has been performed using novel carbonaceous electrodes. The carbonization of the TT and PW has been achieved under an inert atmosphere at high temperatures and the resulting CTT and CPW were then modified on SPEs. Four different immobilization strategies were employed to optimize the best conditions for the use of CTT and CPW for the electrochemical enzymatic oxidation of glucose. This study demonstrates an understanding of the use of carbonaceous electrodes in bioelectrochemical applications using a comparative approach and can pave the way towards the use of such materials in biomass to bioelectronics applications. All immobilization strategies show good analytical performance from which SPE/CTT-Fc/GOx/Chit was the most promising configuration with LOD and LOQ values of 0.97 mM and 3.2 mM, respectively operating up to 10 mM glucose. Further optimization and real sample analysis should be performed to investigate the use of the CTT and CPW as alternative conductive carbonaceous materials in biosensor design. If used as a glucose biosensor, the developed enzymatic electrode could be very promising for many applications such as medical sensors or food safety analysis.

CRediT authorship contribution statement

Şevki Furkan Küçükayar: Methodology, Validation, Investigation, Visualization, Writing – original draft. **Veli Şimşek:** Validation, Investigation, Writing – original draft, Supervision. **Mustafa Oguzhan Caglayan:** Conceptualization, Methodology, Supervision. **Zafer Üstündağ:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing. **Samet Şahin:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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