

Characterization of different FAD-dependent glucose dehydrogenases for possible use in glucose-based biosensors and biofuel cells

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Received: 5 October 2011 / Revised: 6 December 2011 / Accepted: 10 December 2011 / Published online: 6 January 2012
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Abstract In this study, different flavin adenine dinucleotide (FAD)-dependent glucose dehydrogenases (FADGDHs) were characterized electrochemically after “wiring” them with an osmium redox polymer [Os(4,4'-dimethyl-2,2'-bipyridine)₂(PVI)₁₀Cl]⁺ on graphite electrodes. One tested FADGDH was that recently discovered in *Glomerella cingulata* (*GcGDH*), another was the recombinant form expressed in *Pichia pastoris* (*rGcGDH*), and the third was a commercially available glycosylated enzyme from *Aspergillus* sp. (*AspGDH*). The performance of the Os-polymer “wired” GDHs on graphite electrodes was tested with glucose as the substrate. Optimal operational conditions and analytical characteristics like sensitivity, linear ranges and current density of the different FADGDHs were determined. The performance of all three types of FADGDHs was studied at physiological conditions (pH 7.4). The current densities measured at a 20 mM glucose concentration were 494±17, 370±24, and 389±19 $\mu\text{A cm}^{-2}$ for *GcGDH*, *rGcGDH*, and *AspGDH*,

respectively. The sensitivities towards glucose were 2.16, 1.90, and 1.42 $\mu\text{A mM}^{-1}$ for *GcGDH*, *rGcGDH*, and *AspGDH*, respectively. Additionally, deglycosylated *rGcGDH* (*dgrGcGDH*) was investigated to see whether the reduced glycosylation would have an effect, e.g., a higher current density, which was indeed found. *GcGDH*/Os-polymer modified electrodes were also used and investigated for their selectivity for a number of different sugars.

Keywords Glucose biosensor · FAD · Glucose dehydrogenase · Os-polymer · Deglycosylation · Biofuel cell

Introduction

Glucose monitoring systems that measure the blood glucose level of diabetic patients are a necessity for diabetes management. Amperometric biosensors have therefore gained much attention in the field of blood glucose monitoring and clinical analysis, but also food industry and environmental monitoring. A variety of different amperometric glucose biosensors has been developed and commercialized for the last two to three decades, but still a lot of effort is put into further development and improvement of their performance [1–5].

Glucose electro-oxidizing anodes are also of importance for glucose-based biofuel cells, which are proposed as in vivo power sources for pacemakers, micropumps, and so forth [6–18]. The key issues for a biofuel cell application are the establishment of new, robust bioelectrocatalytic systems, improvement of power output and stability and high cell performance in real samples [10, 19]. Among today's glucose-oxidizing enzymes, which are used as biocatalysts for biosensors or biofuel cell anodes, glucose oxidase (GOx) has been the most widely used redox enzyme due to its thermostability and high selectivity for glucose.

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In the early days of glucose biosensor development, the reaction catalyzed by GOx on, or close to, the electrode surface was followed through electrochemical monitoring of either the liberated H₂O₂, or the consumed O₂. However, both reactions suffer from the applied, extreme potentials opening up the sensor system for interfering reactions [20, 21]. This problem was circumvented by the introduction of artificial redox mediators [1, 3, 5, 14, 22], using a lower potential range that favors a higher selectivity in biological fluids. With efficient artificial mediators available also other redox enzymes, e.g., glucose dehydrogenase, which are insensitive to oxygen, became interesting for biosensors and biofuel cells [20, 21].

Two types of glucose dehydrogenases have been utilized for glucose-based biosensors and biofuel cells. The first is a quinoprotein glucose dehydrogenase having pyrroloquinoline quinone as bound cofactor (PQQGDH), which can use different electron acceptors as redox mediators except oxygen, and have an acceptable good electron transfer kinetics [4]. There are two kinds of PQQGDHs; one is intracellular and soluble and the other one is tightly bound to the outer surface of the membrane. Membrane bound PQQGDH has high glucose selectivity but requires suitable detergents for solubilization and purification [23], while soluble PQQGDH exhibits a low substrate specificity and lacks thermal stability [24, 25]. The other type is nicotinamide adenine dinucleotide (NAD) dependent glucose dehydrogenase that exhibits higher substrate specificity and stability than PQQGDH. There are, however, several disadvantages with sensor systems based on NAD-dependent dehydrogenases. The cofactor is not strongly bound to the enzyme and needs to be added, it is not long-term stable in either its oxidized form or in its reduced form and the electrochemistry suffers from very high overvoltages and side reactions causing electrode fouling [22]. Therefore, sensor systems based on such enzymes need either an NADH oxidizing enzyme such as NADH dehydrogenases (EC 1.6.99-diaphorase) or a 2e⁻, 2H⁺ acceptor, i.e., a quinone type mediator, to be present in the system to be able to oxidize NADH to NAD⁺ at a low potential [22].

