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Enhanced hemoglobin electroactivity on carbon in electrolytes or binders containing water-miscible primary alcohols

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ABSTRACT

Herein we demonstrate for the first time, the commonly overlooked impact of water-miscible primary alcohols on hemoglobin (Hb) electroactivity. When Hb is cast into biosensor films, the binder solvent is often an alcohol; however, the impact of alcohols on Hb electroactivity is unknown. It is well-established that Hb electroactivity is inhibited by the protein structure and that alcohols disturb this structure; thus, probing the alcohol-Hb relationship is vital to evaluate electrochemical results using binder-immobilized Hb. Using differential pulse voltammetry on glassy carbon in neutral pH phosphate buffer, we show a twofold higher Hb response when the Nafion binder contains alcohol, indicating the alcohol significantly changes Hb electroactivity. Significant changes are seen for alcohol concentrations as low as 10%. When binders are necessary for a Hb-containing film, a low methanol content is preferred; ethanol and propanol should be avoided.

Conversely, Hb quantification is 53× higher in alcohol-containing electrolytes, due to changes to both the Hb and carbon. Methanol-containing electrolyte exhibits an ultra-low 0.78 nM Hb detection limit. This detection limit is twofold lower than any previously reported for solution-based Hb detection and on par with surface-immobilized Hb, while having the advantages of simplicity, ease of application and low cost.

1. Introduction

Electrochemical biosensors, which convert a biological event into a readable electronic signal, are an active research area with applications in diagnostics, biotechnology, and environmental studies [1]. The promising potential of electrochemical biosensors stems from their speed, sensitivity, and low-cost [1,2] and these systems are becoming more portable and affordable as electronics become smaller and more common. A commercially successful example is the blood glucose monitor [1]. As well, new developments in electrochemical biosensors further contribute to the exciting area of lab-on-a-chip devices [2].

Detecting Hb is important due to its cytotoxic effects and the role hemoglobin (Hb) plays in various diseases [3] such as anemia [4]. Electrochemical-sensing of Hb may provide an alternative to the commonly used fluorimetric, colorimetric, and chemiluminescence methods [5–7], while offering enhanced portability and cost-effectiveness [8]. Additionally, electrochemical sensing systems offer high sensitivity and low detection limits comparable to optical methods [9,10].

Hb is a blood protein capable of redox activity associated with the oxidation and reduction of iron within the heme prosthetic group [11–13], similar to that seen with other heme-containing proteins such as cytochrome *c* [14], myoglobin [15,16], and horseradish peroxidase [17]. However, direct electron transfer between Hb and an electrode is generally considered to be difficult because the four redox-active hemes are buried in the interior, hydrophobic regions of the protein [18,19]. While Hb can be detected in solution [12,20,21], it is more common to use an electrochemical mediator [11,22], to denature by unfolding Hb [23], or to immobilize Hb directly onto the electrode [11,24] to observe its electroactivity. Nafion binder is often used to immobilize Hb [25]; typically, the Nafion contains an alcoholic solvent in the suspension but the impact of the alcohol on Hb electrochemistry is unknown.

Various electrodes have been used for electrochemical Hb detection, particularly silver [26,27] and metal oxides [24,28]; however, the low cost and high abundance of carbon makes it an attractive and promising material for biosensor electrodes. To improve the electron transport and/or binding of Hb to the electrode surface, the carbon may be modified by: quantum dots [20,29], mediators [11,22], carbon nanotubes [13,24], or embedding the Hb into clay [11] or polymer films [11]. On unmodified carbon, literature observations of Hb electroactivity have been inconsistent. Some groups reported no Hb electroactivity using pyrolytic graphite [16,30], glassy carbon [31], and graphite [32], while others had success using carbon nanotubes [13,24], carbon black [19], glassy carbon [12,21], graphene [12], and pyrolytic graphite [33]. It is interesting to note that Shen and Hu [30] and Zhao

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et al. [16] observed no or very poor direct Hb electrochemistry on pyrolytic graphite, while Cao and Hu [33] observed a pair of redox peaks on the same material.

