Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.

Table S1. Purification of active *Am*PDH1 N75 mutants, expressed in shaking flasks.

*Am*PDH1	N75G Y11		N75Q Y34		N75H Y23		N75K X90		wt Z27	
	U	U mg^{-1}	U	U mg^{-1}	U	U mg^{-1}	U	U mg-^{1}	U	U mg^{-1}
Harvest	50.3		25.0		23.7		13.7		59.2	
12 ml Phenyl-Sepharose	25.7		11.2	6.8	9.1	5.4	5.6	4.3	19.8	22.8
1 ml DEAE-Sepharose	9.9	36.1	6.5	25.1	6.6	19.2	4.5	11.6	13.2	34.3

Table S2. Oligonucleotide primers used in site-saturation and site-directed mutagenesis.

	Name	Sequence
*Am*PDH1 N75X	fw*Am*PDHN75	5'-TACACGACGATTCCTCAAGACG-3'
	rv*Am*PDHN75X	5'-CTTGAGGAATCGTCGTGTAKNNCCAATC-3'
*Am*PDH1 N75G	fw*Am*PDHN75	5'-TACACGACGATTCCTCAAGACG-3'
	*rvAm*PDHN75G	5'-CTTGAGGAATCGTCGTGTAGCCCCAATC-3'
*Am*PDH1 N175G	fw*Am*PDHN175G-B	5'-CAGCGGCATTTCCTTCAACGAC -3'
	rv*Am*PDHN175G-B	5'-TGAAGGAAATGCCGCTGTACG-3'
*Am*PDH1 N175Q	fw*Am*PDHN175Q-B	5'-CAGCCAAATTTCCTTCAACGAC -3'
	rv*Am*PDHN175Q-B	5'-TGAAGGAAATTTGGCTGTACG-3'
*Am*PDH1 N252Q	fw*Am*PDHN252Q	5'-GCTTCTGGACAAGGAACC-3'
	rv*Am*PDHN252Q	5'-CCTTGTCCAGAAGCCGAG -3'
*Am*PDH1 N75G N175Q	fw*Am*PDHN175Q-B	5'-CAGCCAAATTTCCTTCAACGAC -3'
	*rvAm*PDHN175Q-B	5'-TGAAGGAAATTTGGCTGTACG-3'
*Am*PDH1 N75G N175Q N252Q	fw*Am*PDHN252Q	5'-GCTTCTGGACAAGGAACC-3'
	rv*Am*PDHN252Q	5'-CCTTGTCCAGAAGCCGAG-3'

Materials and Methods

Expression and purification of *A. meleagris* pyranose dehydrogenase 1 in *P. pastoris*

The pre-culture was incubated for one day at 30 °C and 110 rpm in two baffled shaking flasks with 150 mL of BMGY (2% peptone, 1% yeast extract, 100 mM potassium phosphate buffer, pH 6.0, 1.34% yeast nitrogen base without amino acids, 0.4 µg mL-1 biotin, 1% glycerol). The initial "basal salts" medium volume in the reactor was 4 L. Compressed air was used for aeration and ammonium hydroxide was added to maintain pH 5. A 41–48 h batch/fed-batch phase with glycerol was followed by methanol induction. The supernatant was harvested 69–97 h after induction by centrifugation at 4,000 rcf for 20 min at 4 °C (Sorvall Evolution RC, Kendro Laboratory Products, Newtown, CT, USA). AmPDH1 N75G was expressed at 30 °C and 110 rpm in six baffled shaking flasks with 200 mL of BMGY medium. Methanol induction was started after one day and the supernatant harvested as above after additional 119 h.