In addition to the above-mentioned enzymes, thermostable FAD-dependent glucose dehydrogenases (FADGDH) have begun to gain attention in the field of bioelectrochemistry. Different thermostable FADGDHs have been reported [26–32]. FADGDH (EC 1.1.99.10, D-glucose: acceptor 1-oxidoreductase) was first discovered in 1951 in *Aspergillus oryzae* [30] but remained a little investigated enzyme. Since the usage of FADGDH as electrode catalyst for glucose biosensors [26] and for biofuel cell anodes [33] was promoted, more attention was drawn to it and new members of this type of enzyme were identified from different fungal species [34–36]. However, very few reports have been published on FADGDH-based electrodes. The advantages of FADGDHs for glucose biosensors are high turnover rates and substrate

selectivity, good stability, a lower redox potential than that of PQQGDHs and an oxidative half-reaction of the reduced FAD that is unaffected by molecular oxygen. Due to these properties FADGDHs are interesting candidates for the construction of oxygen insensitive amperometric glucose biosensors and glucose biofuel cell anodes. In this study, three FADGDHs were characterized with respect to biosensor and biofuel cell applications. The recently discovered enzyme from *Glomerella cingulata* (*GcGDH*) and the recombinantly in *Pichia pastoris* produced *GcGDH* (*rGcGDH*), were compared with the commercially available GDH from *Aspergillus* sp. (*AspGDH*). An Os-polymer [Os(4,4'-dimethyl-2,2'-bipyridine)₂(PVI)₁₀Cl]⁺, which was previously found to be optimal for *GcGDH* [37], was used in this study to “wire” the FADGDHs. Furthermore, the effect of the glycosylation in respect to the behavior on the electrode was also investigated using *rGcGDH* and its deglycosylated form (*dgrGcGDH*) as an example.

Materials and methods

Chemicals and materials

The enzyme preparation of glucose dehydrogenase from *G. cingulata* (*GcGDH*) with a molecular mass of 95–135 kDa (different glycoforms) was a liquid preparation with a protein concentration of 7.4 mg mL⁻¹ and a specific activity of 878 U mg⁻¹ and prepared according to [38]. Recombinantly, in *P. pastoris*, expressed *GcGDH* (*rGcGDH*) 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 U mg⁻¹ and was prepared according to [39]. Deglycosylated, recombinantly expressed *dgrGcGDH* with a molecular mass of 67 kDa was a liquid preparation with a protein concentration of 15 mg mL⁻¹ and a specific activity of 795 U mg⁻¹. For the deglycosylation, *rGcGDH* was treated with Endo Hf (0.1 mg per mg *rGcGDH*, New England Biolabs) and α -mannosidase (0.1 mg per mg *rGcGDH*, Sigma-Aldrich) and subsequent purification by size exclusion chromatography. *AspGDH* from *Aspergillus* sp. (molecular mass=97 kDa, volumetric activity 6,500 U mL⁻¹) was obtained as a powder and a kind gift by Genzyme, UK (www.genzymediagnosics.com). Osmium redox polymer [Os(4,4'-dimethyl-2,2'-bipyridine)₂(PVI)₁₀Cl]⁺, was synthesized as reported previously in [40].

Potassium phosphate dibasic was purchased from Sigma-Aldrich (Stockholm, Sweden). Poly(ethyleneglycol) (400) diglycidyl ether (PEGDGE) was purchased from Polysciences (Warrington, PA, USA). D(+)-Glucose, D(+)-cellobiose, D(+)-lactose, D-gluconic acid, D(+)-mannitol, D(+)-mannose, 2-deoxy-D-glucose, D(+)-galactose, D(+)-maltose, D(+)-xylylitol, D(-)-fructose, fucose, D(+)-trehalose, and D-sorbitol were from Sigma-Aldrich (Stockholm, Sweden). D(+)-Xylose, rhamnose, and acetaminophen were from ICN Biomedicals, Inc.

(Columbus, OH, USA). Arabinose was from Sigma (St Louis). D(+)-Glucosamine and sucrose were from Chemicon (Malmö, Sweden). The water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA). Standard solutions were prepared by dissolving appropriate amounts in the buffer used as carrier in the flow system (see below) and degassed before use to avoid air bubble formation in the flow system.

