# Electrostatic Control of the Electron Transfer Enabling Binding of Recombinant Glucose Oxidase and Redox Polyelectrolytes

Ioanis Katakis, Ling Ye, and Adam Heller*

*Department of Chemical Engineering*
*The University of Texas at Austin*
*Austin, Texas 78712-1062*

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Controlling the formation of adducts between polyelectrolytes and enzymes is of interest in enzyme immobilization[1-3] and protein purification.[4,5] Controlling the formation of adducts between redox polyelectrolytes and redox enzymes is of specific relevance to electrochemical, e.g., amperometric, biosensors. In these, electrons may flow via a "wiring" redox polymer from reaction centers of the enzyme to an electrode. "Wiring" of an enzyme enables the measurement of its turnover rate as an electrical current.[6]

We report on controlling adduct formation and "wiring" through controlled variation of the isoelectric point of recombinant glucose oxidase rGOx, (β-D-glucose, oxygen oxidoreductase EC 1.1.3.4). Electrostatic interaction between polyanionic or polycationic rGOx (i.e., rGOx with net negative or positive charge) and similarly or oppositely charged redox polyelectrolytes in aqueous solutions controls the formation of complexes and the flow of electrons from the substrate-reduced enzyme to the redox polyelectrolyte. The currents increase when the electrostatic interaction is attractive and diminish when it is repulsive.

Recombinant, yeast-derived rGOx,[7,8] in which the amount of peripheral oligosaccharide is 5 times higher than that in wild-type *Aspergillus niger*-derived glucose oxidase, was used. Part of the oligosaccharide was periodate-oxidized[9,10] to polyaldehyde. By controlling the $IO_4^-$ concentration, reaction time, and temperature, 20–60 (spectrophotometrically assayed[11]) aldehyde functions were formed in each enzyme subunit. The polyaldehyde was reacted with pentaethylene hexamine at a high hexamine/enzyme ratio so as to avoid cross-linking. The resulting poly-Schiff base was reduced with $NaBH_4$ to the polyamine-modified enzyme. Between 120 and 360 secondary and primary amine functions were thus introduced into the periphery of each rGOx molecule, while approximately one-half (36 units $mg^{-1}$) of the initial specific activity was retained. The isoelectric points of the derivatized enzymes ranged from 3 for unmodified rGOx to 9 for the most heavily modified enzyme. Isoelectric focusing (IEF) runs for some of these enzymes are shown in Figure 1, lanes 2–6.

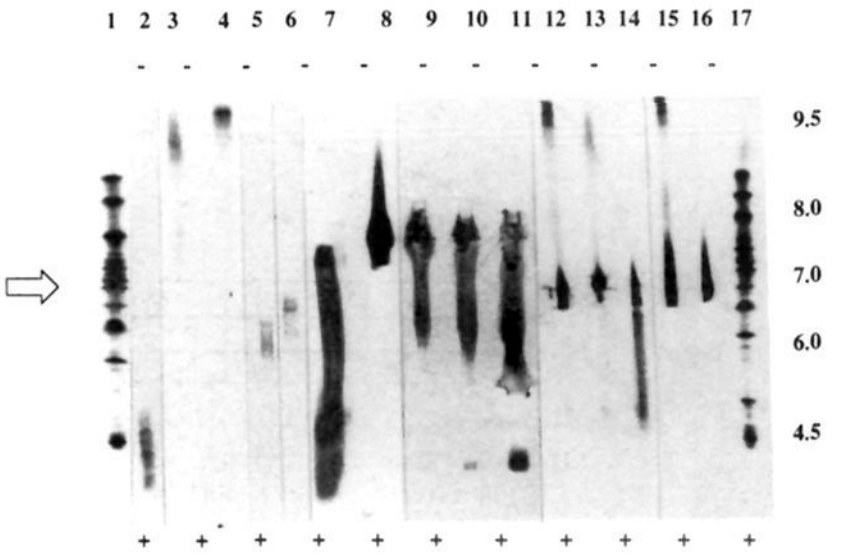


**Figure 1.** Agarose gel isoelectric focusing of chemically altered enzymes and their redox polyelectrolyte complexes. The cathode is at the top and the anode at the bottom. The pH profile is indicated at the right. The arrow indicates the sample application point. Lanes 1,17: IEF standards. Lanes 2–6: 8 μg of rGOx, rGOx-8.5, rGOx-9, rGOx-7, and rGOx-6, respectively. Lane 7: 5 μg of POs-SA. Lane 8: 5 μg of POs-EA. Lanes 9–11: complexes of POs-EA with 75, 77, 80 wt % rGOx, respectively. Lanes 12–14: complexes of POs-SA with 62, 44, and 29 wt % rGOx-9, respectively. Lanes 15 and 16: complexes of POs-EA with 44 and 15 wt % rGOx9, respectively.