The enzyme was purified from the harvested supernatant by adding $(NH_4)_2SO_4$ to 35% saturation at 4 °C and hydrophobic interaction chromatography at room temperature (750 mL of Phenyl-Sepharose fast flow, 15 mL min^{-1}). After washing with sodium phosphate buffer (50 mM, pH 6.5, 35% saturation $(NH_4)_2SO_4$) until OD_{280} was below 200 mAU, the enzyme was eluted in one column volume (CV) gradient from 35% to 0% sat. $(NH_4)_2SO_4$. Active fractions were pooled and the buffer exchanged with Bis-Tris buffer (50 mM, pH 6.5) to a conductivity of less than 4 mS cm^{-1}, using a Viva-Flow 50 Module (Sartorius AG, Göttingen, Germany). Subsequently, anion exchange chromatography (60 mL of DEAE-Sepharose fast flow, 10 mL min^{-1}) was conducted. After washing with 4 CVs with Bis-Tris buffer (50 mM, pH 6.5), PDH was eluted in a step-wise gradient (0.5 CV 0-6%, 2 CVs at 6%, 3 CVs 6-13% elution buffer Bis-Tris 50 mM, pH 6.5, 1 M NaCl). Fractions with high enzyme activity were pooled and $(NH_4)_2SO_4$ was added to 35% saturation. The pooled fractions were applied to a second hydrophobic interaction chromatography (60 mL of Phenyl-Source, 1 mL min^{-1}). Washing and elution was done as in the first step but with a 35% to 0% $(NH_4)_2SO_4$ saturation gradient of 5-8 CVs overnight. The resulting fractions were pooled for highest specific activity, washed with sodium phosphate buffer (50 mM, pH 6.5) and concentrated by ultracentrifugation (10 kDa Amicon Ultra-15 Centrifugal Filter Units, Millipore Corp., MA, USA). If necessary, the resulting enzyme solution was further purified by gel filtration (180 mL of Superose12, 1 mL min^{-1}). Purified and concentrated enzyme solutions were stored at 4 °C.

The recombinant expression of N-glycosylation site mutants and the wild type (*wt*) enzyme were compared in simultaneous expression in an Infors HT Multifors 6x0.5 L bioreactor system, according to the Invitrogen "*Pichia* Fermentation Process Guidelines". The pre-culture was incubated for 24 h in 30 mL of YPD medium with Zeocin™ (100 mg mL^{-1}) in baffled shaking flasks at 30 °C and 110 rpm. Glycerol batch cultivation was followed by glycerol fed-batch (0.4 mL h^{-1}, 21 h). Subsequently, the methanol feed was primed and the feed rate set to 0.2–0.4 mL h^{-1} after dissolved oxygen levels peaked again. Samples were taken and the supernatant separated from the pellet (3 min, 14,000 rpm). Wet biomass, protein concentration and enzyme activity at standard conditions were determined.

Table S3. Purification of *Am*PDH1 N75G, N175Q, DM and TM.

***Am*PDH1 N75G**

6x200 ml in shaking flasks

	Total Activity U	**Vol. activity U ml^{-1}**	**Spec. Activity U mg^{-1}**	**Yield %**	**Purity increase x-fold**
Harvest	153	0.11	3.7	100	1
Phenyl-Sepharose 780 ml	123	0.59	7.6	80	2
DEAE-Sepharose 60mL	50	0.28	52.9	32	14
Phenyl-Source 60 ml	**40**	**200.6**	**29.3**	**26**	**8**

***Am*PDH1 N175Q**

5L bioreactor

	Total Activity U	**Vol. activity U ml^{-1}**	**Spec. Activity U mg^{-1}**	**Yield %**	**Purity increase x-fold**
Harvest	960	0.29	0.6	100	1
Phenyl-Sepharose 780 ml	1357	3.39	5.3	141	10
DEAE-Sepharose 60mL	314	2.86	11.6	33	21
Phenyl-Source 60 ml	110	**476.5**	**39.9**	**11**	**73**

***Am*PDH1 N75G N175Q (DM)**

5L bioreactor

	Total Actity U	**Vol. activity U ml^{-1}**	**Spec. Activity U mg^{-1}**	**Yield %**	**Purity increase x-fold**
Harvest	1275	0.4	0.5	100	1
Phenyl-Sepharose 780 ml	1508	5.59	4.5	118	10
DEAE-Sepharose 60mL	597	2.3	8.3	47	18
Phenyl-Source 60 ml	538	1345	30.1	42	63
Superose 180 ml	217	**543.7**	**32.2**	**17**	**68**

