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Mediated spectroelectrochemical determination of holo-transferrin reduction potential using a flow cell with disposable screen-printed indium-tin oxide electrode

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ABSTRACT

A convenient spectroelectrochemical method for the determination of holo-transferrin reduction potential is described. All materials and accessories are commercially available and the results are in agreement with previous reports that required custom-built hardware. The method requires minimal preparation with simple sample handling, small solution volume, cost-effective electrode replacement and automated measurement. This method can easily be applied to the study of similar systems in aqueous media and might be of particular importance in the momentous field of glycomics, where large numbers of protein isoforms need to be characterized.

1. Introduction

Transferrin (Tf), an iron-carrying monomeric glycoprotein of molecular weight ~80 kDa, is composed of a single polypeptide chain consisting of 679 amino acid residues arranged in two domains, the N-terminal and the C-terminal lobe connected by a short-linker peptide [1,2]. Because each domain contains a Fe(III)-binding site, four isoforms of Tf can be distinguished, depending on their iron content: no Fe(III) bound (apo-Tf), one Fe(III) ion bound to the N-terminal lobe (Fe_N-Tf), one Fe(III) ion bound to the C-terminal lobe (Fe_C-Tf), and both binding sites occupied (Fe_2-Tf or holo-Tf). The protein backbone of Tf is heavily glycosylated with well-characterized complex carbohydrate structures [1]. Glycosylation has been reported to impair Fe(III) binding and affect Fe(III)-Tf isoform distribution in vitro [3]. It has also been shown that Tf glycoforms with alternating glycan structures are characterized by varied surface exposure of hydrophobic patches, resulting in varied hydrodynamic properties which may affect its function and stability [4].

The mechanism of iron binding and release by Tf is redox-mediated [1]. Therefore, it is of interest to determine the effect of Tf glycosylation on its reduction potential. Previous works by Kretchmar et al. [5] and Kraiter et al. [6] have provided a sound theoretical background for determination of Fe_2^{III}-Tf reduction potential. However, the experimental setup required custom-built hardware and complex procedures that make it difficult to reproduce measurements. The objective of the present work is to establish a convenient spectroelectrochemical method for determination of Fe_2^{III}-Tf reduction potential using commercially available accessories. Specifically, a spectroelectrochemical flow cell was chosen because of its small sample volume, easy sample loading and maintenance of anaerobic conditions. A disposable screen-printed indium-tin oxide (ITO) electrode was selected due to its suitable potential window, transparency, quick preparation and simple yet affordable replacement. This method is intended to be used to study the reduction potentials of Tf glycoforms, but can easily be applied to the study of other redox-active biomolecules and might be of particular importance in the momentous field of glycomics, where large numbers of protein isoforms need to be characterized and simple, high-throughput methods are in demand.

2. Material and methods

2.1. Reagents

All experiments were conducted in 0.1 M PIPES buffer (pH 7.4) and 0.5 M KCl at room temperature. Double-distilled water and chemicals of the highest purity were used. Native human apo-Tf (Biorbyt) was dissolved in water and then washed out with the buffer in Amicon Ultra centrifugal filter devices (10,000 MWCO, Merck Millipore). A stock solution of holo-Tf (Fe_2^{III}-Tf) was prepared by the addition of freshly prepared ferric dinitrilotriacetate ($Fe(NTA)_2$) in slight double molar excess to the solution containing approximately 0.5 mM apo-Tf and 10 mM HCO_3^- [7]. The mixture was left overnight and then washed out

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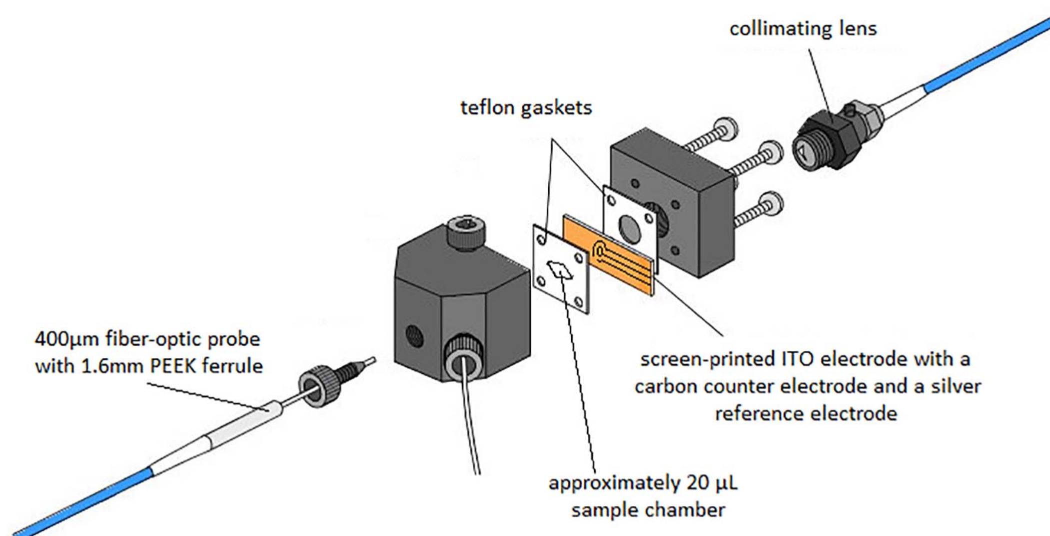

Fig. 1. A schematic representation of the spectroelectrochemical cell, modified from product information by ALS Co., Ltd., Japan. Used with permission.

twice with the buffer in the Amicon Ultra device. A stock solution of methyl viologen dichloride (MV^{2+}, Acros Organics), used as an electrochemical mediator, was prepared by dissolving a weighed amount in the buffer. The concentration of the MV^{2+} stock solution was verified spectrophotometrically ($\varepsilon_{257} = 20.7 \times 10^3\ M^{-1}\ cm^{-1}$, pH = 10) [8]. Final working solutions consisted of 0.36 mM Fe_2^{III}-Tf, 0.49 mM MV^{2+}, 0.5 M KCl and 150 µM HCO_3^- (from atmospheric CO_2) [9].

