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A novel approach for oxidation analysis of therapeutic proteins

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ABSTRACT

Measuring and monitoring of protein oxidation modifications is important for biopharmaceutical process development and stability assessment during long-term storage. Currently available methods for biomolecules oxidation analysis use time-consuming peptide mapping analysis. Therefore, it is desirable to develop high-throughput methods for advanced process control of protein oxidation. Here, we present a novel approach by which oxidative protein modifications are monitored by an indirect potentiometric method. The method is based on adding an electron mediator, which enhances electron transfer (ET) between all redox species and the electrode surface. Specifically, the procedure involves measuring the sharp change in the open circuit potential (OCP) for the mediator system (redox couple) as a result of its interaction with the oxidized protein species in the solution. Application of Pt and Ag/AgCl microelectrodes allowed for a high-sensitivity protein oxidation analysis. We found that the $Ru(NH_3)_6^{2+/3+}$ redox couple is suitable for measuring the total oxidation of a wide range of therapeutic proteins between 1.1 and 13.6%. Accuracy determined by comparing with the known percentage oxidation of the reference standard showed that percentage oxidation determined for each sample was within ±20% of the expected percentage oxidation determined by mass spectrometry.

Oxidation is a major degradation pathway for protein therapeutics [1]. It can be promoted by light exposure, transition metals, and peroxide formed or induced by excipients or by the presence of oxygen during the manufacturing and storage process [2].

It is well known that methionine is one of the most readily oxidized amino acid constituents of proteins. Methionine residues can be oxidized to methionine sulfoxide when exposed to increased levels of hydrogen peroxide, hydroxyl radicals, hypochlorite, chloramine, and peroxynitrile or singlet oxygen [3]. In addition to methionine, the amino acids that are particularly susceptible to oxidation include cysteine, histidine, tyrosine, and tryptophan and may also involve proline, lysine, and arginine [4,5]. Tryptophan oxidation products have been reported in bovine α-crystallin [6]. The oxidation of cysteine residues in the zinc-binding domain of proteins not only leads to the formation of disulfide bond(s) but may also create Cys-SO_2H and Cys-SO_3H [2].

Several studies have shown a clear correlation between the degree of protein oxidation and its biological activity [7]. Methionine oxidation was shown to decrease bioactivity and stability, affecting the product's clinical application or shelf life [8]. This oxidation, which depends on its location in the protein structure, can pose the risk of affecting the activity of the protein, exacerbating protein aggregation, and/or increasing the risk of immunogenic response.

The oxidation of proteins is currently determined by peptide mapping analysis [8], which is a low-throughput technique. Moreover, the method is not suited for measuring oxidation of excipients such as Tween because the assay is specific for proteins. Therefore, the development of an alternative quantitative method for measuring oxidation of proteins and raw materials is highly desired.

Changes in the extracellular environment can be monitored by measuring the redox potential [9]. This parameter has been shown to play a key role in the quality of fermented dairy products and was successfully applied for the analysis of food raw materials, dietary supplements, and blood [9]. The applicability and advantage of potentiometric measurements with ion-selective electrodes in pharmaceutical analysis has been demonstrated [10,11]. Although redox potentiometry has been used during the

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manufacturing of dairy products [10], there are no studies on the application of redox sensors for protein oxidation analysis.

Recently, an indirect potentiometric method has been introduced [12–19]. Indirect potentiometry is based on the sharp change of electrode potential by a mediator system as a result of its interaction with oxidized species. The main advantage of the proposed method is the simultaneous use of both forms of the mediator system—oxidized and reduced states, that is, hexacyanoferrate(III)/hexacyanoferrate(II). Because no current flows externally, potentiometry does not alter the concentration of the species in the sample and makes it to be a nondestructive method.

Indirect potentiometric analysis has been shown to enable the assessment of the oxidant/antioxidant status of biological fluids [12,13], radicals and radical scavengers in biological matrices [14], and antioxidant activity [15,16]. Another example of a highly sensitive indirect potentiometric method for various oxidizing species such as bromate, chromium(VI), hydrogen peroxide, and oxychlorine is based on using bromine or chlorine as an intermediate [17]. The intermediate is generated during the reaction of the oxidative species with an Fe(III)–Fe(II) redox mediator containing bromide or chloride [17].

