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Bioelectrochemical Behavior of the Composite PVP-Os/chitosan as a Mediator with Different Types of Enzymes at Graphite Electrode

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Abstract

Chitosan was cross-linked to an osmium redox polymer, poly(4-vinylpyridine) osmium bipyridyl [PVP-Os-$(bpy)_2$-Cl], to form PVP-Os-$(bpy)_2$-Cl/chitosan composite known to make a porous and hydrophilic film with an enzyme. In this work we demonstrate such a composite is a useful trestle, for hosting various sugars oxidizing enzymes to construct biosensors. Glucose sensing ability has been proven with the following glucose oxidizing redox enzymes; *Aspergillus niger* glucose oxidase (*An*GOX), *Myriococcum thermophilum* cellobiose dehydrogenase, (*Mt*CDH), glycosylated *Agaricus meleagris* pyranose dehydrogenase (g*Am*PDH), fragmented deglycosylated *Agaricus meleagris* pyranose dehydrogenase (fdg*Am*PDH), and *Aspergillus* sp. glucose dehydrogenase (*As*pGDH), as well as recombinant *Glomerella cingulata* glucose dehydrogenase (r*Gc*GDH).

Keywords: Composite PVP-Os-$(bpy)_2$-Cl/chitosan; Chitosan; GDH; PDH; CDH

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Introduction

Recently researchers have been focused on introducing new methods for development of electrochemical biosensors based on redox enzymes. Their efforts have been directed towards introducing a mediator with an efficient electrons transfer rate both with the enzyme active site as well as with the electrode, since a direct electron transfer mechanism between the electrode and the enzyme active site is in most cases not efficient. This is a consequence of that enzyme active site is deeply buried inside the enzyme structure. In addition it is in principle impossible to fix all enzyme molecules orientated with the active site facing the electrode surface. To facilitate electron transfer and significantly increase current density for electrochemical biosensors, it is common to use a mediator that is either present in solution or covalently attached to a polymer or a sol-gel matrix [1]. Previous study [2] showed that the ideal immobilization process has to be cheap, quick, enzyme friendly, beneficial or feasible for a wide range of biomolecules, and provide with a requirements for successful immobilization of enzyme through a biocompatible and inertness matrix, to retain the native structure of the enzyme and its biological activity. Thus, the common choice was by using polysaccharide-based supports, for the construction of biosensor. Hence chitosan was proven to be such a successful matrix [3]. Chitosan is a linear polysaccharide that has been well studied [4]. Its structure consists mainly of (1-4) linked 2-amino-2-deoxy-β-D-glucopyranose units. It is a natural polymer that can be obtained through partial de-acetylation of chitin [5,6]. The chitosan backbone has a matchless feature, due to the presence of primary amine functionalities in C-2 position of glucosamine residues, which play important roles in the chitosan functionality that can be exploited for biofabrication [7,8]. Previously was found

