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PAPER

Improved glucose oxidation catalytic current generation by an FAD-dependent glucose dehydrogenase-modified hydrogel electrode, in accordance with the Hofmeister effect

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Abstract

Herein, we describe the effect of varying anions in an electrolyte solution on current generation by a redox hydrogel electrode. The electrode surface is coated with a thin film of hydrogel matrix, consisting of an osmium (Os) redox polymer including tethered Os complexes, polymer backbone, and a redox enzyme. In this case, the enzymes employed are flavin adenine dinucleotide-dependent glucose dehydrogenase (FAD-GDH), which catalyzes glucose oxidation, and the result was compared with that reported earlier for glucose oxidase (GOx). The hydrogel matrix facilitates efficient electron transfer from glucose to the electrode via collision of the Os complexes and thus acts as a mediator. The degree of impact of anions on current generation is characteristic of the Hofmeister series. Chaotropic anions, such as nitrate and chloride, increase and decrease the catalytic current produced by FAD-GDH and GOx hydrogel electrodes, respectively. Such anions can adsorb onto the cationic region of the FAD-GDH surface and induce a negative charge, which enhances electrostatic interactions between the enzyme and the positively charged Os polymer. Kosmotropic anions, such as sulphate and phosphate increase the catalytic current due to hydrogel shrinkage, which increases the relative concentrations of both enzyme and mediator within the hydrogel architecture due to an increase in density. High-performance electrode design depends on understanding the impact of ion identity on catalytic current responses of redox hydrogel electrodes.

1. Introduction

A redox hydrogel electrode is a type of immobilized-enzyme electrode fabricated by crosslinking a redox enzyme and redox polymer using polyethylene glycol diglycidyl ether (PEGDGE). One of the most successful types of redox polymers includes $Os^{2+/3+}$-complexes tethered to a polymer backbone of polyvinyl imidazole or polyvinyl pyridine (osmium (Os) polymer). The Os complex acts as a mediator, shuttling electrons from the enzyme to the electrode via Os complex collision [1–3]. Together, the crosslinked enzyme and Os polymer form a thin redox hydrogel matrix film—containing a high density of enzyme and mediator—on the electrode's surface. The enzyme efficiently generates a catalytic current, regardless of its orientation.

The redox hydrogel can achieve a catalytic current with a magnitude on the order of mA cm^{-2} due to the close apposition of the enzyme and Os polymer adduct, producing high current density without hydrogel detachment from the electrode surface. Redox hydrogel technology is applicable to the manufacture of bio-electrochemical devices, such as continuous monitoring biosensors [2] and enzymatic biofuel cells [4, 5]. Recently, certain organic redox molecules (quinones and phenothiazines) have been used as an alternative to the abovementioned Os complexes [6–9]. Formation of the enzyme-Os polymer adduct is facilitated by electrostatic interactions between the negatively charged enzymes and the positively charged polymer-tethered Os complexes. High electrolyte concentrations weaken such electrostatic interactions, decreasing the catalytic current. Therefore, the Os polymer and enzyme are covalently linked with a cross-linker [1, 10]. Even in the presence of crosslinking, high ionic strength conditions decrease the catalytic

current, which is attributable to two potential mechanisms [1, 10, 11]: (a) a shielding effect leading to a more linear (less coiled) form of the Os polymer, thereby decreasing Os electron-transfer activity, and (b) counteracting the effect of enzyme-Os polymer electrostatic interactions.

Interestingly, not only ionic strength but also ionic identity affects catalytic current output. Previously, we demonstrated that the addition of each of seven distinct ions to the phosphate buffer solution decreased the catalytic current output of a hydrogel electrode incorporating glucose oxidase (GOx) [12]. Even at the equivalent ionic strength, the magnitude of current decrease induced by distinct ions differed, in a manner consistent with the Hofmeister series [12]. This series orders ions according to their influence on protein stability and solubility, impacting a wide range of macromolecular and biological phenomena [13, 14]. On one end of the series continuum are the so-called kosmotropes, which are strongly hydrated (have large solvation shells), interacting to a greater extent with the water in the solution, thereby increasing the effective protein concentration, resulting in 'salting-out' effects on proteins and macromolecules (i.e. increasing stability but decreasing hydrophobic solubility, favoring precipitation). On the other end of the series are the so-called chaotropes, which interact only weakly with water, but strongly with protein hydrophobic pockets, resulting in 'salting-in' effects (i.e. destabilizing proteins but increasing their solubility, opposing precipitation). Anions have a stronger effect than cations and are usually ordered as follows (from most to least kosmotropic): citrate^{3-} > $SO_4{}^{2-}$ > $HPO_4{}^{2-}$ > F^- > Cl^- > Br^- > $NO_3{}^-$ > $ClO_4{}^-$. Unsurprisingly, ionic effects on enzyme stability and activity often coincide with those predicted by the Hofmeister series [14, 15].