2.2. Instrumentation

A CH Instruments CHI-660D potentiostat and an ALS SEC-F spectroelectrochemical flow cell equipped with Avantes 400 µm fiber-optic probe were used for spectroelectrochemical (SEC) measurements. Disposable screen-printed electrodes (Dropsens DRP-ITO10), consisting of an ITO working electrode, carbon auxiliary electrode and a silver reference electrode, were used for electrochemical measurements. Spectra were recorded on an Ocean Optics S2000 diode array spectrophotometer with a deuterium/tungsten source. A BASi 0.015″ teflon gasket was used to set the optical path length and the corresponding cell volume was approximately 20 µL (Fig. 1). The actual optical path length was determined spectrophotometrically from the known initial concentration of Fe_2^{III}-Tf ($\varepsilon_{465} = 4.8 \times 10^3\ M^{-1}\ cm^{-1}$) [10,11].

2.3. Calculation

The theory of mediated SEC determination of Fe_2^{III}-Tf reduction potential is well established [5,6,8,10,12–16]. Briefly, upon application of potential to a solution containing Fe_2^{III}-Tf and MV^{2+} as a mediator, the reduction of colorless MV^{2+} to the blue-colored radical $MV^{\cdot+}$ occurs at the electrode. The resulting $MV^{\cdot+}$ in turn reduces red-colored Fe_2^{III}-Tf to colorless Fe_2^{II}-Tf in the solution, and this subsequently dissociates to Fe^{2+} and apo-Tf. It is known that the reduced mediator radical $MV^{\cdot+}$ can dimerize in the solution [8] and the corresponding equilibrium is considered in the final calculation of the Fe_2^{III}-Tf reduction potential. Full details of all calculations are given in [11].

2.4. Measurement

Prior to the measurements, the working solutions were deaerated by bubbling with pure argon for a minimum of 15 min, and then transferred to the SEC cell with a gas-tight syringe. The cell was extensively washed and purged with deaerated buffer before loading the protein sample. The screen-printed electrodes were preconditioned before each experiment by running baseline cyclic voltammetry scans in the range 0 to − 900 mV vs. Ag/AgCl until a reproducible and featureless voltammogram was obtained. A baseline spectrum in the deaerated buffer was also taken before each experiment. The electrodes were replaced when either irremovable optical or electrochemical impurities were noticed on the working electrode during baseline measurements or increased cell resistivity was observed due to decomposition of the screen-printed film.

In the SEC experiments, spectra of the working solutions are taken during a slow potential sweep. For each measurement, the concentration of $MV^{\cdot+}$ can be calculated from the absorbance at 604 nm (A_{604} in Fig. 4a) and the corresponding cell equilibrium potential vs. SHE reference, E_{cell}, can then be calculated from the known concentrations of MV^{2+} and $MV^{\cdot+}$ [11]. The decrease in concentration of Fe_2^{III}-Tf from the initial value, $[Fe_2^{III}\text{-Tf}]_0$, can be followed simultaneously at 465 nm. Each measurement at 465 nm must be background-corrected to account for the absorbance of $MV^{\cdot+}$ and its dimer at that wavelength (A_{465}* in Fig. 4a). For this purpose, a separate identical SEC experiment with MV^{2+} alone is required [5,6,13]. From the known values of $[Fe_2^{III}\text{-Tf}]$ at each E_{cell}, the values of $[Fe_2^{II}\text{-Tf}]$ are calculated and a Nernst plot for the reduction of Fe_2^{III}-Tf can be produced from the known values of E_{cell}, $[Fe_2^{III}\text{-Tf}]$ and $[Fe_2^{II}\text{-Tf}]$, according to the equation [11]:

$$E_{cell} = E^{0'}(Fe_2^{III/II}\text{-Tf}) + \frac{59.2\ mV}{n_{app.}} \log \frac{[Fe_2^{III}\text{-Tf}]}{[Fe_2^{II}\text{-Tf}]}. \quad (1)$$

In order to remove noise from the spectral data, a 51-point second-order Savitzky-Golay polynomial filter was applied and the obtained absorbance values were used for calculations [17,18]. Data obtained for $E_{cell} > -660$ mV vs. Ag/AgCl, for which the fraction of the oxidized Fe_2^{III}-Tf is > 0.9, were not used for the Nernst plot due to the small changes in A_{465}* yielding erratic values of $[Fe_2^{III}\text{-Tf}]/[Fe_2^{II}\text{-Tf}]$. Initial values of A_{465}* for which the concentration of the reduced mediator is negligible ($A_{604} < 0.001$) were averaged to obtain the optical path length.

3. Results and discussion

3.1. Spectroelectrochemistry of the mediator

The evaluation of the ITO screen-printed electrode as an electrochemical interface for the reduction of MV^{2+} and in turn the reduction of Fe_2^{III}-Tf is given in [11]. In order to evaluate the SEC properties of the cell, and also to provide background correction for the working

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