[4] that chitosan has excellent membrane forming abilities, good biocompatibility, high permeability towards water, non-toxicity, and high mechanical strength. Osmium-based redox polymers have been used for decades e.g., in [9-11] to ''wire'' different enzymes, membranes, and bacterial cells [12-15] to various electrodes, such studies were performed either to construct or improve the performance of biosensors. Due to the a capability of osmium-polymer for facile, reversible electron transfer in combination with the ease to control their redox potential through either changing the ligand or the polymer backbone structure, poly (4-vinylpyridine) (PVP), polyvinylimidizole (PVI) based osmium polymers were successfully used for mediated electron transfer [16]. Hence, Os-polymers form, through electrostatic binding of the enzyme, a 3D hydrogel matrix suitable for immobilizing enzymes at the electrodes surface, that can be further stabilized through cross-linking [17,18]. Thus co-immobilization of enzymes with mediators attached to a flexible hydrophilic backbone in the matrix-film, results in a good communication between both the enzyme-polymer redox centers as well as the polymer redox centers-electrode surface that in turn results in an efficient bioelectrocatalytic activity. More recently, Shin and his co-workers succeeded in using the polymer PVP-Os-$(bpy)_2$-Cl to form a composite that was covalently connected to chitosan producing a reactive matrix well adapted for enzyme immobilization. Such composite showed a porous form that positively reflected on the electron transfer rate, and revealed a significant enhancement in the current density for glucose oxidation based on immobilization of glucose oxidase (GOx) in such a composite [19]. Here, we introduce the use of the same Os-polymer, PVP-Os-$(bpy)_2$-Cl, after being covalently attached to chitosan through either glutaraldehyde or poly(ethylene glycol) diglycidyl ether as cross-linker, to form a three dimensional structure with mediating properties, which is mixed with six different sugar oxidizing enzymes. These were glucose oxidase from *Aspergillus niger* (*An*GOx), cellobiose dehydrogenase from *Myriococcum thermophilum* (*Mt*CDH), two different varieties of pyranose dehydrogenase from *Agaricus meleagris* [20], one variant overexpressed in *Pichia pastoris* and therefore substantially glycosylated (g*Am*PDH) [21] and the second variant that was enzymatically deglycosylated and further, fragmented a result of long-term storage in buffer (fdg*Am*PDH) [21] and FAD dependent glucose dehydrogenase from *Aspergillus* sp. (*As*pGDH) [22], and another FAD dependent glucose dehydrogenase from *Glomerella cingulate* recombinantly expressed in *Pichia pastoris* (r*Gc*GDH) [22,23]. These enzymes have been selected for this study for the following reasons: for instance *An*GOx, *Asp*GDH, and r*Gc*GDH have demonstrated to have a good affinity for glucose [22]. When the effect of deglycosylation of g*Am*PDH was investigated, the deglycosylated form that was stored in buffer at 4°C was fragmented into a highly catalytically active form fdg*Am*PDH and a small inactive polypeptide. To estimate the effect of the molecular weight of the enzyme on the response, the two forms allow different diffusion rates of the substrate, and the different capability in the electron transfer rate between the active site and the electrode accordingly [21]. For *Mt*CDH it is known from previous experience, that this enzyme has a good affinity towards lactose as substrate, but lower affinity for glucose [24]. Here we wanted to investigate whether there is an improvement in its response for glucose according to this new approach. These six different enzymes have been successfully immobilized into the matrix trestle film, and it has been shown that there is a possibility to exploit this protocol for improving glucose sensing.

Materials and methods

Chemicals and equipment

The redox polymer poly (4-vinylpyridine) osmium bipyridyl, PVP-Os-$(bpy)_2$-Cl was synthesized according to a reported procedure and obtained as a powder [19]. Glutaraldehyde (25% in water solution), chitosan (low molecular weight, degree of deacetylation 85-90%) were obtained from Sigma Aldrich Co., St. Louis, Mo, USA and glucose oxidase (from *Aspergilus niger, An*GOx, 50.000 units, 10 mg/ml) were obtained from the Sigma-Aldrich, where one unit will oxidize 1.0 µmole of β-D-glucose to D-gluconic acid and H_2O_2 per min at pH 5.1. Glucose dehydrogenase from *Glumerella cingulata* recombinantly expressed in *Pichia pastoris* (r*Gc*GDH) with a molecular mass of 88-131 kDa (glycoforms) was a liquid preparation with a protein concentration of 15 mg mL^{-1}, and a specific activity of 836 Umg^{-1} [22]. Glucose dehydrogenase from *Aspergillus* sp. (*As*pGDH) (molecular mass 97 kDa, volumetric activity 6,500 Uml^{-1}), it was a gift by Genzyme (www.genzymediagnostics.com), 50 Gibson, Sekisui UK Ltd. Pyranose dehydrogenase from *Agaricus meleagris* (*Am*PDH) was recombinantly expressed in *Pichia pastoris*, and therefore heavily glycosylated (gPDH). A portion was then later-deglycosylated with endoglycosidase H (Endo H from New England Biolabs, Bionordiska AB, Stockholm, Sweden) to form dg*Am*PDH, according to a previously described protocol [20]. The deglycosylated form (dg*Am*PDH) was found to spontaneously fragment to form a catalytically highly active form (fdg*Am*PDH) when stored at 4ºC. The activities of g*Am*PDH and fdg*Am*PDH were determined according to a standard photometric assay reported in [21,25]. Cellobiose dehydrogenase from *Myriococcum thermophilum* (*Mt*CDH) was obtained as a solution with a protein concentration of 5.3 mg/ml and a volumetric activity of 9.8 Uml^{-1} (DCP assay, pH 5.5, 30ºC). 0.1 M phosphate buffer solution (PBS, pH 7.4), was prepared from sodium dihydrogen phosphate, purchased from BDH Analar, VWR International Ltd., Poole, BH15 1 TD, England, and disodium hydrogen phosphate dehydrate from Sigma-Aldrich. Glutaraldehyde (GA) and β-D (+) glucose from Sigma-Aldrich, poly-(ethylene glycol)-diglycidyl ether (PEGDGE) was purchased from Polysciences (Warrington, PA, USA). All aqueous solutions were prepared with water purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Cyclic voltammetry experiments were performed with an Auto lab PGSTAT30 (Utrecht, The Netherlands) equipped with GPES 4.9 software using a three-electrodes configuration with an Ag|AgCl (KCl sat.) reference electrode, a platinum foil counter electrode and the modified graphite electrodes (40765 Graphite rod, ∅ 3.05 mm, 0.073 cm^2 geometric surface area, Alfa Aesar, Germany), as working electrode. Before use the graphite electrodes were polished on wet fine emery paper type (Tufback Durite, P1200) and then carefully rinsed with Milli-Q water. Nitrogen gas was

