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Amperometric Flow Injection Analysis of Glucose and Galactose Based on Engineered Pyranose 2-Oxidases and Os Polymers for Biosensor Applications

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Abstract

In the present study, wild type and three mutants of pyranose 2-oxidase (PyOx), which showed improved properties for D-galactose oxidation, were investigated for their oxidising ability when immobilised on graphite electrodes. Four different flexible Os polymers with formal potentials ranging between -0.140 and 0.270 V vs. $Ag|AgCl_{0.1\ M\ KCl}$ were applied together with the various forms of PyOx to wire graphite electrodes using polyethylene glycol diglycidyl ether as crosslinking reagent. The pH profiles for the electrodes modified with wild type and all PyOx mutants in combination with Os polymers were investigated with both glucose and galactose respectively, since the PyOx variants showed improved catalytic activity for galactose. All modified electrodes showed highest response in the pH range between 8.5-10 and K_M, I_{max} values for the both substrates glucose and galactose were determined. To prove the catalytic activity, the biosensors were also characterized with cyclic voltammetry. The protein amount 0.26 U was found optimum for PyOx-WT, 0.36 U for PyOx-MT1, 0.41 U for PyOx-MT2 and 0.28 U for PyOx-MT3 and analytical characterization of the enzyme electrodes was performed for glucose and galactose under optimized conditions.

Keywords

Biosensor, biofuel cell anode, pyranose oxidase, osmium redox polymers, glucose, galactose.

1 Introduction

Pyranose oxidase (PyOx, pyranose:oxygen 2-oxidoreductase, E.C. 1.1.3.10, glucose 2-oxidase) oxidizes glucose as well as other monosaccharides that are found as constituents of hemicelluloses including D-xylose, D-galactose, and L-arabinose, albeit with lower catalytic efficiencies. PyOx is a relatively large flavin adenine dinucleotide (FAD) containing

glycoprotein (ca. 300,000 kDa) produced by white rot fungi, with the FAD covalently attached to the polypeptide backbone [1, 2]. PyOx catalyzes a Ping Pong Bi Bi type reaction that consists of a reductive half reaction in which an aldopyranose substrate reduces the FAD cofactor to yield $FADH_2$ and 2-dehydroaldose, as the result of oxidation of the sugar at position C-2, and the ensuing oxidative half-reaction, which involves the re-oxidation of $FADH_2$ by an electron acceptor (Figure S1) [3-5]. During this oxidative half-reaction a C-4a-hydroperoxyflavin intermediate is formed when oxygen is used, the first evidence of such an intermediate for a flavoprotein oxidase [6]. PyOx can also use 2 e^-, H^+ acceptors such as various quinones, and 1 e^- acceptors such as complexed metal ions and radicals as its electron acceptor [7-9]. The ability of PyOx to react with alternative electron acceptors and a range of sugar substrates can be employed in various applications [10-16]. The ability of PyOx to oxidise the sugar at the C-2 position from which follows that PyOx is active on both anomeric forms of sugars, in contrast to glucose oxidase (GOx), the most commonly studied sugar-oxidising enzyme in bioelectrochemistry, and in biosensor and biofuel cell applications, which only reacts with ß-D-glucose, and most other sugar oxidizing enzymes makes PyOx special but has only to a small extent thus far been a focus in bioelectrochemistry.

The crystal structure of PyOx [17, 18] provides detailed information about the residues interacting with the sugar substrate in the active site. As a consequence, site-directed mutagenesis at these residues allows analysis of structure–function relationships, and also possible improvements by semi-rational protein design. Guided by the crystal structure, saturation mutagenesis was used to exchange all of the active-site residues one by one, and screen for improvements in the catalytic activity with D-galactose and alternative electron acceptors. One of these active-site variants, V546C, was reported [19, 20] to show significantly improved catalytic properties attractive for various biotechnological applications. In homogeneous steady-state characterization, the resulting variants

(T169G/H450G/E542K/V546C,S113E/T169G/H450G/Q461R/V546C and S113E/T169G/H450G/Q461R/E542K/V546C) show a significant increase in activity when D-galactose is used as electron donor and either 1,4-benzoquinone or the ferricenium ion was used as solution-phase electron acceptor over the wild-type PyOx, thus showing promise for use in enzyme electrodes for determination of galactose, should the enzyme and electron acceptor be co-immobilized on electrodes [19, 20].