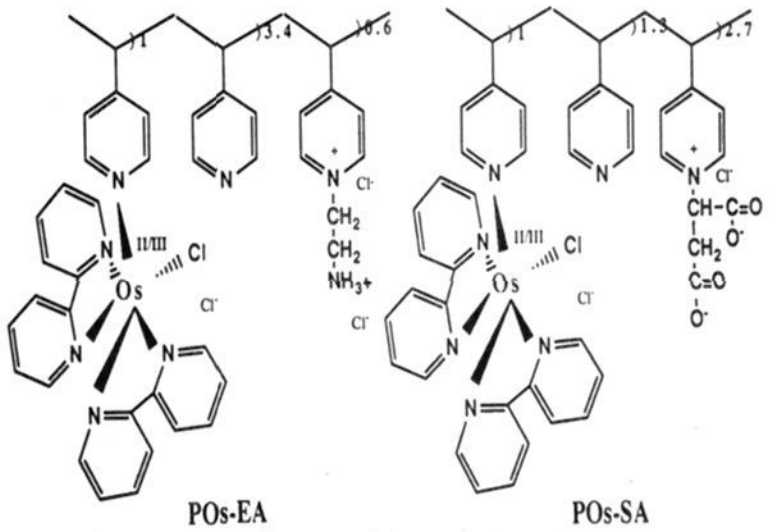


**Figure 2.** Structures and compositions of the redox polyelectrolytes.

By reacting 70 kDa poly(vinylpyridine) complexed with $[Os(bpy)_2Cl_2]^{II/III}$ with bromoethylamine, the water-soluble redox polycation POs-EA (Figure 2, left) was formed,[12-14] and by reacting it with bromosuccinic acid, the water soluble polyanionic zwitterion POs-SA (Figure 2, right) was prepared. The IEF runs of these polymers are shown in Figure 1, lanes 7 and 8. POs-EA, being a polycation at any pH, does not focus when migrating to the cathode. POs-SA focuses at pH 4.8, where protonation of free pyridine rings of the poly(vinylpyridine) backbone balances the excess negative charge associated with the succinate functions.

Complexing of enzymes and polyelectrolytes was observed through the IEF migration patterns[15] of Figure 1, lanes 9–16. When a complex was not formed, e.g., between native rGOx and POs-SA, both of which are polyanions at pH 7, the two components migrated and focused independently at their respective isoelectric points of 3 and 4.8. However, when a complex was formed, the

(1) (a) Beitz, J.; Schellenberger, A.; Lasch, J.; Fischer, J. *Biochim. Biophys. Acta* **1980**, *612*, 451. (b) Colovchenko, N. P.; Kataeva, I. A.; Akimenko, V. K. *Biokhimiya* **1992**, *57*, 1031. (c) Decher, G.; Essler, F.; Hong, J. D.; Lowack, K.; Schmitt, J.; Lvov, Y. *Polym. Prepr., Am. Chem. Soc. Div. Polym. Chem.* **1993**, *34*, 745.

(2) (a) Furusaki, S.; Asai, N. *Biotechnol. Bioeng.* **1983**, *25*, 2209. (b) Lesins, V.; Ruckenstein, E. *Colloid Polym. Sci.* **1988**, *266*, 1187.

(3) (a) Roth, C. M.; Lenhoff, A. M. *Langmuir* **1993**, *9*, 962. (b) Wehtje, E.; Adlercreutz, P.; Mattiasson, B. *Biotechnol. Bioeng.* **1993**, *41*, 171.

(4) (a) Dautzenberg, H.; Koetz, J.; Linow, K.-J.; Philipp, B.; Rother, G. *Polym. Prepr., Am. Chem. Soc. Div. Polym. Chem.* **1991**, *32*, 594. (b) Ezzell, S. A.; Middleton, J. C.; McCormick, C. L. *Polym. Prepr., Am. Chem. Soc. Div. Polym. Chem.* **1991**, *32*, 573. (c) Kokufuta, E.; Shimizu, H.; Nakamura, I. *Macromolecules* **1981**, *14*, 1178. (d) Kokufuta, E.; Takahashi, K. *Polymer* **1990**, *31*, 1177.