Electrode preparation and equipment

For the flow injection measurements, the electrodes were mounted into a flow-through amperometric cell of the wall jet type [41] containing a platinum wire counter electrode and an Ag|AgCl_{0.1 M KCl} reference electrode (+288 mV vs. NHE). The potential of the working electrode vs. the reference electrode was kept at the required value using a three-electrode potentiostat (Zäta Elektronik, Höör, Sweden). The electrode response was registered with a recorder (BD 112, Kipp & Zonen, Delft, The Netherlands). Samples were injected with an injector (Rheodyne, type 7125 LabPRO, Cotati, CA, USA) supplied with an injection loop of 50 μL . A 50 mM potassium phosphate buffer solution at pH 7.4 was used as the carrier in the flow system and pumped at a flow rate of 0.5 mL min⁻¹ (Minipuls 2, Gilson, Villier-le-Bel, France). Cyclic voltammetry experiments were performed with an Autolab PGSTAT 30 (Utrecht, The Netherlands) or an EG&G potentiostat/galvanostat model 273 A (Princeton Applied Research, Oak Ridge, TN, USA) using modified graphite electrodes as working electrode, a saturated calomel reference electrode (SCE), and a platinum foil counter electrode. Argon was purged through the solutions for at least 15 min prior to the experiments. All the results presented below are referred to Ag|AgCl_{0.1 M KCl} reference electrode.

Graphite rods (Ringsdorff Werke, Bonn, Germany) were prepared as described previously in [42, 43]. The geometric

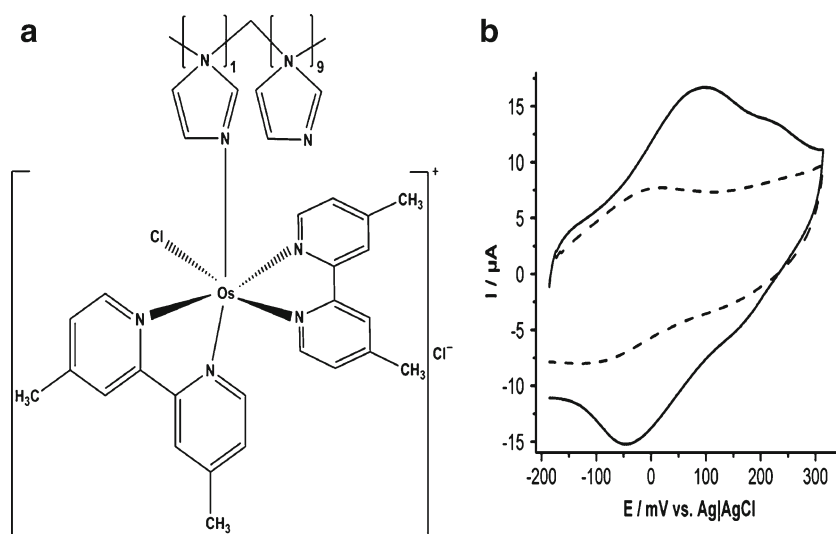
surface area of the graphite rods is 0.071 cm² and the roughness factor is approx. 5 [44]. The Os-polymer was dissolved in Milli-Q water at a concentration of 10 mg mL⁻¹. One microliter of an enzyme solution with a protein activity of 6.5 U μL^{-1} was spread evenly on the top of the polished electrode. After 5 min, 2 μL of Os-polymer solution (10 mg mL⁻¹) followed by 1 μL of freshly prepared PEGDGE solution (10 mg mL⁻¹ in water) was placed on top of the first layer of the electrode. The electrodes were kept overnight at 4°C under constant humidity for complete cross-linking [43, 45].

Results and discussion

Cyclic voltammetry of osmium redox polymer

The electrochemical behavior of the Os-polymer [Os(4,4'-dimethyl-2,2'-bipyridine)₂(PVI)₁₀Cl]⁺ was characterized using cyclic voltammetry in the presence and absence of GcGDH in 50 mM potassium phosphate buffer at pH 7.4 without substrate. The structure of this Os-polymer is shown in Fig. 1a. The polymer was selected previously out of six different Os-polymers covering a broad potential range, because it gave the highest current density [37]. The formal redox potentials (E°) of the polymer was determined to be 21 mV vs. Ag|AgCl_{0.1 M KCl} for [Os(4,4'-dimethyl-2,2'-bipyridine)₂(PVI)₁₀Cl]⁺. This value agrees well with the literature values [40] and with our previous results [14]. The rather low E° value minimizes the effects of interfering components by preventing their direct electrochemically oxidation at the electrode surface at high potential. Due to these preliminary results and the fact that the polymer has been already used to successfully “wire” different redox enzymes in biosensors and biofuel cells [14, 46, 47], it was selected for this study. The possibility to successfully “wire”