The impact of dissolved anions in decreasing glucose oxidation current output from the GOx hydrogel electrode can be summarized as follows [12]:

(a) Kosmotropic and chaotropic anions induce hydrogel shrinkage (increasing relative enzyme and Os polymer adduct densities and thus catalytic current) and swelling (which has the opposite effect), respectively [13, 16–18].
(b) Chaotropic ions adsorb onto the enzyme surface (impeding substrate access to the enzyme reaction site).
(c) Polyvalent kosmotropic ions (e.g. citrate, phosphate, and sulfate) tend to weaken the electrostatic interactions between the negatively charged enzyme and the positively charged Os polymer.

Perchlorate ions are an exception that do not conform to the behavior predicted by the Hofmeister series. They cause extreme dehydration of the GOx electrode hydrogel, preventing mass transport within this matrix, thus decreasing catalytic current [12].

Due to factors (a) and (c), the GOx hydrogel electrode exhibits decreased catalytic current generation at high phosphate buffer concentrations [12]. However, we previously demonstrated increased catalytic current generation under such conditions when a flavin adenine dinucleotide-dependent glucose dehydrogenase (FAD-GDH) hydrogel electrode is used [19].

This study examines the effect of distinct anions on the performance of an FAD-GDH hydrogel electrode [20]. This enzyme has attracted considerable attention by virtue of being unaffected by dissolved oxygen [21]. Furthermore, the electrocatalytic activity of FAD-GDH is much higher than that of GOx in the Os polymer hydrogel system [20]. Recently, the effects of electrolytic ions on the stability of FAD-GDH have been reported [22, 23] (e.g. kosmotropic ions stabilize FAD-GDH via a compaction effect). The designing of high-performance redox hydrogel electrode necessitates an understanding of the impact of distinct ions on catalytic current generation. Therefore, the relationship between dissolved anion identity and electrochemical responses of FAD-GDH and deglycosylated FAD-GDH (d-FAD-GDH) hydrogel electrodes was investigated, particularly in the context of behaviors predicted by the Hofmeister series.

2. Methods

All reagents were of analytical grade and all solutions were prepared with distilled water. Biocatalyst solution composition: 25.2 mg ml^{-1} FAD-GDH enzyme from *Aspergillus terreus* (Ikeda Tohka Industries Co., Ltd [24]), 6 mg ml^{-1} poly(1-vinylimidazole) (PVI) complexed with Os(bipyridine)$_2$Cl ($E = 0.22$ V vs Ag|AgCl) as the redox polymer (PVI-Os), and 8 mg ml^{-1} PEGDGE (molecular weight 500; Sigma-Aldrich) as the crosslinker, at a mass ratio of 45 enzyme:45 PVI-Os:10 PEGDGE. The synthesis of PVI-Os proceeded as previously described, including partial quaternization to improve water solubility [1]. The preparation of d-FAD-GDH proceeded as previously described [20].

A 3 mm diameter glassy carbon disk electrode was made hydrophilic via plasma oxidation (10 min) prior to the addition of 4.5 μl of the hydrogel solution by pipetting and drying at 4 °C for 18 h. The total electrode surface hydrogel loading was fixed at 600 μg cm^{-2}.

A series of pH 7.0 electrolyte solutions were prepared by mixing 0.1 M each of disodium hydrogen phosphate and sodium dihydrogen phosphate solutions containing 0.5 M of a specific sodium salt (A: citrate,

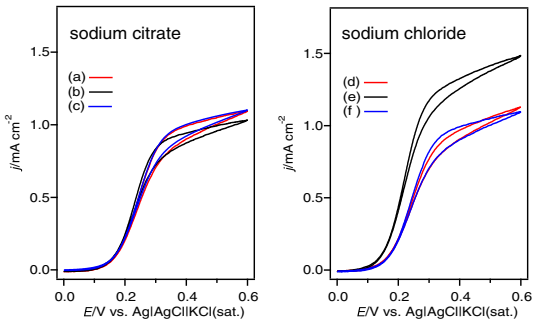

Figure 1. Cyclic voltammograms demonstrating sequential FAD-GDH hydrogel electrode glucose oxidation performance in the presence of: 0.2 M glucose only (a) and (d), with added 0.5 M Na-citrate (b), with added 0.5 M NaCl (e), and a repeat of 0.2 M glucose only (c) and (f).