There has been intensive research into the use of mixed ligand transition metal complexes co-ordinated to polymers for co-immobilization of biorecognition elements and electron donors/acceptors for application to biosensor [21-23] and biofuel cell [24-31] device development. This research has focused on osmium-based redox polymers as they exhibit several advantages such as lower ionization energy, which leads to a subsequent stabilization of higher oxidation states, lower redox potentials and greater extension of the metal d orbitals, leading to enhanced metal-ligand back bonding and providing increased complex stability [21, 32, 33]. From the first applications of these redox polymers for reagentless mediated biosensing [34, 35], they attract much attention due to their efficient electron shuttling properties combined with the polymeric structure promoting a stable adsorption as well as the possibility for multiple layers of immobilized protein molecules, biological membranes or bacterial cells on the electrode surface [36-38]. So far they have been used for immobilizing many biological catalysts including sugar oxidizing enzymes glucose oxidase, oligosaccharide dehydrogenase, galactose oxidase, PyOx, pyranose dehydrogenase, PQQ and FAD glucose dehydrogenase, cellobiose dehydrogenase, fructose dehydrogenase etc. [39-54], various peroxidases [55-57], multicopper blue oxidases such as laccase and bilirubin oxidase [28, 58-63] and whole bacterial cells [64-67] and biological membranes [68, 69].

Galactose is of considerable importance to the human organism, contributing directly to vital information and control processes in the body [70]. It also functions as a fundamental

and structural substance for cells, cell walls, and intracellular matrix. The quantitative determination of galactose is thus of great importance in clinical chemistry, food and fermentation industries [70-72]. In this research, in order to develop enzyme electrodes with improved sensitivity to galactose, the activity of wild type pyranose oxidase (PyOx-WT) and that of three different pyranose oxidase mutants (PyOx-MTs) were examined by wiring with four different redox polymers for use in enzyme electrodes for determination of galactose.

2. Experimental

2.1 Reagents

Glucose and galactose were purchased from Sigma-Aldrich (St. Louis, MO USA). Polyethyleneglycol diglycidyl ether (PEGDGE) was purchased from Polysciences (Warrington, PA, USA). Redox polymers 1-3 were provided from the School of Chemistry, National University of Ireland Galway and redox polymer 0 was synthesized according to a previously published protocol [74]. The structures of the redox polymers are presented in Figure S2. The PyOx MTs, with improved activity for galactose, were obtained according to a previously published protocol [20]. PyOx with active-site variant are as follows:

PyOx MT-1: T169G/H450G/E542K/V546C,

PyOx MT-2: S113E/T169G /H450G/Q461R/V546C,

PyOx MT-3: S113E/T169G/H450G/Q461R/E542K/V546C.

2.2 Instrumentation

Electrochemical studies were performed in an amperometric wall-jet cell connected to a flow injection analysis (FIA) system [75]. The wall-jet electrochemical flow-through cell contains three electrodes, a working electrode made of graphite rods (type RW001, 3.05 mm diameter, Ringdorf Werke GmbH, Bonn, Germany), a reference electrode $Ag|AgCl_{0.1\ M\ KCl}$

and a counter electrode made of a platinum wire. An injector (Rheodyne, Cotati, CA, USA), lab balances (Sartorius, Göttingen, Germany), a peristaltic pump (Gilson, Villier-le-Bel, France), pH meter (Metrohm 744, Metrohm Filderstadt, Germany), pipettes (Eppendorf Hamburg, Germany), a potentiostat (Zäta Elektronik, Höör, Sweden), a strip chart recorder (Kipp & Zonen, Delft, The Netherlands) were used in experiments. For cyclic voltammetry studies, an electrochemical analyzer BAS 100A (Bioanalytical Systems, West Lafayette, IN, USA) was used with saturated calomel electrode (SCE) as reference electrode and platinum foil as counter electrode. A 50 mM Tris buffer of pH 8.5 was used as electrolyte and it was thoroughly out-gassed with argon prior to experiments to maintain an inert atmosphere.

2.3 Preparation of the PyOx/redox polymer modified electrodes

Graphite rods were cut in approximately 6 cm long pieces and polished on wet emery paper (P2000) to create a flat and smooth surface. After rinsing them thoroughly with Milli-Q water, they were dried at room temperature. Aliquots of PyOx WT or PyOx MTs were drop-cast on the top of the polished end of the graphite electrode using a Hamilton syringe. After about 15-20 min, an aliquot of 2 µL of redox polymer solution (10 $mg.mL^{-1}$ in Milli-Q water) was mixed with the remaining drop on the active surface of the electrodes using the micro syringe tip. Finally, 1 µL of a freshly prepared PEGDGE solution (10 $mg.mL^{-1}$ in Milli-Q water) was spread on the top of the electrodes and the electrodes were left overnight at 4°C at constant humidity for complete cross-linking reaction. Before using the electrodes in the FIA system, electrodes were intensively rinsed with Milli-Q water in order to remove any weakly attached components. The PyOx modified graphite electrode was fitted into a Teflon holder and inserted into a flow-through amperometric cell of the wall-jet type and kept at a constant distance (ca. 3 mm) from the carrier solution inlet port. The wall-jet cell was connected on-line to a flow injection analysis system, in which the carrier buffer flow was maintained at a

constant flow rate of 1 mL min^{-1} with a peristaltic pump. An injector consisting of an electrically controlled six-port injection valve and a 50 µL injection loop was used. The output signal was recorded on a strip chart recorder. All measurements were performed at room temperature. Every injection was repeated three times and at least 2 electrodes of the same type were tested to check the reproducibility.