purged through the solution cell for at least 20 min prior the CV measurements.

Electrodes preparation

The PVP-Os/chitosan composite was prepared by mixing 10 µL of an aqueous solution of PVP-Os (5 mg ml^{-1} in water) with 50 µL of a 1% chitosan solution (in 1% acetic acid solution, pH 4.5) followed by either the addition of 5 µL of a 0.5% GA (in water solution) or the addition of 2.5 µl of freshly prepared 10 mg ml^{-1} PEGDGE-water solution. The PVP-Os polymer solution or the PVP-Os/chitosan composite were well mixed with 5 µL of enzyme solution to prepare a mixture composite of (enzyme/PVP-Os) as well as (enzyme/PVP-Os/chitosan) as reported in [19]. The solution matrix was mixed well and kept overnight at 4°C before use. 1 µL of the hydrogel matrix was drop coated on the surface of a graphite electrode and, left to dry for at least 1-2 h at room temperature before use.

Results and Discussion

The *An*GOx was already investigated in a previous study [19]. In this study the investigation was extended to include a number of other sugar oxidizing enzymes, r*Gc*GDH, *Asp*GDH, g*Am*PDH, fdg*Am*PDH, and *Mt*CDH to find out whether the catalytic behavior of these electrodes based on composite PVP-Os/chitosan with the different enzymes will benefit from the composite.

PVP-Os/chitosan/*An*GOx and PVP-Os/*An*GOx films

Composite films of PVP-Os/chitosan and PVP-Os polymer based *An*GOx have been used with GA as a cross-linker to modify graphite electrodes, and the cyclic voltammograms (CVs) were recorded in 0.1 M phosphate buffer solution (PBS) pH 7 (blue color) and in 15 mM glucose (red color) for two sets, A and B at a scan rate of 5 mV/s **(Figure 1).** Both the PVP-Os/chitosan/GOx and PVP-Os/GOx based modified electrodes show well-defined bioelectrocatalytic behaviors, where the PVP-Os/chitosan/GOx electrode **(Figure 1A)** exhibits seven times higher response, compared with that of the PVP-Os/GOx electrode **(Figure 1B)**. These results were estimated from a series of experiments using three equivalently prepared electrodes for both types of modified electrodes. The results are in high agreement with previously obtained results [19].