Fig. 1 **a** Structure of the Os-polymer [Os(4,4'-dimethyl-2,2'-bipyridine)₂(PVI)₁₀Cl]⁺. **b** Cyclic voltammogram of the Os-polymer on graphite electrode: (solid line) without enzyme, (broken line) with cross-linked GcGDH. Experiments were performed in 50 mM phosphate buffer at pH 7.4 and the scan rate was 10 mV s⁻¹



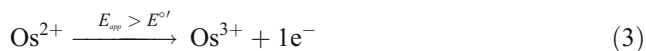
the other FADGDHs to graphite rod electrodes was therefore anticipated [43]. The mechanism of interaction and electron transfer between enzymes and Os-polymers is well described in the following reports [45, 48–50]. The FADGDHs belong to the same structural family of GMC oxidoreductases (glucose-methanol-choline oxidoreductase) as GOx [51]. Similar to GOx, the FADGDHs oxidize glucose at the C1 position and only the β -form is oxidized and $2e^-/2H^+$ are transferred from glucose to the bound FAD, Reaction 1. The electron transfer occurs between FADGDHs and Os-polymers through the following reactions.



Then the Os-complexes attached to the polymer, acting as $1e^-$ non- H^+ acceptors, reoxidize the reduced enzyme. The sum of the reoxidation reaction is given by Reaction 2.



Finally, the reduced Os-complexes are reoxidized by the electrode having an applied potential (E_{app}) higher than the $E^{\circ'}$ of the redox polymer, reaction (3).



In an ideal setup, the enzyme is perfectly electrically connected to the electrode and the measured current (the analytical response signal) represents the actual turnover rate of the enzyme [43]. The turnover rate and likewise the current increase linearly with the diffusional flux of the substrate and with the substrate concentration until the maximum turnover rate of the enzyme is reached [43].

To observe any interaction between the Os-polymer and FADGDHs, cyclic voltammograms (CVs) were recorded for electrodes made with Os-polymer in the presence and absence of *GcGDH* in 50 mM phosphate buffer at pH 7.4 without substrate. The PEGDGE cross-linker was used for cross-linking of both types of electrodes. The Os-polymer exhibited a single redox couple as shown in the CVs in Fig. 1b. When coimmobilized with *GcGDH* (Fig. 1b), the wave was slightly shifted into a more negative potential region reflecting the electrostatic interaction between redox polymer and enzyme. This effect was also seen in previous work [14, 43, 52].

For the work with the different FADGDH-modified electrodes in the flow system, the proper working potential was chosen according to the CVs shown in Fig. 1b. The amperometric measurements were thus performed at +175 mV in all experiments reported below, so that there should be sufficient electrochemical driving force to keep the Os-polymer completely in its oxidized form.

Effect of pH

The pH dependence of the FADGDH/Os-polymer modified graphite electrodes was tested in the electrochemical flow-through cell by varying the pH of the phosphate carrier buffer solution between 6.0 and 8.5. Figure 2a shows the