3. Results and discussions

3.1 Optimization of applied potentials, comparison of Os polymers towards PyOx catalytic activities and comparison of PyOx-MT towards glucose and galactose catalysis.

The formal potentials (E°') of the Os(II/III) transition for the redox polymers depends on the co-ordination sphere in the metal-complex structure (see Fig. S1). To obtain best response and to facilitate the transfer of electrons from reduced $PyOx_{FAD}$ to the redox polymer and then from the redox polymer to electrodes, it is important to establish the E°'-value of each polymer. A 6 mM glucose solution was used as substrate solution and the applied potential was varied stepwise over a potential range and hydrodynamic amperometric response was measured for each applied potential. The E°'-values is estimated as the potential of half-maximum current in the hydrodynamic amperometric response and the applied potentials for all redox polymers was selected to be a potential where maximum current is achieved (between 80 to 140 mV more positive of the formal potential) as presented in Table 1.

To compare the effect of the redox polymer choice on the response to the sugar substrates, four different redox polymers were investigated. The analytical response to glucose obtained from the electrodes modified with the various combinations of PyOx/redox is shown in Figure 1A. The bound FAD of PyOx-WT has an E°'-value of approximately -150 mV [17, 18]. Therefore, redox polymers with an E°' higher than -150 mV are expected to

accept electrons from the reduced enzyme active site and further transfer it to the electrode. As revealed in Fig. 1A the response to glucose for PyOx with redox polymers 0 and 1 is low as a result of the low thermodynamic driving force for electron transfer to these redox polymers in their oxidized states to reoxidize reduced PyOx, whereas redox polymers 2 and 3 deliver current to the electrode as a result of oxidation of glucose by PyOx and electron transfer through the redox polymers to the electrode. When the PyOx-MTs were wired with Os polymer 0 and 1, no detectable responses to glucose were obtained. This may be caused by the unfavourable kinetics of these variants for the substrate glucose (see below). Heller suggested that the E°' of the mediator should be about 50 mV more positive than that of the enzyme for optimal performance [76], a suggestion supported by the results in Figure 1A. From Figure 1A, it is clear that for all PyOx variants co-immobilized with redox polymer 3 the highest response is obtained compared to that for all other redox polymers. To compare the catalytic response of PyOx-MTs towards galactose and glucose, the electrodes were therefore prepared by co-immobilizing the PyOx-MTs and redox polymer 3. The results in Figure 1B show that all the mutants have higher catalytic response for galactose compared to that obtained with PyOx-WT, which shows a relatively high response toward glucose. The mutants were engineered to enhance the response for galactose [19, 20] and these results show that the engineering and mutation of the PyOx enzyme were successful in improving response to galactose when the enzymes are co-immobilized with redox polymers as electron acceptors, to provide enzyme electrode biosensors.

3.2 Cyclic voltammetry

Cyclic voltammetry (CV) was performed to verify activity of the enzymes immobilized on the graphite electrode. To observe any interaction between the osmium redox polymers and PyOx, CVs were recorded for electrodes made with redox polymer in the presence and absence of PyOx enzyme at a scan rate of 1 $mV.s^{-1}$. The results presented

in Figure 2A show the voltammogram for an electrode modified redox polymer 3 alone compared to that for an electrode modified with redox polymer 3 and PyOx-MT1. It was observed that when redox polymer 3 was co-immobilized with PyOx-MT1, the CV was slightly shifted into a more negative direction and a decrease in the peak current occurs, possibly reflecting interaction between redox polymer and enzyme, as a similar response was observed in our previous studies where pyranose dehydrogenase was co-immobilized with redox polymers [41]. Similar results were also obtained for all of the other osmium polymers (results not shown). The results presented in Figure 2B again show that when PyOx-MT1 is co-immobilized with redox polymer 3, the catalytic activity towards galactose was higher than that for glucose.