PVP-Os/chitosan/fdg*Am*PDH and PVP-Os/fdg*Am*PDH films

The electrochemical behaviors of all the other enzymes were instead incorporated into the PVP-Os/chitosan and PVP-Os polymer through PEGDGE, and not GA. On the basis of previous investigations that PEGDGE preserves enzymatic activity and produce biosensors with both similar or better sensitivity and response time than those with GA fixation [26]. Additionally, our results revealed better improvement in the catalysis signals by using the PEGDGE linker. This phenomenon could be explained by giving an appropriate time for a composite matrix to react and to control the best orientation of the redox centers of the enzyme active sites, at which maximal catalytic behavior can be obtained. The cyclic voltammetric results for the various glucose oxidizing enzymes are shown in (Figures 1-6). in the absence and presence of 15 mM glucose in PBS at pH 7.4. A solution of 15 mM glucose was chosen, to obtain a clear difference in catalytic signals in

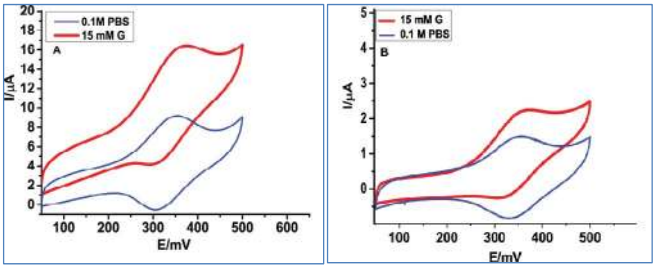

Figure 1 Cyclic voltammograms of (A) PVP-Os/chitosan/*An*GOx and (B) PVP-Os/ *An*GOx, in 0.1 M PBS pH 7.4 (blue color) and in 15 mM glucose (red color), vs. Ag|AgCl (KCl sat.) 5 mV/s scan rate.

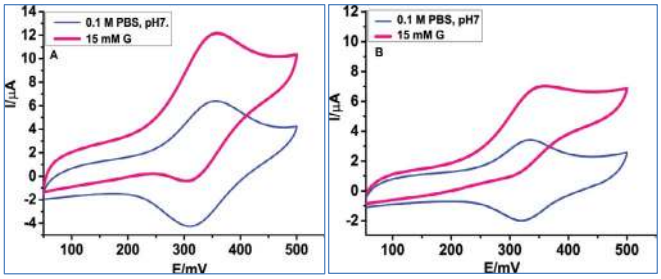

Figure 2 Cyclic voltammograms of (A) PVP-Os/chitosan/fdg*AmPDH*, and (B) PVP Os/fdg*AmPDH* in 0.1 M PBS pH 7.4 and in 15 mM glucose vs Ag|AgCl (KCl sat.), and a 5 mV/s scan rate.

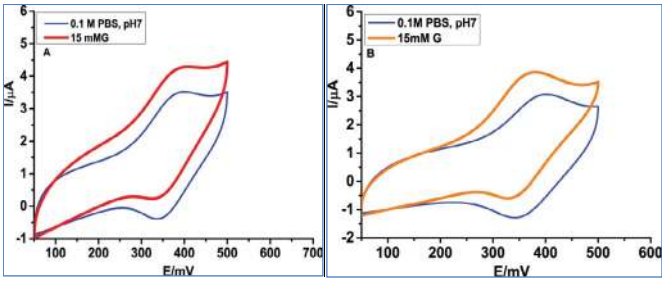

Figure 3 Cyclic voltammograms of (A) PVP-Os/chitosan/g*Am*PDH, and (B) PVP-Os/ g*Am*PDH, at scan rate 5 mV/s and 15 mM glucose in 0.1M PBS pH7.4 vs. Ag|AgCl (KCl sat.).

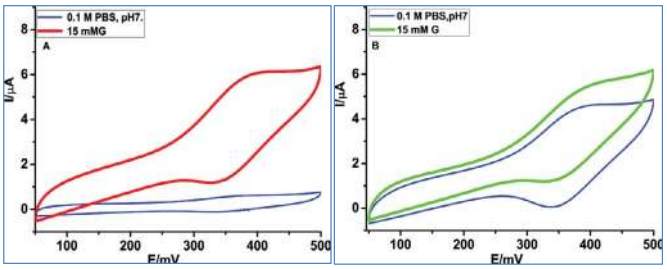

Figure 4 Cyclic voltammograms of (A) PVP-Os/chitosan/r*GcG*DH, and (B) PVP-Os/rGcGDH, at scan rate 5 mV/s, in 0.1 M PBS pH 7.4 and in 15 mM glucose vs. Ag|AgCl (KCl sat.).