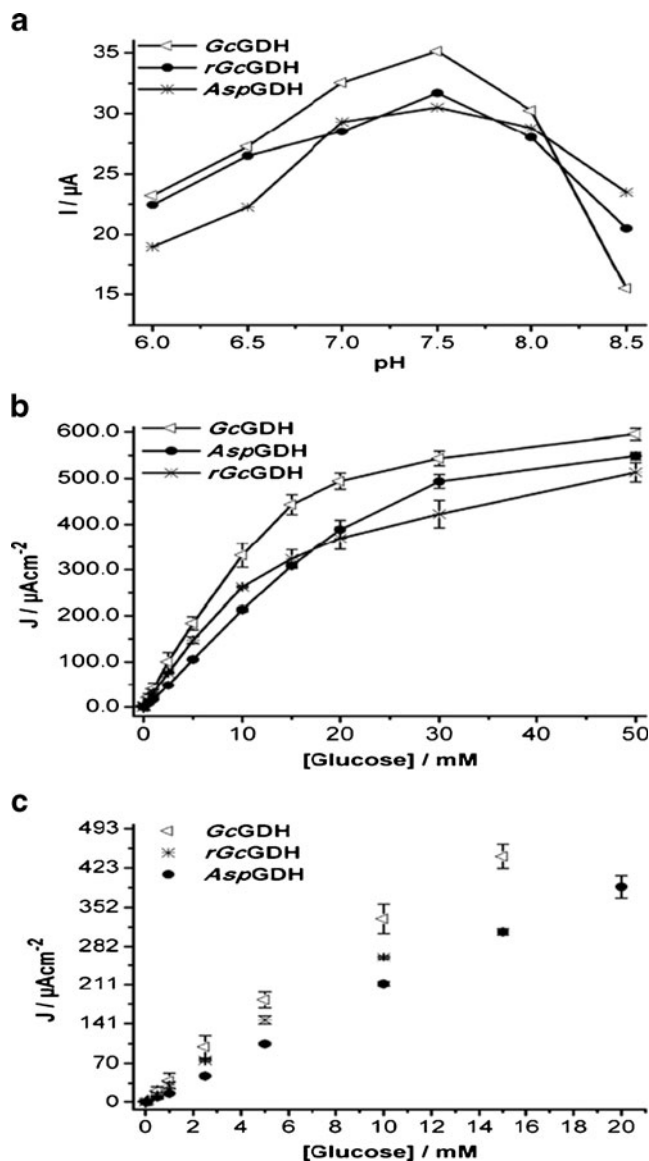


Fig. 2 a Effect of pH on the response of the *GcGDH*/Os-polymer, *rGcGDH*/Os-polymer and *AspGDH*/Os-polymer modified graphite electrodes. Experiments were performed in 50 mM phosphate buffer containing 20 mM glucose solution. The flow rate was 0.5 mL min^{-1} and the working potential was set to +175 vs. $\text{Ag|AgCl}_{0.1 \text{ M KCl}}$. b Calibration graphs (current densities) of *GcGDH*/Os-polymer, *rGcGDH*/Os-polymer and *AspGDH*/Os-polymer modified graphite electrodes. Experiments were performed in 50 mM phosphate buffer at pH 7.4 containing various glucose concentrations. The flow rate was 0.5 mL min^{-1} and the working potential was set to +175 mV vs. $\text{Ag|AgCl}_{0.1 \text{ M KCl}}$. c Linear range of the glucose calibration curves of *GcGDH*/Os-polymer, *rGcGDH*/Os-polymer and *AspGDH*/Os-polymer modified graphite

results obtained by injecting samples of 20 mM glucose. Compared to the other two GDHs, *AspGDH* exhibits a slightly broader pH profile between pH 7.0–8.0 and therefore also less sensitive to pH drifts of an electrode, but for all three measured GDHs the pH optimum was found to be pH 7.5. At this pH, which is close to the pH of physiological conditions, *GcGDH* (35.1 μA) showed the highest activity, as revealed by the response current, followed by *rGcGDH* (31.7 μA), and *AspGDH* (30.5 μA).

Analytical characteristics

Calibration graphs for glucose using three modified graphite electrodes based on *GcGDH*, *rGcGDH*, and *AspGDH* (in equal amounts in terms of activities) along with the Os-polymer are presented in Fig. 2b and the analytical characteristics are summarized in Table 1. The values of the apparent Michaelis–Menten constants (K_M^{app}) and maximum current (I_{max}) were calculated by fitting the data to the Michaelis–Menten equation using least square regression (Table 1). The value of K_M^{app} is a reflection of both the affinity of the enzyme for the substrate and, as the enzyme is immobilized in the three-dimensional hydrogel on the electrode surface, it also reflects any mass-transfer resistance of the substrate in the polymer. A low K_M^{app} indicates thus either a high affinity of the enzyme for the substrate or a fast diffusion through the polymer, while a high K_M^{app} indicates a low affinity but also a high mass-transfer resistance [53]. In this study, the polymer is the same and therefore assumed to have a similar mass-transfer resistance for all tested enzymes. The lowest K_M^{app} value of 13.6 mM and therefore the highest affinity towards glucose was obtained for the *GcGDH*-based biosensor, followed by the *rGcGDH*-based biosensor with a K_M^{app} value of 17.4 mM and the *AspGDH*-based biosensor with a K_M^{app} value of 23.4 mM. All three tested FADGDHs exhibited high I_{max} values of between 50 and 60 μA . The catalytic current values divided by the geometric surface area of the electrode result in the following current densities of 494 ± 17 , 370 ± 24 , and 389 ± 19 $\mu\text{A cm}^{-2}$ for the *GcGDH*-, *rGcGDH*-, and *AspGDH*-based biosensors, respectively, for 20 mM glucose (and using an injection volume of