3.3 pH profile

To study the effect of pH on the activity of enzymes, the pH values of the running buffer (50 mM Tris buffer between pH 7.0 and 8.5 and 50 mM ethanolamine buffer between pH 9.0 and 11.0) was varied. The buffers were thoroughly out-gassed under vacuum before use to prevent the appearance of bubbles in the FIA system. Glucose and galactose solutions (6 mM) were prepared in an appropriate buffer and injected (50 µL) into the carrier stream. The results in Figure 3A again demonstrate that the highest current response was obtained when PyOx-WT was mediated by redox polymer 3, and was so for all pH values. As already shown in Figure 1A, the PyOx-MTs responded better in terms of output current when wired with Os polymer 2 and 3 using galactose as substrate so the pH profiles were only studied with Os polymer 2 and 3. There is a sharp increase in current response around pH 8.5 for PyOx-WT and PyOx-MTs, which increased until pH 10. Even though the pH of 10.0 in 50 mM ethanolamine buffer was found to be the optimum for maximum current response this buffer system could not be used for characterization because exposing the electrode for a long time at such an alkaline pH would lead to denaturation and inactivation of the enzyme and

additionally the sugars may get directly oxidized more easily at high pHs. Therefore, 50 mM, Tris buffer with pH 8.5 was selected as optimal operating medium. Moreover, the experimental data showed that when redox polymer 3 was used as mediator, higher current values could be obtained over the whole studied pH range.

3.4 Optimization of enzyme amount

The effect of the amount of enzyme on the electrode response was determined using redox polymer 3. Different amounts of PyOx-WT and PyOx-MTs were used to modify the graphite electrodes along with redox polymer 3. These graphite electrodes were tested when used as working electrodes in the FIA system with 6 mM galactose for PyOx-MTs and 6 mM glucose for PyOx-WT injections (Figure 4). The optimum amount of protein immobilized on the electrode was found to be 3 μL (0.26 U) for PyOx-WT, 2 μL (0.36 U) for PyOx-MT-1, 4 μL (0.41 U) for PyOx-MT-2 and 2 μL (0.28 U) for PyOx-MT-3. The optimum enzyme amount was used for preparation of electrodes with PyOx-WT and PyOx-MTs for further studies.

3.5 Analytical performance

The calibration curves for galactose and glucose with redox polymer 3 immobilized with PyOx-MT1, PyOx-MT2 and PyOx-MT3 on graphite electrodes were recorded in Tris buffer at pH 8.5. The enzyme electrodes were placed in the cell, and various concentrations of galactose and glucose dissolved in Tris buffer (pH 8.5) were injected into the flow system. The results of the calibration curves obtained using the biosensors based on redox polymer 3 immobilized with PyOx-MT1, PyOx-MT2 and PyOx-MT3 are presented in Figure 5 and Table 2 showed the linear ranges of the calibration curves for all types of the enzyme-modified electrode. The linear ranges for PyOx-MT1 was 0.1–8 mM galactose whereas PyOx-MT2 and PyOx-MT3 showed similar linear range (0.1–10 mM galactose). The detection limits for galactose were 2.9, 3.2 and 3.4 μM for PyOx-MT1, PyOx-MT2 and

PyOx-MT3 respectively. The detection limits of PyOx-MT1, PyOx-MT2 and PyOx-MT3 for glucose were 4.5, 9.0 and 8.5 µM respectively which were higher than that for galactose. The results reveal that all the mutants not only showed improved catalytic resoponse for galactose but also lower detection limits. All developed biosensors showed good reproducibility of the analytical response.

The parameter, K_M is a measure of the affinity of an enzyme for its substrate, which is inversely proportional to the affinity, and I_{max} is the maximum rate of the enzyme reaction. K_M can also altered due to diffusional limitations of substrate or electron transport to the electrode surface. K_M and I_{max} can be calculated for immobilized enzymes by fitting the experimental data into an electrochemical Michaelis-Menten plot (current vs. substrate concentration) [77]. The parameters K_M and I_{max} were determined for glucose and galactose from the calibration curves and reported in Table 2. The results show that there was no significant changes in the K_M values of PyOx-WT and PyOx-MTs for glucose. In contrast, the K_M values of PyOx-MTs are significantly lower for galactose, compared to that for PyOx-WT which indicates that the substrate affinity of the mutants for galactose is better than that of PyOx-WT.

4. Conclusions

In homogeneous steady-state characterization, the resulting mutants (T169G/H450G/E542K /V546C/S113E/T169G/H450G/Q461R/V546C and S113E/T169G/H450G/Q461R/E542K/ V546C) showed a significant increase in activity when D-galactose was used as electron donor and Os polymers were used as electron acceptor. The catalytic efficiency increased up to 30-fold. The PyOx-MTs, which showed improved activity for D-galactose as a substrate were used for biosensing of galactose. They were co-immobilized with different redox polymers with formal potentials ranging between -0.140 and 0.270 V. The PyOx-WT and the three PyOx-MTs were characterized by their pH profile,

substrate specificity and enzyme loading experiments. The parameters of the biosensing system, I_{max} and K_M, for glucose and galactose were also determined. With the results it can be concluded that these optimized PyOx-based biosensors mediated using osmium based redox polymers can be used as bioanodes for biosensor and biofuel cell studies.