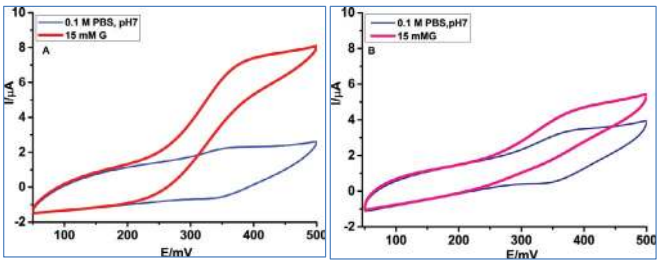

Figure 5 Cyclic voltammograms of (A) PVP-Os/chitosan/*Asp*GDH, and (B) PVP-Os/*Asp*GDH, at scan rate 5 mV/s and 15 mM glucose in 0.1 M PBS pH 7.4 vs Ag|AgCl (KCl sat.).

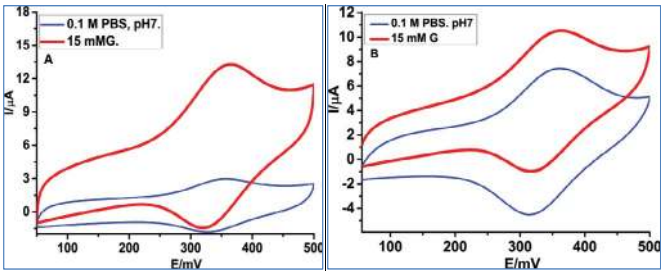

Figure 6 Cyclic voltammograms of (A) PVP-Os/chitosan/*Mt*CDH, and (B) PVP-Os/*Mt*CDH, at scan rate of a 5 mV/s. And 15 mM glucose in 0.1M PBS pH 7.4 vs. Ag|AgCl (KCl sat.).

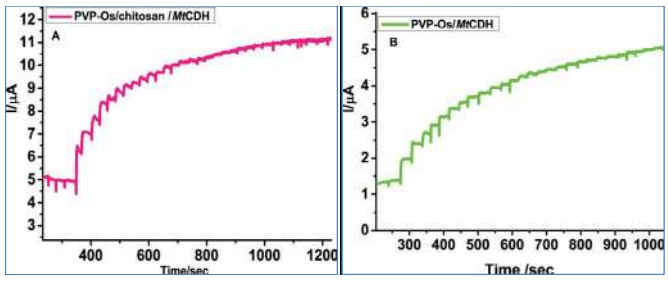

Figure 7 Amperometric measurement of (A) PVP-Os/chitosan/*Mt*CDH, and (B) PVP-Os/*Mt*CDH, recorded in the consecutive addition of 300, 600 mM glucose to the 10 mL of 0.1 M PBS pH 7.4 at 0.45 V, vs. Ag|AgCl (KCl sat.).

presence/absence of glucose, based on previous investigations reported for *An*GOx [1,19] alongside our investigations that involved the following enzymes; e.g., fdg*Am*PDH, g*Am*PDH [21], r*GcG*DH and *Asp*GDH [22]. On the other hand, *Mt*CDH was also investigated in this study under the same conditions as the other enzymes, even though this enzyme has demonstrated less affinity for glucose [24].

Hence, the composite films PVP-Os/chitosan and PVP-Os were blended with fdg*Am*PDH to make two sets of electrodes. The voltammetric responses were recorded for these two sets at 15 mM glucose and 0.1 M PBS, pH 7.4 (Figure 2). Both the PVP-Os/chitosan/fdg*Am*PDH (Figure 2A) and PVP-Os/fdg*Am*PDH (Figure 2B) modified graphite electrodes exhibited high catalytic currents for glucose oxidation. However, the PVP-Os/chitosan/fdg*Am*PDH electrode exhibited a 6 fold increase in the anodic peak value (I_{pa}) in the presence of 15 mM glucose i.e., 12 µA, while the I_{pa} for that obtained from the PVP-Os/fdg*Am*PDH electrode was just increased two fold, to give 7 µA. Moreover, the PVP-Os/chitosan/fdg*Am*PDH electrode revealed a noticeable decrease in the anodic potential (E_{pa}) from 291 mV to a 224 mV, but the PVP-Os/fdg*Am*PDH showed a less decrease in the E_{pa} value end with from 293 to 245 mV.