B: sulphate, C: phosphate, D: fluoride, E: chloride, F: nitrate, or G: perchlorate). Finally, solutions were carefully adjusted to pH 7.0 using 0.1 M dipotassium hydrogen phosphate or potassium dihydrogen phosphate solutions. Rotating-disk cyclic voltammetry was performed using an electrochemical analyzer (Bioanalytical Systems, BAS 50 W). The electrode was rotated at 2000 rpm using a rotator (RDE-2, BAS). A platinum wire counter-electrode and Ag|AgCl|KCl(saturated) reference electrode were used. All measurements were performed in pH 7.0 phosphate buffer at 25 °C in a total volume of 20 ml. A scan rate of 20 mV s^{-1} was used in all experiments.

3. Results

The effect of anions on the FAD-GDH hydrogel electrode was elucidated by comparing rotating-disk cyclic voltammograms (CVs) obtained in the presence and absence of various sodium salts. Steady-state currents were highly stable at experimental time scales. The standard CV (pH 7.0 phosphate buffer containing 0.2 M glucose only) demonstrates a glucose oxidation current of 1 mA cm^{-2} at 0.3 V (figure 1, left panel, curve (a)). After rinsing the hydrogel electrode with distilled water, it was submerged in an identical buffer, now also containing 0.5 M sodium citrate (figure 1, left panel, curve (b)). The hydrogel electrode was rinsed with distilled water, and then it was again submerged in the initial buffer to obtain a recovery curve (figure 1, left panel, curve (c)) nearly identical to curve (a). Thus, the effect of sodium citrate on electrode performance—potential-dependent current increase beyond 0.3 V—is time-independent and reversible. The impact of sodium citrate suggests an interaction between the negatively charged enzyme and the positively charged electrode surface: as anode potential increases, more enzymes may collect on the electrode surface, thereby increasing current output.

The effect of sodium chloride on the current output was examined in a similar manner (figure 1, right panel). Relative to a standard CV (curve (d)), the presence of sodium chloride increased the steady-state current (curve (e)), with an onset potential of approximately 0.15 V. The onset gradient slope suggests a decrease in surface electron-transfer resistance. After rinsing the hydrogen electrode with distilled water, the recovery curve (figure 1, right panel, curve (f)) was obtained, which again resembled curve (d). Reversibility suggests that sodium salt identity-induced catalytic current modulation is not due to irreversible structural changes to the enzyme/Os polymer, but due to reversible conformational changes within the hydrogel. This phenomenon is similar to that observed for the GOx-hydrogel electrode.

Figure 2 summarizes CVs of the FAD-GDH hydrogel electrode (left panel) and the d-FAD-GDH hydrogel electrode (right panel) in 0.1 M phosphate buffer containing 0.2 M glucose in the presence of different sodium salts at a concentration of 0.5 M (including fluoride, phosphate, sulfate, citrate, chloride, nitrate, or perchlorate).

Figure 3 summarizes current densities at 0.6 V of the CVs represented in figure 2 and the data for GOx from the previous report [12], ordered according to the Hofmeister series [15, 25], with kosmotropic to chaotropic ions aligned from left to right. Residual current is defined as the ratio of catalytic current density in 0.1 M phosphate buffer containing 0.2 M glucose and 0.5 M sodium salt, to that in the same solution lacking sodium salt.

Most notably, while certain sodium salts increase the catalytic glucose oxidation current generated by FAD-GDH or d-FAD-GDH hydrogel electrodes, they decrease the catalytic current generated by the GOx hydrogel electrode.

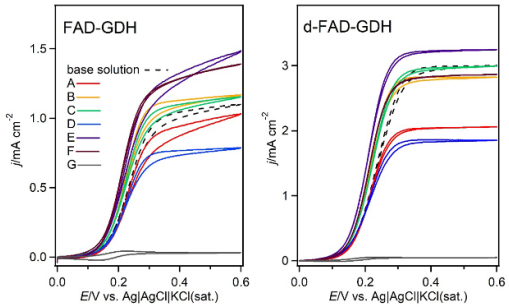

Figure 2. Cyclic voltammograms demonstrating FAD-GDH (left panel) and d-FAD-GDH (right panel) hydrogel electrode glucose oxidation performance in phosphate buffer containing 0.2 M glucose in the absence (base solution, black broken line) or presence of various 0.5 M sodium salts ((A) citrate, (B) sulphate, (C) phosphate, (D) fluoride, (E) chloride, (F) nitrate, or (G) perchlorate).