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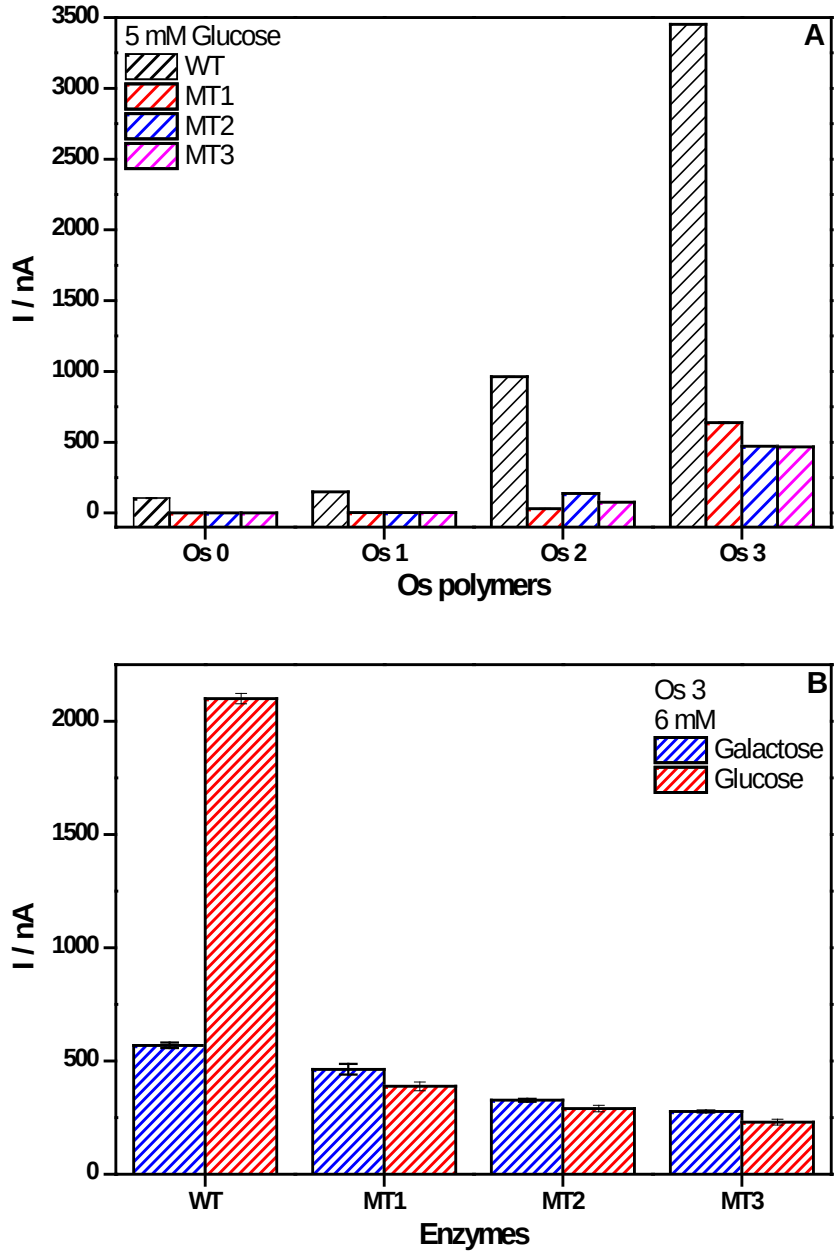

Figure 1. Comparison of Os polymers towards PyOx-WT and PyOx-MTs catalytic activities (A) and comparison of PyOx-MTs for glucose and galactose catalysis (B). Experiments were performed in 50 mM Tris buffer at pH 8.