PVP-Os/chitosan/g*Am*PDH and PVP-Os/g*Am*PDH films

The g*Am*PDH modified electrodes based on a PVP-Os/chitosan and PVP-Os polymer, respectively, were investigated with cyclic voltammetry and the responses for both electrodes were recorded as was previously described for the fdg*Am*PDH modified electrodes. However, it was found that there was not much difference in the catalytic behavior at the two different electrodes **(Figure 3)**. Since the observed difference in the values of I_{pa} at the PVP-Os/chitosan/g*Am*PDH and PVP-Os/g*Am*PDH films were just 4.5 µA and 4 µA, respectively, that may be attributed to the higher molecular weight of g*Am*PDH compared to that of fdg*Am*PDH, which might cause slow mass transport in the matrix-film of the hydrogel in spite of the porous nature of the composite PVP-Os/chitosan film **(Figures 3A and 3B)** as well as to the more deeply buried active site [21].

PVP-Os/chitosan/r*GcG*DH and PVP-Os/r*Gc*GDH films: When using r*GcG*DH in combination with the composites PVP-Os/chitosan, and PVP-Os polymer on graphite electrodes, the voltammetric responses showed catalytic current signals for both modified electrodes at a value of 6.5 µA and 6 µA respectively, see (Figures 4A, 4B). However, a 6 times higher I_{pa} was observed for the PVP-Os/chitosan/r*Gc*GDH based electrode in the presence of 15 mM glucose compared with no glucose (Figure 4A), on the other hand such a difference was only once for the PVP-Os/r*Gc*GDH based electrode (Figure 4B). On the other hand the modified electrode of PVP-Os/chitosan/r*Gc*GDH showed more pronounced decrease in the E_{pa} value, compared with a less negative shift in the E_{pa} at the PVP-Os/r*Gc*GDH based electrode.

PVP-Os/chitosan/*Asp*GDH and PVP-Os/*Asp*GDH films: When using *Asp*GDH in combination with PVP-Os/chitosan and PVP-Os polymer on graphite electrodes, the catalytic behavior of the recorded cyclic voltammograms showed a well sigmoidal behavior for the electrodes also containing chitosan (Figure 5), which refers to the good communication between the FAD containing active site of the enzyme and the polymer [1]. The catalysis in the presence of chitosan in a 15 mM glucose solution the I_{pa} showed a 5 times higher signal with a noticeable negative shift in the E_{pa} value (Figure 5A) in comparison to the PVP-Os/*Asp*GDH modified electrode that gave just one time increase in the I_{pa} and a less negative shift in the E_{pa} **(Figure 5B).**

PVP-Os/chitosan/*MtC*DH and PVP-Os/*MtC*DH film: *Mt*CDH was incorporated in the matrix film of PVP-Os/chitosan and PVP-Os polymer, to modify graphite electrodes, and the catalytic behavior of CVs for glucose oxidation were registered and showed excellent defined catalytic signals, with a 10 fold raise in the response signal, as well as a high decrease in the E_{pa}, where the I_{pa} was equal to 14 µA in the presence of 15 mM glucose (Figure 6A) whilst the I_{pa} of PVP-Os/*Mt*CDH was shown just 11 µA within

a 3 fold increase in the catalysis signal, and a less shift in the E_{pa} see (**Figure 6B)**

Amperometric measurement for glucose sensing responses at both PVP-Os/chitosan and PVP-Os based on *Mt*CDH