***Am*PDH1 N75G N175Q N252Q (TM)**

5L bioreactor

	Total Activity U	**Vol. activity U ml^{-1}**	**Spec. Activity U mg^{-1}**	**Yield %**	**Purity increase x-fold**
Harvest	602	0.18	0.3	100	1
Phenyl-Sepharose 780 ml	590	2.36	3.4	98	10
DEAE-Sepharose 60mL	347	3.66	19.5	58	57
Phenyl-Source 60 ml	**178**	**446.1**	**39.6**	**30**	**115**

Results and Discussion

Fig. S1. Coomassie stained SDS-PAGE of purified *Am*PDH1 *wt* (1), *Am*PDH1 N75G (2), *Am*PDH1 N175Q (3), *Am*PDH1 DM (4) and *Am*PDH1 TM (5), glycosylated (a, c and e) and deglycosylated by Endo Hf (b, d and f), after purification (a and b), 20 days (c and d) and 71 days (e and f) later. The number of days stored at 4 °C at 7–22 mg mL^{-1} is given for each sample.

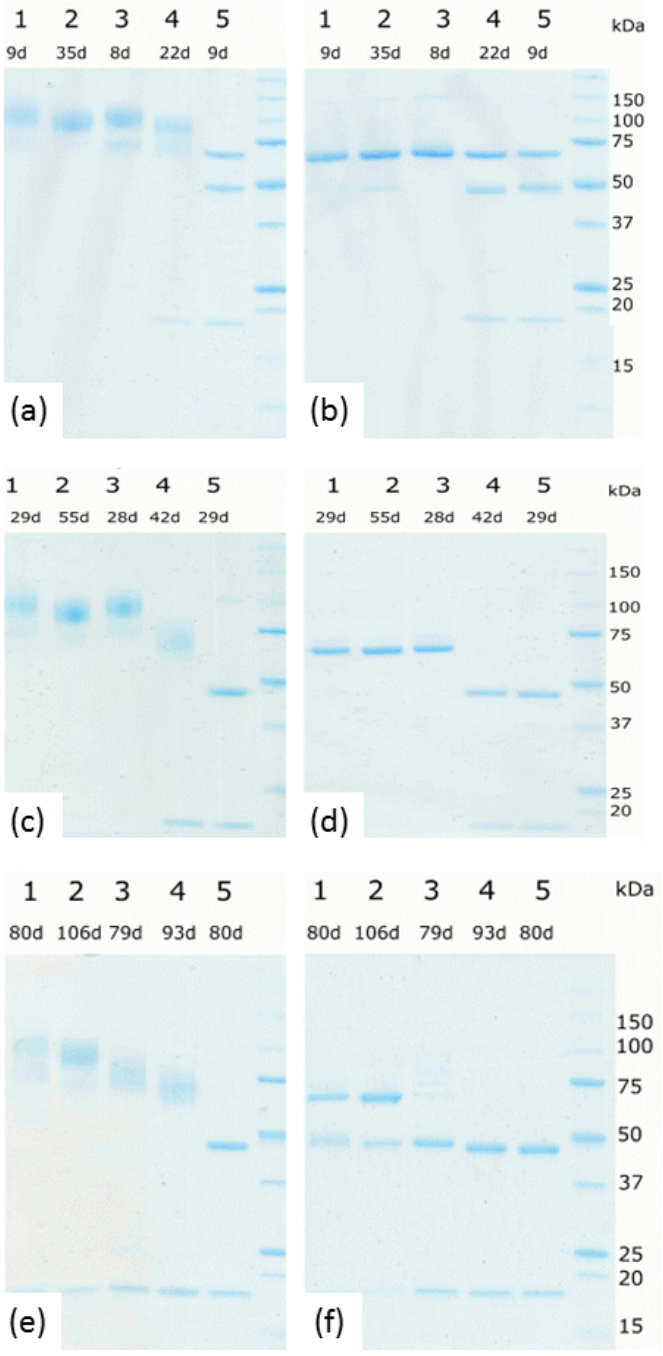
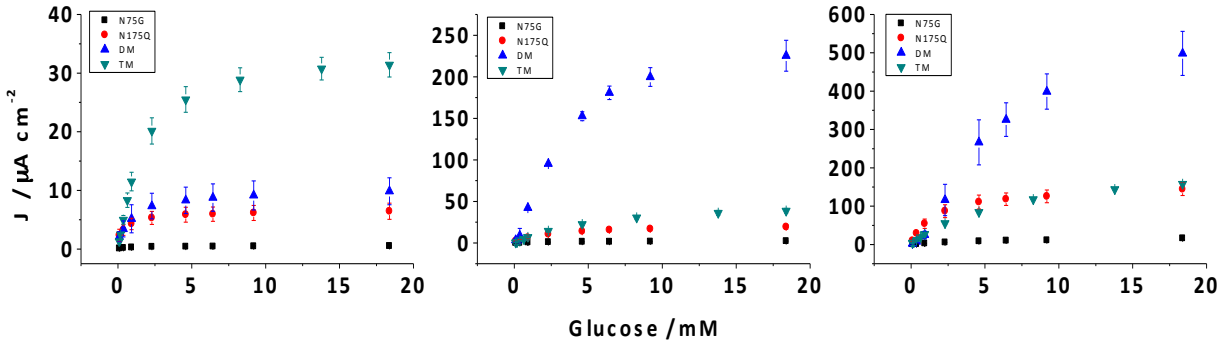