50 μL and a flow rate of 0.5 mL min^{-1}). In Fig. 2b, the calibration curves for *GcGDH* and *rGcGDH* have a similar shape, which demonstrate that the catalytic constants of the two enzymes are almost similar. The catalytic constants in solution k_{cat} and k_{cat}/K_M for *GcGDH* and *rGcGDH* are 380 ± 6 , 418 ± 4 s^{-1} and 2.0×10^4 , 2.5×10^4 $\text{M}^{-1}\text{s}^{-1}$ respectively [38]. Only the current density and thus the I_{max} for the *rGcGDH* electrode is lower, which might be due to a lower enzyme loading on the electrode. The different calibration curve for *AspGDH* suggests a higher K_M^{app} for glucose and also demonstrates different catalytic constants.

Figure 2c shows the linear part of the calibration curves obtained for glucose for all three tested biosensors. At higher concentrations, the calibration curves showed a deviation from linearity due to substrate saturation. The sensitivity, linear range and detection limits for glucose were calculated from the calibration curves for the biosensors and are presented in Table 1. The highest sensitivity for glucose (2.16 $\mu\text{A mM}^{-1}$) was obtained with the biosensor based on *GcGDH*. The biosensors based on *rGcGDH* and *AspGDH* showed slightly lower sensitivities for glucose: 1.90 and 1.42 $\mu\text{A mM}^{-1}$ respectively. The linear range for biosensors based on *GcGDH* and *AspGDH* were between 0.005–18 mM glucose and 0.005–20 mM glucose, respectively. The *rGcGDH*-based biosensor showed a smaller linear range of 0.005–12 mM glucose. The detection limit for glucose (3.4 μM) was almost identical for all three biosensors. In conclusion, *GcGDH* showed better sensitivity and lower limit of detection and comparable linear range with already published reports on FADGDHs [27, 28].

The selectivity of the *GcGDH*/Os-polymer-based system was tested with different substrates. Sugars such as galactose, fructose, mannose and maltose as well as compounds like ascorbic acid, uric acid and acetaminophen, which might be present in blood samples were used to identify possible cross-reactivities [54]. These interfering substances normally appear in very low concentrations in human blood (e.g., ascorbic acid, uric acid, and acetaminophen less than 1 mM) [55]. Nien et al. in his study showed that the current responses of these interferences such as ascorbic acid, uric acid and acetaminophen were about ~0%, ~0%, and 3%, respectively, based on

Table 1 Comparison of different parameters for *GcGDH*/Os-polymer, *AspGDH*/Os-polymer, *rGcGDH*/Os-polymer, and *dgrGcGDH*/Os-polymer modified electrodes

Enzymes	K_M^{app} (mM)	I_{max} (μA)	Sensitivities ($\mu\text{A mM}^{-1}$)	Linear ranges (mM)	R^2
<i>GcGDH</i>	13.6 ± 1.5	56.0 ± 2.4	2.16 ± 0.07	0.005–18	0.994
<i>AspGDH</i>	23.4 ± 4.3	58.3 ± 2.9	1.42 ± 0.10	0.005–20	0.991
<i>rGcGDH</i>	17.4 ± 0.9	48.8 ± 1.2	1.90 ± 0.05	0.005–12	0.998
<i>dgrGcGDH</i>	13.9 ± 3.1	51.5 ± 2.6	2.15 ± 0.13	0.005–16	0.993

Experiments were performed in a 50 mM phosphate buffer at pH 7.4 containing various glucose concentrations. The flow rate was 0.5 mL min^{-1} and the applied potential was set to +175 mV vs. $\text{Ag|AgCl}_{0.1 \text{ M KCl}}$

the sensing current of 6.0 mM glucose as 100% [56]. Nineteen such sugars were tested with *GcGDH*/Os-polymer-based modified electrodes using a sugar concentration of 20 mM. The results obtained are shown in Table 2. The highest relative activities compared to glucose were measured for 2-deoxy-D-glucose (37.2%) and xylose (22.7%). Xylose was already identified as the second best substrate in homogeneous solution (glucose, $K_M=19$ mM, $k_{cat}=380$ s⁻¹; xylose, $K_M=24$ mM, $k_{cat}=60$ s⁻¹) [38]. The high reactivity with 2-deoxy-D-glucose shows that the modification at C-2 only moderately influenced the binding or the oxidation of glucose at C-1. For other substrates including galactose (0.56%), mannose (0.45%), and maltose (0.29%), which can be present in blood samples, the relative activity varied between 0.1% and 3% (Table 2). Towards lactose, gluconic acid, mannitol, rhamnose, sorbitol, and fructose, *GcGDH* showed no detectable activity. An almost similar response profile to all these compounds was also observed for *AspGDH*-based system (results not shown). One can therefore conclude that these FADGDHs have at least equal selectivity for glucose compared with that of GOx and much better than PQQGDH, which is a critical parameter for use in diagnostics. Of the investigated, electrochemically active compounds only acetaminophen gave a strong response (28.9%) at the applied potential but

acetaminophen is not a substrate of GDH. It might get reduced as an electron acceptor and most of its activity might be from direct oxidation at the electrode at this applied potential [56].