One important consideration for Hb electroactivity on carbon electrodes is the presence of carbon surface functionalities. In the literature, there is disagreement whether carbon-oxygen surface functionalities improve [16,21] or inhibit [12] Hb electroactivity. There are suggestions that more C-O groups, such as alcoholic or phenolic groups, are responsible for promoting the electron transfer, perhaps by changing polypeptide conformations [21]. More recent evidence suggests less oxidized carbons have a larger Hb electroactivity [12]. Our recent results indicate that ether and carbonyl surface groups inhibit Hb electroactivity, while lactones and phenols appear to have little inhibitive effect [34].

Another important variable separating Hb-active and Hb-inactive carbons is whether a binder is used to hold the materials on the electrode. Often, the binder is liquid Nafion [24] since it is commercially available and is convenient for casting into thin films on electrodes. Spectroscopic data showed that the Hb structure can be influenced by both Nafion and ethanol [24]. Liquid Nafion typically contains an alcohol such as methanol [25], ethanol [24], or 1-propanol [35]; however, it is well-known that alcohols changes the Hb structure [36–38]. Denaturation of the protein's conformational framework was observed by changes in the peptide bond, and via fluorescence as tryptophan residues normally found in the hydrophobic Hb interior are exposed to solution [38]. These changes in the Hb structure likely facilitate Hb electroactivity due to the protein denaturation making the hemes more accessible to electron transfer with the electrode; however, the impact of alcohol-induced changes to Hb electroactivity has not previously been examined.

Until this work, the effect of Nafion's alcoholic solvent was overlooked when this binder was used to immobilize Hb; we propose that it may be a significant factor when considering films made using alcohol-containing binders. When creating Nafion thin films, the alcoholic solvent is in contact with both the Hb and the carbon surface, potentially modifying either or both and leading to changes in Hb electroactivity. Solvent effects on Hb electroactivity have been seen previously, with dimethyl sulfoxide improving Hb electroactivity [39], whereas glycerol decreases it [40]. This is likely because dimethyl sulfoxide denatures Hb, modifying the heme environment as evidenced by shifting the Soret absorption band [41], while glycerol does not [40]. Since the heme is the site of the Hb electroactivity, modifications to the heme may be facilitating Hb electrochemical reduction.

The impact of water-miscible primary alcohols on the direct electroactivity of Hb has never been studied. This paper shows the significant increase in Hb electroactivity on glassy carbon upon exposure to an alcoholic solvent. This knowledge is used to propose a simple system for Hb detection, achieving a sensitivity of $0.209\,\mu A\,nM^{-1}$ ($3240\,\mu A\,L\,g^{-1}$) and a detection limit of 0.78 nM ($5.0 \times 10^{-5}\,g\,L^{-1}$). We also show that the alcoholic content of a Nafion suspension can strongly impact the measured Hb electrochemical response and caution must be used when considering electrochemical results with systems using this Nafion. We provide suggestions on the choice of alcohol-containing Nafion to limit possible interferences.

2. Material and methods

2.1. Electrochemical experimentation

All electrochemical measurements were made using a Bio-Logic VMP3 multipotentiostat connected to a three-electrode system in an all-glass one-compartment electrochemical cell. The three electrodes were: a platinum mesh counter electrode, a Hg/Hg_2SO_4 reference electrode (saturated K_2SO_4, measured as 0.692 V versus the standard hydrogen electrode), and a glassy carbon working electrode (GC, CHI 104, 3.0 mm carbon diameter, 6.35 mm total diameter including the inert Kel-F shell). The Hg/Hg_2SO_4 reference electrode was used to avoid possible chloride contamination leading to the well-known allosteric chloride effect on Hb [42]. The 0.1 M phosphate buffer (PB, pH 7.08) made from K_2HPO_4 (ACS Reagent, Sigma Aldrich, ≥98%) and KH_2PO_4 (Sigma Life Science, ≥99.0%) in a 64.5%–35.5% mole ratio dissolved in 18.2 MΩ·cm water.