(5) (a) Park, J. M.; Muhoberac, B. B.; Dubin, P. L.; Xia, J. *Macromolecules* **1992**, *25*, 290. (b) Ganzler, K.; Greve, K. S.; Cohen, A. S.; Karger, B. L.; Guttman, A.; Cooke, N. C. *Anal. Chem.* **1992**, *64*, 2665. (c) Nguyen, T. Q. *Makromol. Chem.* **1986**, *187*, 2567. (d) Luckman, P. F.; Ansarifar, M. A. *Br. Polym. J.* **1990**, *22*, 233.

(6) Heller, A. *J. Phys. Chem.* **1992**, *96*, 3579.

(7) De Baetselier, A.; Vasavada, A.; Dohet, P.; Ha-Thi, V.; De Beukelaer, M.; Erpicum, T.; De Clerck, L.; Hanotier, J.; Rosenberg, S. *Bio/Technology* **1991**, *9*, 559.

(8) Frederick, K. R.; Tung, J.; Emerick, R. S.; Masiarz, F. R.; Chamberlain, S. H.; Vasavada, A.; Rosenberg, S.; Chakraborty, S.; Schopter, L. M.; Massey, V. *J. Biol. Chem.* **1990**, *265*, 3793.

(9) Yasuda, Y.; Takahashi, N.; Murachi, T. *Biochemistry* **1971**, *10*, 2624.

(10) Nakamura, S.; Hayashi, S.; Koga, K. *Biochim. Biophys. Acta* **1976**, *445*, 294.

(11) Sawicki, E.; Hauser, T. R.; Stanley, T. W.; Elbert, W. *Anal. Chem.* **1961**, *33*, 93.

(12) Gregg, B. A.; Heller, A. *J. Phys. Chem.* **1991**, *95*, 5976.

(13) Katakis, I.; Heller, A. *Anal. Chem.* **1992**, *64*, 1008.

(14) Ye. L.; Hammerle, M.; Olsthoorn, A. J.; Schuhmann, W.; Schmidt, H.-L.; Duine, J.; Heller, A. *Anal. Chem.* **1993**, *65*, 238.

(15) Katakis, I.; Davidson, L.; Heller, A., unpublished results.

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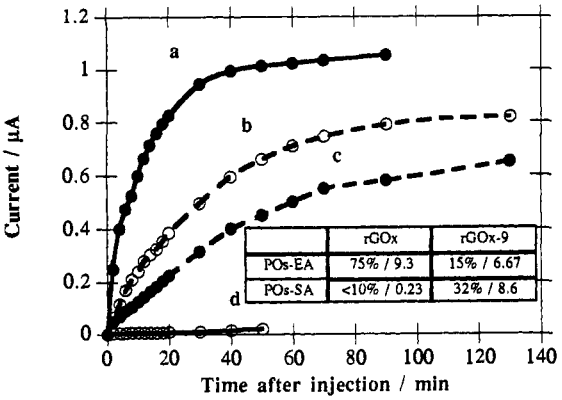


**Figure 3.** Time dependence of the current of modified graphite electrodes (6.15-mm diameter, in 33 mM phosphate buffer, 0.15 M NaCl, pH 7.3, 21.5 °C) after injections of rGOx (3.1 $\mu$g mL$^{-1}$, 0.43 units mL$^{-1}$) and rGOx-9 (11 $\mu$g mL$^{-1}$, 0.36 units mL$^{-1}$). (a) rGOx:POs-EA, (b) rGOx-9:POs-SA, (c) rGOx-9:POs-EA, (d) rGOx:POs-SA. Argon atmosphere, stirred 10-mL cells, electrodes poised at 0.45 V (SCE). The binding capacity as wt % of the enzyme and the current densities normalized for the injected enzyme activities ($\mu$A cm$^{-2}$ unit$^{-1}$ mL) are shown in the inset.

enzyme and the polyelectrolyte, whether cationic or anionic, migrated and focused together (lanes 9, 14, and 16). The enzyme binding capacity, which we define as the highest enzyme:polymer ratio at which all of the enzyme is polyelectrolyte-complexed, was determined through IEF runs such as those shown in Figure 1, lanes 9–16. When this capacity was exceeded, migration and focusing of free enzyme at its normal isoelectric point was observed (lanes 10, 11, 12, 13, and 15). The enzyme binding capacities varied between 3.0 for POs-EA complexed with rGOx (lane 9) and 0.0 for POs-SA complexed with the same enzyme. The capacity of POs-EA for binding the aminated enzyme with an isoelectric point of 9 (rGOx-9) was 0.2 (lane 16) and that for POs-SA was 0.4 (lane 14).