Stability tests

The operational stability is one of the key parameters of a biosensor. The long-term stability is not only beneficial to biosensor transport and storage but also helps to decrease the per measurement costs [55]. The operational stabilities of the *GcGDH*/Os-polymer and the *AspGDH*/Os-polymer

Table 2 Substrate specificity of *GcGDH*/Os-polymer modified electrode using 20 mM solution of each sugar

Substrate (20 mM)	% of Glucose activity
Glucose	100
2-Deoxy-D-glucose	37.2
Xylose	22.7
Glucosamine	3.00
Cellobiose	1.83
Lactose	0
Gluconic acid	0
Mannose	0.45
Mannitol	0
Sorbitol	0
Galactose	0.56
Sucrose	0.12
Maltose	0.29
Arabinose	0.52
Xylitol	0.16
Rhamnose	0
Fucose	0.26
Trehalose	0.18
Fructose	0

Experiments were performed in a 50 mM phosphate buffer at pH 7.4. The flow rate was 0.5 mL min⁻¹ and the applied potential was set to +175 mV vs. Ag|AgCl_{0.1 M KCl}

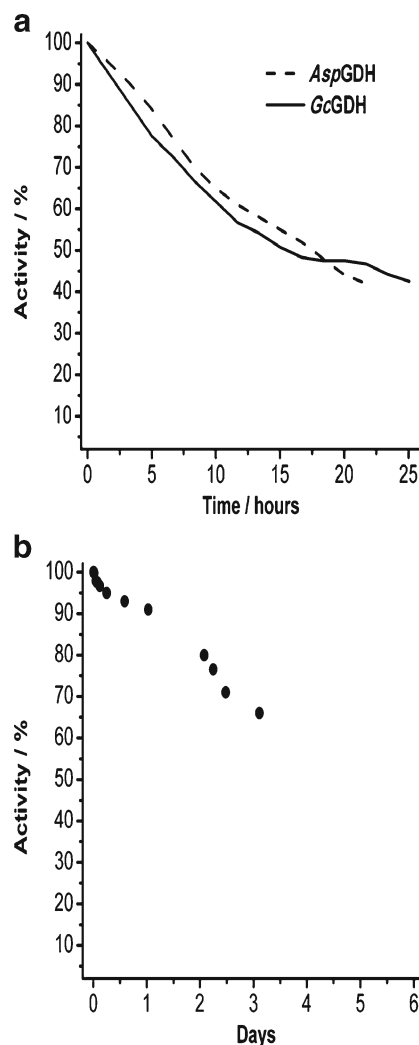


Fig. 3 Stability tests: **a** Variation of the response current for glucose with time for *GcGDH*/Os-polymer and *AspGDH*/Os-polymer modified electrodes in a constant flow of 5 mM glucose solution throughout the duration of test up to 25 h. Experiments were performed in 50 mM phosphate buffer at pH 7.4. The flow rate was 0.5 mL min⁻¹ and the working potential was set to +175 mV vs. Ag|AgCl_{0.1 M KCl}. **b** Variation of the response current with time for *GcGDH*/Os-polymer modified electrode with consecutive injections of 5 mM glucose as substrate for 6 days. Experiments were performed in 50 mM phosphate buffer at pH 7.4. The flow rate was 0.5 mL min⁻¹ and the applied potential was set to +175 mV vs. Ag|AgCl_{0.1 M KCl}

modified electrodes were investigated using a constant flow of a 5 mM glucose solution throughout the duration of the test. Figure 3a shows the results of these stability tests performed for 25 h. It should be noted that there was a slow decrease in the current response of both modified electrodes in the first 15 h, which further decreased for the *AspGDH*/Os-polymer-based electrode, but less strongly for the *GcGDH*/Os-polymer-based electrode. After 25 h with a constant flow of 5 mM glucose, both modified electrodes kept almost 40% of their initial response to glucose.