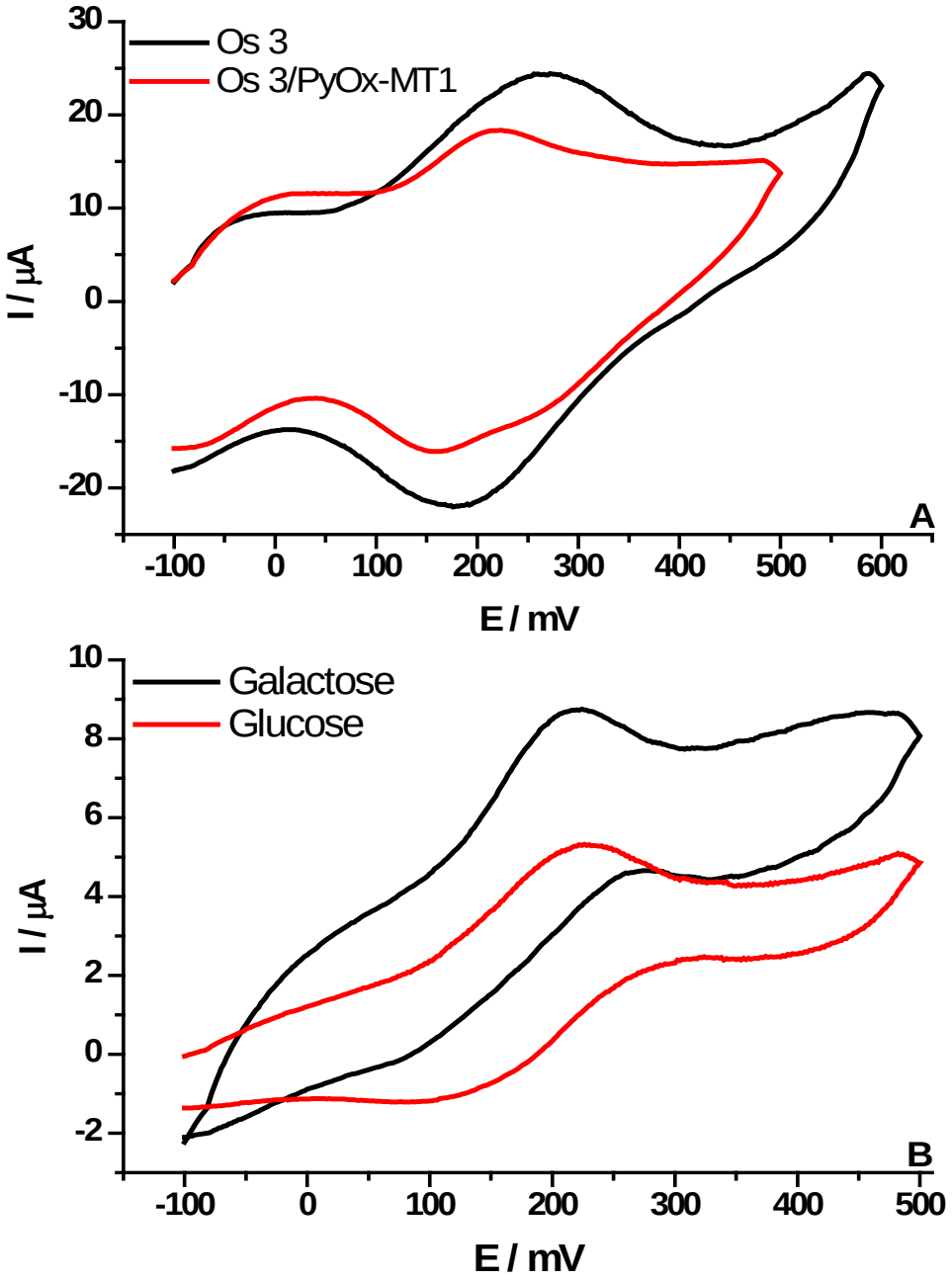

Figure 2. Cyclic voltammograms of (A) Os polymer 3 modified electrode (black line) and PyOx-MT1/Os polymer 3 modified electrode (red line) in the absence of substrate and (B) PyOX-MT1/Os polymer modified electrodes in the presence of galactose (black line) and glucose (red line). Experiments were performed in 50 mM Tris buffer at pH 8.5 at scan rate of 1 mV s^{-1}.