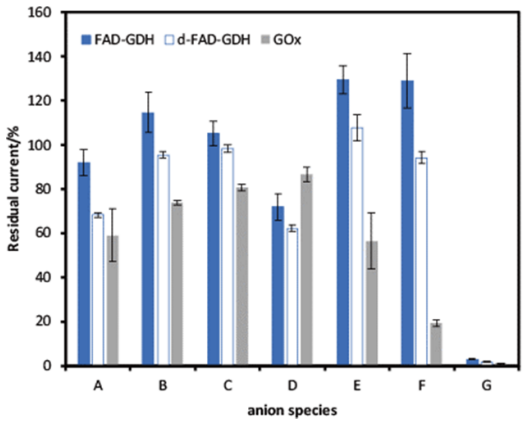

Figure 3. Effects of various 0.5 M sodium salts on glucose oxidation current at 0.6 V across different (FAD-GDH, d-FAD-GDH, and GOx) hydrogel electrodes. (A) citrate, (B) sulphate, (C) phosphate, (D) fluoride, (E) chloride, (F) nitrate, and (G) perchlorate.

4. Discussion

Excluding perchlorate (figure 3(G))—which significantly suppressed catalytic current (as also noted previously [12]), perhaps through Os complex interaction and hydrogel dehydration—chaotropic anions, such as chloride and nitrate (figures 3(E) and (F)) produced opposite trends in current generation by (d-)FAD-GDH versus GOx hydrogel electrodes. Chaotropic ions did not decrease the catalytic current generated by FAD-GDH and d-FAD-GDH hydrogel electrodes. However, the catalytic current generated by the GOx hydrogel electrode in the presence of chaotropic ions decreased, and it increased as ions became more kosmotropic [12].

For the FAD-GDH hydrogel electrode, enzyme negative surface charge is enhanced by adsorption of chaotropic anions onto its hydrophobic portion, which may improve electron transfer between the enzyme and the positively charged Os complex. Considering the law of matching water affinities, a chaotropic anion easily forms ion pairs with chaotropic cations; and a kosmotropic anion, with kosmotropic cations [26]. For instance, a carboxyl group moiety on the enzyme surface, which is a kosmotropic anion, forms ion pairs with kosmotropic cations, and a positively charged amino group, which is a chaotropic cation, easily forms ion pairs with chaotropic anions [27]. Figure 4 indicates surface charge distribution of GOx (*Aspergillus niger,* 1 GAL [28]) versus FAD-GDH (*Aspergillus flavus,* 4YNT [29]) enzymes, demonstrating the abundant negative charge of GOx relative to the more neutral FAD-GDH and therefore, suggesting a role for these different charge states in the differing effects of anions on GOx and FAD-GDH hydrogel electrodes. Although the FAD-GDH enzyme represented in figure 4 is derived from a different strain than that employed in this study, it has 60% amino-acid sequence homology to the FAD-GDH from *Aspergillus terreus.* FAD-GDH has a cationic region close to the active site, and therefore, chaotropic anions such as Cl^- and NO_3^- tend to adsorb on this area. Besides charged groups, uncharged polar groups have also been found to be binding sites for anions [30]. This leads to an increase in the negative surface charge of the enzyme, improving affinity to the positively charged mediator. Such interactions between proteins and chaotropic anions have been reported previously [31]. The current decrease in the GOx hydrogel electrode may be attributed to the adsorption of

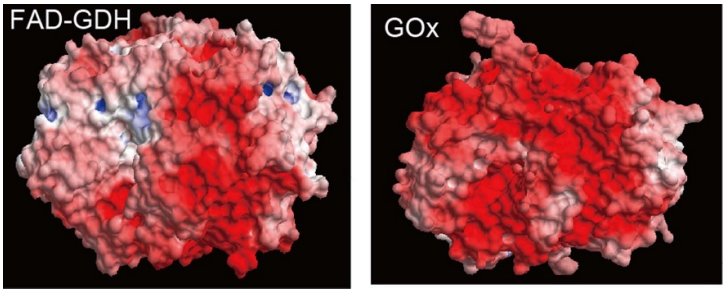

Figure 4. Three-dimensional rendering of redox enzymes (left: FAD-GDH, right: GOx) relevant to the present study, indicating surface charge distribution. Red indicates negative charge, blue indicates positive charge, and white indicates no charge. Rendering software: CueMol 2 ver 2.2.3.443.