We chose *Mt*CDH among the studied enzymes for further studies as an appropriate model to estimate the amperometric parameters of the biosensors, since this enzyme i) has shown higher catalytic signals among those studied, ii) has shown a distinct glucose sensing even though this enzyme has a less affinity for glucose in comparison with lactose, this might provide us with a sufficient evidence for succeeding this approach, for the capability of making glucose biosensor based on such enzyme. Hence, the amperometric measurements were obtained for the PVP-Os/chitosan/*Mt*CDH and PVP-Os/*Mt*CDH based modified electrodes, via a sequential addition of 250 µL of 300 mM then 600 mM of glucose each 25 s to the 10 mL of 0.1 M PBS, pH 7.4 under continuous stirring, applying 0.45 V vs. Ag|AgCl (KCl sat.). The corresponding amperometric responses are shown in **(Figure 7)**. As can be seen from the two plots of current versus [glucose] the anodic current increases as the glucose concentration is increased and the catalytic current reaches a steady state value. The catalytic current of the PVP-Os/chitosan/*Mt*CDH modified electrode revealed a much higher enhancement signal **(Figure 7A)** than that of PVP-Os/*Mt*CDH (Figure 7B). Calibration curves for glucose sensing capability were estimated accordingly; see **(Figures 8A and 8B).** The catalytic current values were divided by the geometric surface area of the modified graphite electrode, resulting in current density plots vs. glucose concentration, the PVP-Os/chitosan electrode based on *Mt*CDH **(Figure 8A)** showed (154 ± 0.1) µA cm^{-2} and 0.86 µA mM cm^{-2} current density and sensitivity, respectively. In contrast the PVP-Os/MtCDH modified electrode (Figure 8B) showed only 68.05 ± 0.3 µA cm^{-2} and 0.234 µA mM cm^{-2}. The apparent Michaelis-Menten constant (K_M^{app}) was estimated from Michaelis-Menten equation rearrangement of the Lineweaver-Burk plot [27]. It is known that the value of K_M^{app} reflecting both the affinity of the enzyme towards substrate and any mass transfer resistance of the substrate through the polymer [22]. In this approach *Mt*CDH was immobilized with the same volume of the hydrogel-film, on both types of modified electrode surfaces, viz PVP-Os/chitosan/*Mt*CDH and PVP-Os/*Mt*CDH. Thus any one of the tested electrodes that shows a lower K_M^{app} value refers to an improvement in the affinity of this enzyme for its substrate, which is expected to be, as a result of the porous nature of PVP-Os/chitosan/*Mt*CDH based electrode, that promotes the diffusion rate of substrate through the matrix film. Accordingly, the PVP-Os/chitosan showed a lower K_M^{app} that equal to 30 ± 2 mM **(Figure 8A)** while the K_M^{app} of the PVP-Os based electrode was 120 ± 0.2 mM **(Figure 8B).** This value is similar to that in the literature [24]. It can be deduced that the composite PVP-Os/chitosan caused a significant enhancement in the performance of the biosensor from the evaluated values of K_M^{app}, current density, and sensitivity, all offer evidence for the beneficial behavior of the composite PVP-Os/chitosan bioelectrode. As this composite provides a suitable biocompatible environment, retaining the bioactivity through a stable immobilization of the enzyme with an effective communication between the FAD-containing dehydrogenase domain of *Mt*CDH and the Os-polymer redox centres. Moreover, these results suggest that the hydrophilic properties of chitosan cause a facile transport of all the species

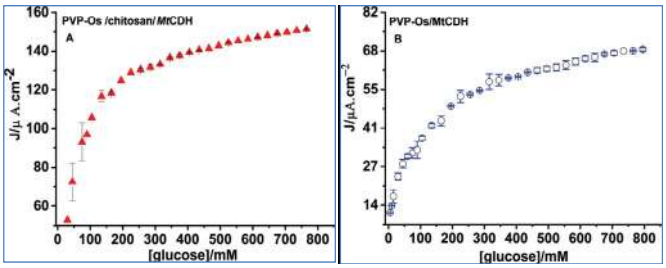

Figure 8 Current density versus glucose concentration of (A) PVP-Os/chitosan/*Mt*CDH, and (B) PVP-Os/*Mt*CDH at 0.45 V, vs. Ag|AgCl (KCl sat.).

involved in the electrocatalytic process that facilitate electronic and ionic transport, through the porous structure of the bio-composite film, resulting in the decrease in the formal potential of the PVP-Os/chitosan and an increase in the catalytic current [1,19].