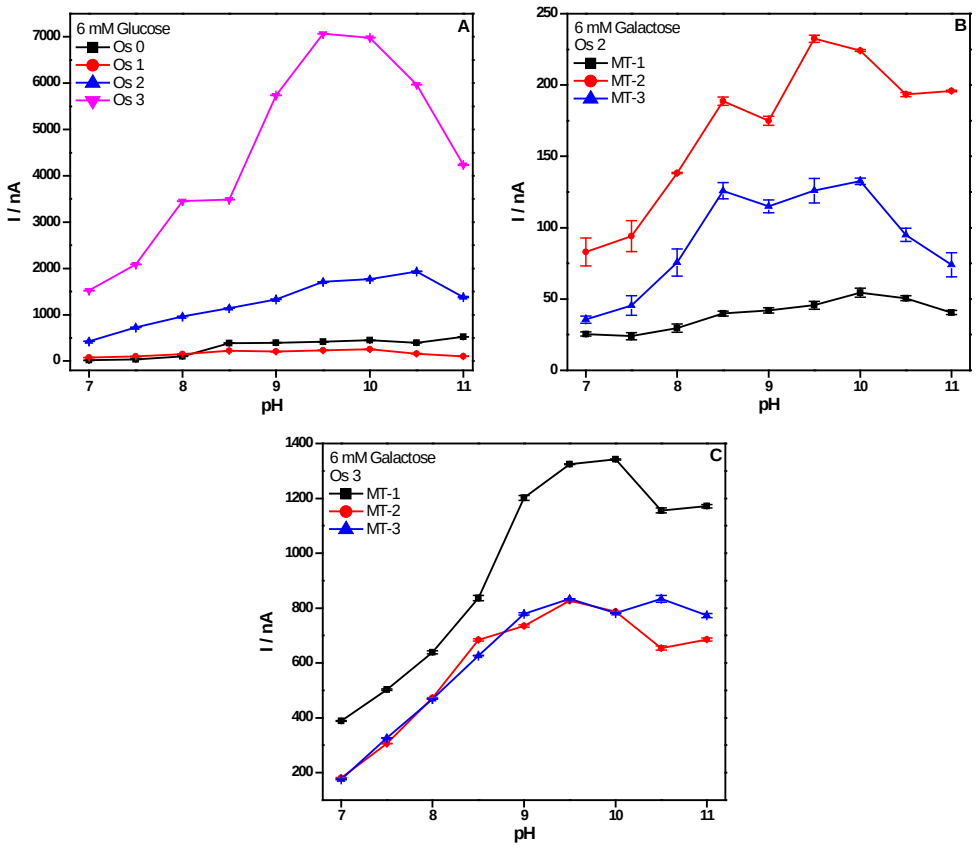

Figure 3. pH profiles for (A) PyOx WT mediated with redox polymer 0, 1, 2, 3 (B) PyOx MTs wired with Os polymer 2 and (C) PyOx MTs wired with Os polymer 3

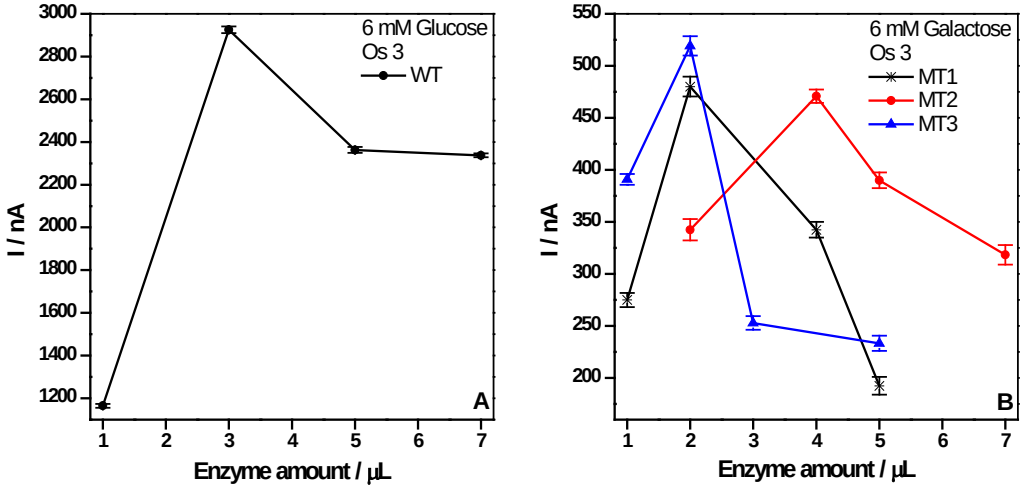

Figure 4. Effect of enzyme loading on the catalysis of (A) glucose on PyOx WT/Os 3 modifies electrodes and (B) galactose on PyOx MTs/Os 3 modifies electrodes.