Another stability test for the *GcGDH*/Os-polymer-based electrode consisted of consecutive measurements of its response to 5 mM glucose. Figure 3b shows the results of the operational stability test of *GcGDH*/Os-polymer modified electrode under storage conditions (50 mM phosphate buffer, pH 7.4, 4 °C). For this experiment, 50 μ L of a 5 mM glucose solution was injected into the electrochemical cell for 6 days. It was observed that the biosensor kept more than 90% of its initial activity after the first day. One can see a slow and linear decrease in the response of the modified electrode during the first day of experiments and then a sudden break down after the second day. After 6 days, the current response was 56% of its initial response. As a conclusion, it can be pointed out that by simple adsorption of the enzymes in combination with electrostatic binding and covalent cross-linking to the Os-polymer on the graphite surface, one can obtain a modified electrode, which has a good and comparable stability with previously reported stabilities of modified electrodes using Os-polymers [28, 46, 47, 50] and can be operational for several days.

Effect of deglycosylation

To study the effect of deglycosylation on the sensor performance, equal amounts in terms of activities of *rGcGDH* and *dgrGcGDH* were immobilized on graphite electrodes with the same Os-polymer loading and cross-linked using a 10 mg mL⁻¹ of PEGDGE solution. The calibration curves for the enzyme modified electrodes using glucose as substrate are compared in Fig. 4. The results show that there is an increase in current density with the smaller (68 kDa) *dgrGcGDH*. The current densities were 370 \pm 24 and 520 \pm 20 μ A cm⁻² for *rGcGDH* and *dgrGcGDH* respectively. With the deglycosylation of the enzyme the packing in the polymer or the “wiring” by the Os-complexes was improved and an increase in the current density and also in the linear response range was also observed. The linear range was between 0.005 and 12 mM glucose for *rGcGDH*, which was extended up to 16 mM glucose for *dgrGcGDH*. The K_M^{app} value for *dgrGcGDH* was also lower than that of *rGcGDH* (Table 1). The increase in the current density and improvement in the linear range after deglycosylation of the

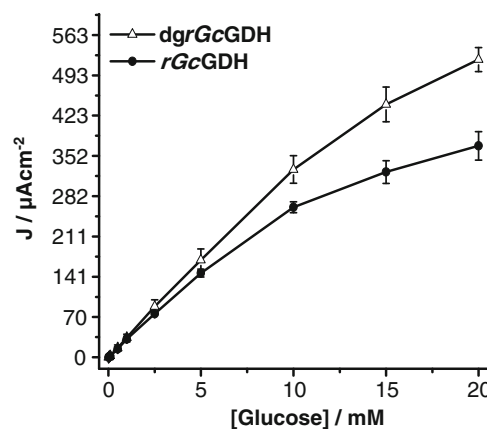


Fig. 4 Comparison of glycosylated *rGcGDH*/Os-polymer and deglycosylated *dgrGcGDH*/Os-polymer modified graphite electrodes. Experiments were performed in 50 mM phosphate buffer at pH 7.4 containing various glucose concentrations. The flow rate was 0.5 mL min⁻¹ and the applied potential was set to +175 mV vs. Ag|AgCl_{0.1 M KCl}

enzyme might be due to a more efficient electron transfer between the bound FADH₂ of *dgrGcGDH* and the Os-polymer, probably by reduced steric hindrance. Additionally, it can be attributed to a higher Os-polymer concentration around the deglycosylated, smaller enzyme in the *dgrGcGDH* electrode due to the smaller size and by an increased formation of electrostatic interactions between the enzyme and the Os-polymer [57, 58].

Conclusion

The investigated FADGDHs/Os-polymer-based electrodes show a high current density, a high sensitivity and a broad linear range towards glucose. The high selectivity of all enzymes towards glucose and the high turnover rates of this substrate suggests the application of these FADGDHs/Os-polymer-based systems for either glucose determination or as bioanodes in glucose biofuel cells. The enzyme/Os-polymer modified electrodes showed a good and comparable stability. The higher current density obtained with deglycosylated *GcGDH* modified electrodes suggests that the downsizing of the enzyme by deglycosylation is a promising way to enhance the electric contact between the enzyme and the electrode, which leads to the improvement of the electron transfer and thus the current density.

Acknowledgments The authors thank the following agencies for the financial support: The Higher Education Commission of Pakistan, The Swedish Research Council (VR, projects 621-2007-4124 and 2010-5031), a Science Foundation Ireland Charles Parsons Energy Research Award, and The European Commission (project “3D-nanobiodevice” NMP4-SL-2009-229255). The supply of the *Aspergillus* sp. GDH by Genzyme Diagnostics is gratefully acknowledged.

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