Table 1. Summary of anion effects on components of the different enzyme-Os polymer hydrogel electrodes.

	GOx	FAD-GDH	Hydrogel (positively charged Os polymer)
Chaotropic anions	Prompting adsorption of Na^+ near substrate-binding site, electrostatically inhibiting access of Os complex	Adsorption near substrate-binding site, promoting access of Os complex	Hydrogel swelling
Kosmotropic anions	Forming ion pair with Na^+ and preventing Na^+ adsorption near the substrate-binding site	No significant effect on FAD-GDH	Hydrogel shrinking

the kosmotropic cation, Na^+, near the substrate-binding site, and the resulting electrostatic repulsive interaction decreases the electron transfer. Since GOx has a much higher negative surface charge compared to FAD-GDH (figure 4), the change in the surface charge by anionic adsorption will be negligible. However, cation adsorption would significantly affect the interaction with the Os complex of the redox polymer.

Anion effects on components of the different enzyme-Os polymer hydrogel electrodes are summarized in table 1. In the presence of citrate, sulfate, phosphate, or fluoride ions (figures 3(A)–(D)), the current generated by the GOx-hydrogel electrode decreased with increasing kosmotropicity and ionic strength. In contrast, effects of kosmotropic anions on FAD-GDH hydrogel electrode performance assume a bell-shaped profile. Kosmotropic anions exert opposing effects on current generation efficiency. Kosmotropic anions have a strong affinity for water molecules, and therefore, they dehydrate and shrink the hydrogel, thereby increasing the catalytic current. However, relative to monovalent ions at the same concentration, polyvalent ions exhibit increased ionic strength, which decreases the electrostatic interactions between enzymes and the Os polymer. Fluoride is classified as kosmotropic based on the positive coefficient of Jones–Dole viscosity B [26]. However, it is positioned at the boundary between kosmotropic and chaotropic ions, and the shrinking effect is not significant. The effect of ionic strength is predominant in the presence of fluoride, resulting in a decreased catalytic current.

In the case of GOx, which electrostatically interacts more favorably with the Os polymer, increasing ionic strength decreases catalytic current generated by the hydrogel electrode. This effect may outweigh the current increase due to hydrogel shrinking. Conversely, in the case of FAD-GDH (less negatively charged than GOx), ionic strength effects are less pronounced and the hydrogel shrinking effect predominates. Thus, decreases or increases in current are determined by the balance between high ionic strength and hydrogel shrinking within the different enzymatic electrode systems.

No opposing anion effect on FAD-GDH and d-FAD-GDH hydrogel electrodes was noted. However, impacts on the FAD-GDH electrode were larger than on the d-FAD-GDH electrode. When considering the interaction between polysaccharide chains and water, solubility, hydration, swelling, and the hygroscopic state of polysaccharide chains are determined by the strength of three types of hydrogen bonds: water–water, polysaccharide–water, and polysaccharide–polysaccharide. Deglycosylation of FAD-GDH can change enzyme surface hydrogen bonding patterns. Considering the Hofmeister series, chaotropic ions can disrupt the hydrogen bonding network between water molecules, while kosmotropic ions do the opposite [32]. Although further studies concerning ionic effects on sugar chain solubility are required, enzyme surface hydrogen bonding may contribute to the differences in electrochemical response.

5. Conclusions

In the presence of specific ions, glucose oxidation catalytic current generation by the FAD-GDH hydrogel electrode was enhanced due to a combination of hydrogel shrinking and an enhanced enzyme surface negative charge. Conversely, in the presence of specific ions, glucose oxidation catalytic current generation by the GOx hydrogel electrode was decreased due to weakened electrostatic interactions between the negatively charged enzyme and the positively charged Os polymer. The current increase in the presence of kosmotropic anions is likely due to increased hydrogel density via dehydration.

In the presence of chaotropic ions, enzyme-Os polymer electrostatic interactions are strengthened by the adsorption onto the enzyme surface, increasing its negative charge, thereby increasing catalytic current.

Clarifying the relationship between ion identity and glucose oxidation catalytic current modulation is relevant to designing an optimal redox polymer for the redox enzyme of interest, or selecting the optimal (poly)electrolytes for biofuel cells not intended for use with implantable/wearable devices. This research demonstrates that adjusting enzyme-redox polymer interactions and rationally altering enzyme surface or redox polymer structure can contribute toward designing a high-durability electrode for use within living organisms.

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