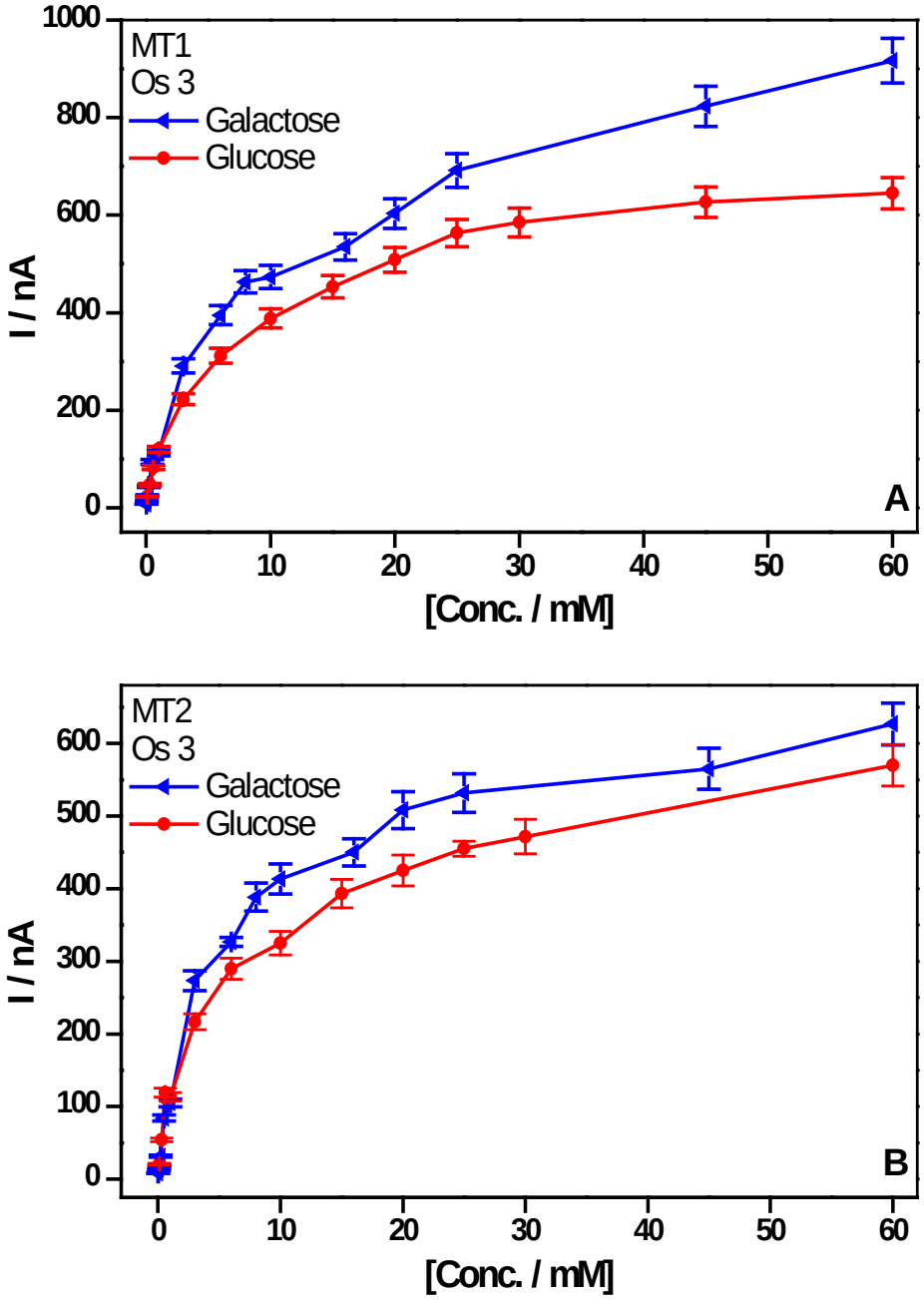

Figure 5. Calibration curves of PyOx MTs wired with Os polymer 3 on graphite electrodes, obtained by measuring MET using D-glucose and D-galactose in 50 mM Tris buffer at pH 8.5 (A) PyOx MT-2 (B) PyOx MT-3

Table 1. Redox polymer formal and applied potentials vs. $Ag|AgCl_{0.1\ M\ KCl}$

Os polymers	Formal Potential/(mV)	Applied Potential/(mV)
Os 0	-185	-80.0
Os 1	-140	0.00
Os 2	180	320
Os 3	270	350

Table 2. I_{max}, K_M, LOD, LOQ and linear ranges for PyOx enzymes using galactose and glucose as substrates.

Enzyme	I_{max}/(nA)	K_M/(mM)	LOD/(µM)	LOQ/(µM)	Linear range/(mM)
			Galactose		
PyOx-MT1	1249.8	17.39	2.9	9.0	0.1–08
PyOx-MT2	667.3	5.51	3.2	9.8	0.1–10
PyOx-MT3	1031.6	6.42	3.4	10	0.1–10
PyOx-WT	819.9	31.75	--	--	--
			Glucose		
PyOx-MT1	719.3	7.53	4.5	14	0.1–10
PyOx-MT2	613.3	7.03	9.0	27	0.1–15
PyOx-MT3	559.5	8.64	8.5	30	0.1–15
PyOx-WT	1916.6	7.68	--	--	--