Fig. S2. Variation of the response of Os-polymer/mPDHs-drop-coated electrodes to different concentrations of glucose registered in flow-injection amperometry: (■) N75G-; (●) N175Q-; (▲) DM-; (▼) TM-modified electrodes. The mPDHs were cross-linked with various Os-polymers: (1) Os(dmobpy)PVI-; (2) Os(dmbpy)PVI-; (3) Os(bpy)PVI-based polymer. The current density response to the substrate was recorded in 50 mM phosphate buffer (137 mM NaCl, pH 7.4) pumped through the system at a flow rate of 0.5 mL min-1. An applied potential of 288 mV vs. NHE was used for the Os(dmobpy)PVI/mPDH- and 444 mV vs. NHE for the Os(dmbpy)PVI/mPDH- and the Os(bpy)PVI/mPDH-modified electrodes.



Catalytic performance of mPDH-modified electrodes

All of the films containing mPDHs possess an ability to directly communicate with the electrode surface, which can be seen from the CVs and FI data (Fig. S3 and S4). From the CV measurements depicted in Fig. S3, the on-set response to glucose starts at a potential higher than that of the $E^{o\prime}$ of the enzyme, located at around 250 mV *vs.* NHE at pH 7.4. An applied potential of 444 mV *vs.* NHE was chosen for the flow-injection amperometry studies in order to generate steady state current densities (Fig. S4). The DM oxidises glucose with the highest current output compared to the TM and both the SMs with a J_{max} of 7.8 µA cm^{-2}.

The observed current densities to glucose are significantly lower than those obtained with MET through Os-polymer/mPDH-modified films. This is not surprising since in the case of MET a multi-layer conducting three dimensional structure between the mediator and enzyme is formed, through which electrons can be easily be transferred towards the electrode surface[2]. Nevertheless, the DET approach in construction of EBFCs may have advantages because of drawbacks associated with the use of mediators such as toxicity, cell voltage losses and their general instability[1,2,3].

Fig. S3. Cyclic voltammograms by mPDHs-drop-coated graphite electrodes at a scan rate of 1 mV s^{-1} in 50 mM phosphate buffer (137 mM NaCl, pH 7.4) in inert atmosphere (green line) in the absence and (blue line) in the presence of 25 mM glucose: (a) N75G-; (b) N175Q-; (c) DM-; (d) TM-modified electrodes.