A qualitative relationship between electron transfer and adduct formation, i.e., enzyme binding capacity, was observed in glucose electrodes made with either rGOx or with rGOx-9 "wired" with a redox polyelectrolyte to a graphite electrode. In these experiments, POs-EA or POs-SA was adsorbed on the electrode, which was thoroughly washed to remove any nonadsorbed polyelectrolyte. Charge measurements, through slow (0.001 V s$^{-1}$) cyclic voltammetry, showed $(4.0 \pm 0.4) \times 10^{-8}$ equiv of redox centers per cm$^2$ for POs-EA and $(2.9 \pm 0.15) \times 10^{-8}$ equiv cm$^{-2}$ for POs-SA. The electrodes were then dipped in well-stirred, pH 7.3 physiological buffer solutions (0.15 M NaCl, 0.033 M phosphate) containing 0.3 M glucose, poised at 0.45 V (SCE), and rGOx or rGOx-9 was added to produce solutions containing respectively 0.43 or 0.36 unit mL$^{-1}$ of the enzyme. The glucose electrooxidation currents (Figure 3) correlated with the enzyme binding capacity of the redox polyelectrolyte. Assuming that the glucose permeability in the adsorbed film was not greatly altered for the different enzyme:polymer combinations and that the formation of adducts in the IEF agarose gel was paralleled by their formation on the electrode surface, the increase in current with enzyme binding capacity of the redox polyelectrolyte could have resulted from (a) increase in the amount or specific activity of the bound enzyme when electrodes were modified with redox polymers having a higher enzyme binding capacity and/or (b) higher rate of electron transfer from the substrate-reduced enzyme to the redox polyelectrolyte when the electrostatic interaction, manifested in a higher enzyme binding capacity, was stronger.

**Table 1.** Relationship between the Current, Normalized for Bound Enzyme Activity, and the Enzyme Binding Capacity of the Redox Polyelectrolyte

| enzyme | polymer | relative enzyme activity, $A$ | current, $I$ (nA) | activity normalized current, $I/A$ | capacity (%) |
|---|---|---|---|---|---|
| rGOx | POs-EA | 1.0 ± 0.15 | 327 | 327 | 75 |
| rGOx | POs-SA | 0.4 ± 0.15 | 0.89 | 2 | <10 |
| rGOx-9 | POs-EA | 9.2 ± 0.3 | 17.81 | 2 | 15 |
| rGOx-9 | POs-SA | 4.0 ± 0.3 | 53.74 | 13 | 32 |

To distinguish between the two, the relative activity of the electrode-bound enzymes was assayed by measuring the rate of $H_2O_2$ formation through the rGOx-catalyzed oxidation of glucose by $O_2$. For these assays, the conditions where such that neither $O_2$ nor glucose mass transport limited the rate of $H_2O_2$ formation. The carbon electrodes, modified with polymer as described, were dipped for 1 h in a solution of enzyme in physiological buffer (3.1 $\mu$g mL$^{-1}$ and 0.43 unit mL$^{-1}$ in the case of rGOx and POs-EA, 270 $\mu$g mL$^{-1}$ and 9 units mL$^{-1}$ in all other cases) and then thoroughly washed in deionized water in a well-stirred 10-mL beaker. Next, they were inserted, as stationary electrodes, in a 3-mL, $O_2$-blanketed cell having a large (1.5-cm diameter) Pt electrode rotated at 2500 rpm to rapidly stir the 0.25 M glucose physiological buffer solution. The 0.25 M glucose concentration greatly exceeded the apparent Michaelis constants of the electrodes (16–25 mM glucose). With the enzyme electrodes poised at 0 V (SCE), where glucose is not catalytically electrooxidized, and the Pt electrode poised at 0.65 V, where the nonelectrochemical reaction product, $H_2O_2$, is electrooxidized, the relative rates of $H_2O_2$ generation were measured for the four enzyme–polymer pairs. The results are summarized in Table 1. Independent experiments showed that in the dissolved enzyme–redox polyelectrolyte adduct the enzymatic generation of $H_2O_2$ was not inhibited. The current, when normalized for the relative bound enzyme activity, increased with the binding capacity of the polymer. The higher enzyme activity-normalized current of the electrode modified with the electrostatically well-bound POs-EA:rGOx and POs-SA:rGOx-9 pairs and the low current in the repulsive pairs, POs-EA:rGOx-9 and POs-SA:rGOx are explained by a higher rate of electron transfer from the enzyme to the "wire" when a better bound adduct is formed.

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