Scanning electron microscopy of naked graphite and of PVP-Os/chitosan and PVP-Os modified graphite electrodes

Scanning electron microscopy (SEM) of the surface of both the composite PVP-Os/chitosan and PVP-Os polymer based electrodes as well as of the naked graphite surface **Figure 9**. Results of the SEM images exhibit a porous and grainy structure-film for the PVP-Os/chitosan modified surface **(Figure 9)**, at two magnifications, 8000 × , and 2100 × see **(Figures 9A and 9B)** respectively. In comparison the PVP-Os modified surface shows a more homogenous smooth film see **(Figures 10A and 10 B)** at 100 × and 8000 × at A, and B respectively. Further the SEM results of PVP-Os were compatable with the SEM images of bare graphite tested at the same magnifications, see **(Figures 10 C and 10 D)** at 100 × and 8000 × for C and D, respectively. These results were in agreement with previous investigations [19].

Conclusion

PVP-Os was cross-linked to chitosan, through either GA or PEGGDGE. The modified GE based on either the composite mixture of PVP-Os/chitosan or the PVP-Os were successfully studied and characterized. The catalytic voltammetric behavior of the composite PVP-Os/chitosan with all the above investigated enzymes, except g*Am*PDH exhibited an enhanced conversion rate towards the oxidation of glucose. The obtained results are considered to be a good indication of stable incorporation of such enzymes into the porous structure matrix–film that has been demonstrated through the SEM-images, g*Am*PDH showed less conversion rate for glucose oxidation among the investigated enzymes. The reason for that might be attributed to a deeply buried active site in addition to a slow mass transport of the high molecular mass through the porous film compared with fdg*Am*PDH. While *Mt*CDH shows a higher current density, sensitivity, and lower K_M^{app} value based on PVP-Os/chitosan than that of PVP-Os without chitosan. Due to the output results assessment, this approach can be considered as promising candidate for improving the performance of biosensors.

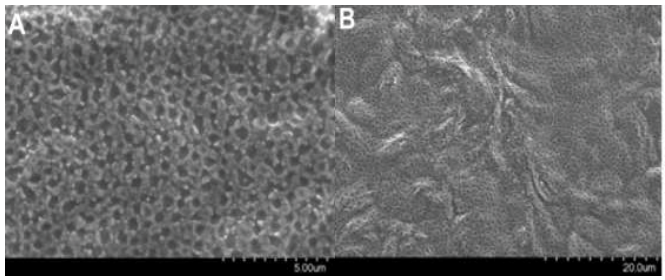

Figure 9 SEM-images shows a grainy and porous structure of the composite GE/PVP-Os/chitosan, at two magnifications (A) 8000 X and (B) 2100 X.

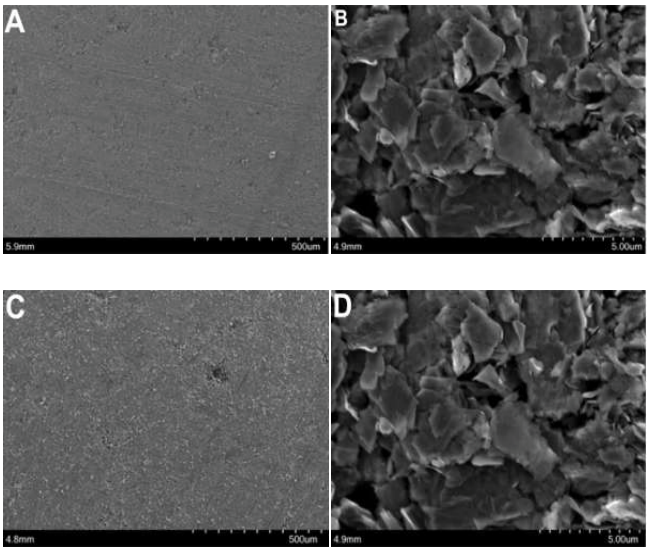

Figure 10 SEM-images showing the non-porous structure for PVP-Os at two magnifications (A) 100X and (B) 8000X compared with a bare GE at the same magnifications (C and D).

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