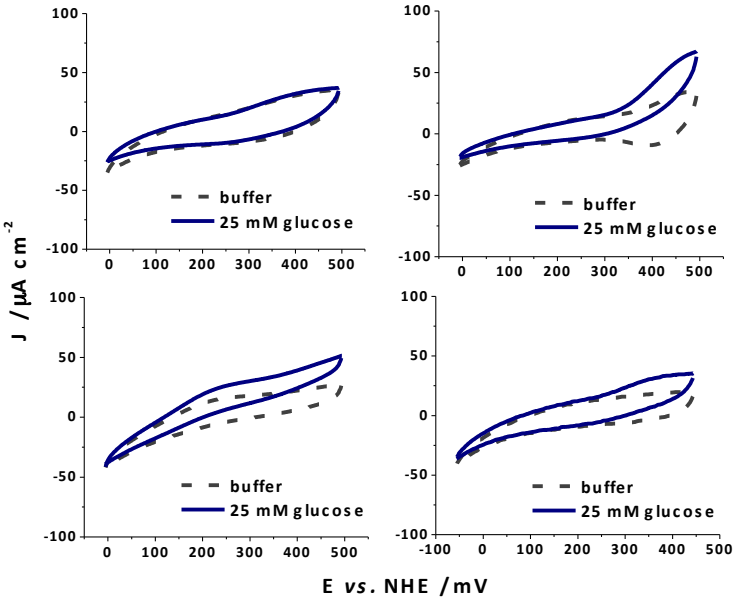
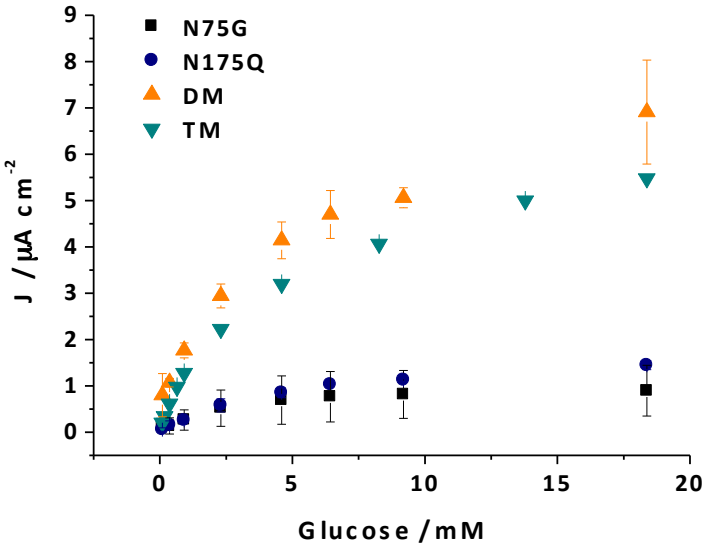


Fig. S4. Amperometric response of mPDH-drop-coated graphite electrodes to different concentrations of glucose measured using the FI system: (■) N75G-; (●) N175Q-; (▲) DM-; (▼) TM-modified electrodes. The current density response was recorded in 50 mM phosphate buffer (137 mM NaCl, pH 7.4) pumped through the system at a flow rate of 0.5 mL min^{-1}. An applied potential of 444 mV *vs.* NHE was utilised.



Operational stability of mPDH-modified electrodes

One of the most challenging aspects in fabrication of EBFCs is maintenance of their long-term stability[4]. With a desired lifetime of several months to years it is generally difficult to produce EBFCs, which will operate for more than 24 h. In case of having the redox enzymes directly adsorbed on the surface of the working electrode, it is often a challenge to maintain the DET response even for a period of one working day. For enzymatically deglycosylated recombinant *Am*PDH the DET response to the same concentration of substrate decreases with each new injection in FI either indicating leaching of the enzyme from the electrode surface or decomposition of the protein[5]. In that case site-directed mutagenesis in combination with recombinant expression may serve as an efficient tool for modulation of the enzyme properties[6]. In the present study the stability of the films prepared by drop-coating of each of the mPDHs onto the surface of the working electrode was examined in the DET mode using the FI system and 5 mM glucose as substrate. A quick decrease in response is observed for the SMs-modified electrodes with a percentage of the remaining current of 9% for the N75G- and 8% for the N175Q-drop-coated films after only 1.5 and 3 h of repeated substrate injections (data not shown). The TM-modified electrodes show a decrease in 67% in current response after only 3 h of repeated substrate injections. Compared to the previously reported data on DET for enzymatically produced dgPDH, a significant improvement is observed in the stability of the films of the DM-modified electrodes. After about 5 h the electrodes modified with DM retain > 87% of the initial current response.

References

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