Prior to polishing, the glassy carbon was wiped clean and submersed in 1 M NaOH for five minutes to remove residual Hb, followed by rinsing with Millipore 18.2 MΩ cm water. The glassy carbon was polished on nylon pads (BASi PK-4 MF-2060 polishing kit, 3 and 1 μm diamond polishes) and rinsed with copious amounts of 18.2 MΩ cm water between polishing steps. The electrochemical cells and counter electrodes were cleaned by wiping with a Kimwipe to remove excess Hb, then washed with distilled water, followed by a five-minute submersion in 1 M NaOH to remove residual Hb, and rinsed thoroughly with 18.2 MΩ cm water.

Differential pulse voltammograms (DPVs) were collected between 300 and −900 mV with a $20\,mV\,s^{-1}$ scan rate. The initial potential was held for 2 s, all subsequent steps had a 50 mV pulse height, 250 ms pulse width, −25 mV step height, and 1250 ms step time. All experiments were performed in triplicate and graphical error bars indicate one standard deviation.

2.2. Examining immobilized Hb (in Section 3.1)

Hb (methemoglobin, Sigma Life Science, lyophilized powder, from bovine blood) was immobilized on a glassy carbon electrode using either (1) a thin film formed from 10 μL of a liquid Nafion suspension containing 1-PrOH (Ion Power Inc., DuPont DE521, *ca.* 50% 1-propanol content) air-dried for 10 min ($GC/Hb/Nf_{suspension}$), (2) a 4.5 × 4.5 mm solid piece of Nafion (Nafion 117, Aldrich Chemical Company Inc., 0.007 inch thick) soaked in water ($GC/Hb/Nf_{solid}$), or (3) a solid piece of Nafion soaked in 50% 1-PrOH ($GC/Hb/Nf_{solid,PrOH}$). The soaked solid Nafion films were pressed dry with Kimwipe immediately before use. 10 μL of $1\,g\,L^{-1}$ Hb was air-dried for *ca.* one hour on the glassy carbon electrode before Nafion application. As a control experiment, 10 μL of Hb was dried onto a bare glassy carbon electrode without any Nafion (GC/Hb).

The $Nf_{suspension}$-modified electrodes were examined in the typical one compartment all-glass electrochemical cell containing 5 mL of 0.1 M PB. The Nf_{solid}-modified electrodes were examined in an electrochemical cell made from a polytetrafluoroethylene Swagelok pipe fitting, described in the Supplementary data (with detailed diagram Fig. S-1). Briefly, the modified working electrode was inserted through a 6.35 mm diameter hole in the Swagelok pipe fitting, while the wider pipe fitting opening contained the reference and counter electrodes and 2 mL of 0.1 M PB electrolyte.

2.3. Examining solution-based Hb (in Section 3.2)

Electrochemical studies of solution-based Hb were also conducted. For these experiments, the 10 mL PB electrolyte also contained $0.2\,g\,L^{-1}$ Hb, and/or one of the following alcohols: methanol (MeOH, Fisher Scientific, 99.9%), ethanol (EtOH, anhydrous, 100%), or 1-propanol (1-PrOH, Caledon Laboratory Chemicals, 99.5%). The electrolyte alcohol content was varied in increments of 10% by volume. Electrolytes containing alcohol were prepared immediately prior to experiments ensuring that evaporation of the alcohol was not an issue and alcoholic concentrations in the aqueous electrolyte were accurate.

UV–vis absorption spectra of PB electrolytes containing Hb and alcohols were collected on a Cary 5000 UV–vis spectrophotometer (Varian Inc.) using disposable Brand plastic cuvettes (4.5 × 23 mm window, path length 10 mm). The samples were measured repeatedly between wavelengths of 250 and 500 nm at a rate of $600\,nm\,min^{-1}$, requiring 38 s per scan due to the instrument's filter change process. Absorption spectra of PB were used as the background.