The mediator approach has also been applied for determining the redox capacity of a complex system such as milk [18]. Measuring the redox potential requires an interface that exchanges electrons with all redox couples in the solution. A novel approach was demonstrated by which adsorbed metal nanoparticles were used for enhancing electron transfer (ET)[1] exchange rates between redox species and electrode surface [19]. The latter had a significant effect on the measurement of the open circuit potential (OCP).

Here, we report that indirect potentiometric analysis was successfully applied for determining levels of oxidation in samples of interest for biopharmaceutical development as a rapid and novel alternative to mass spectrometry. The results demonstrate that a novel assay is capable of monitoring protein oxidation ranging from 1.1 to 13.6% with accuracy of 86.0–113.3% determined relative to the peptide mapping analysis data.

Materials and methods

Materials

Hexaammineruthenium(II) chloride 99%, hexaammineruthenium(III) chloride 99%, potassium hexacyanoferrate(III) 99%, potassium hexacyanoferrate(II) 99%, potassium hexachloroiridate(IV) 99.99%, and potassium hexachloroiridate(III) 99.99% were purchased from Sigma–Aldrich. HPLC-grade water was obtained from Fisher Scientific (Fair Lawn, NJ, USA).

Pt (MI-800 Micro-ORP electrode) and Ag/AgCl, 3 M KCl (MI-401 Micro reference electrode) microelectrodes were purchased from Microelectrodes (Bedford, NH, USA) and were used as indicator and reference electrodes, respectively. Therefore, all potentials are quoted versus Ag/AgCl (3 M KCl) reference electrode. Potentiometric measurements were performed using a pH 700 Series voltmeter from Oakton. A NanoDrop 1000 (Thermo Scientific) was used for protein concentration measurements.

Five different models of monoclonal antibodies were used in this study: STX-100, Tysabri, Daclizumab, Anti-Tweak, and Anti-BDCA2. Table 1 summarizes formulation buffer (FB) compositions of tested proteins. STX-100 is a humanized monoclonal antibody that targets integrin αvβ6. STX-100 exhibits significant antifibrotic activity in preclinical animal models of kidney, lung, and liver disease. The U.S. Food and Drug Administration has previously granted orphan drug designation to STX-100 for chronic allograft nephropathy. Tysabri (natalizumab) is a humanized monoclonal antibody against the cell adhesion molecule α4-integrin. Natalizumab is used in the treatment of multiple sclerosis and Crohn's disease. Anti-Tweak is a humanized monoclonal antibody specific for Tweak useful in the treatment of lupus nephritis. Daclizumab (zenapax) is a therapeutic humanized monoclonal antibody used to prevent rejection in organ transplantation, especially in kidney transplants. Daclizumab works by binding to CD25, the alpha subunit of the interleukin 2 (IL-2) receptor of T cells.

Table 1
Formulation buffer compositions of therapeutic proteins.

Therapeutic protein	Formulation buffer
STX-100	10 mM sodium citrate and 5% (w/v) sucrose (pH 6.1)
Tysabri	10 mM sodium phosphate and 140 mM NaCl (pH 6.1)
Tweak	10 mM sodium succinate and 150 mM L-arginine HCl (pH 5.5)
Anti-BDCA2	10 mM sodium succinate, 150 mM L-arginine HCl, and 0.05% polysorbate 80 (pH 6.0)
Daclizumab	40 mM succinate and 100 mM sodium chloride (pH 6.0)

Potentiometric method for determining protein oxidation

The working electrode (Pt microelectrode) was washed with 0.1 M HCl, followed by HPLC-grade water, before use. Potentiometric measurements were carried out with a two-electrode cell (indicator and reference electrodes).

To prepare a redox couple measuring solution, a hexaammineruthenium(II) chloride stock solution of 0.1 M, 27.30 mg of hexaammineruthenium(II) chloride was added to 1 ml of HPLC-grade water and used within 1 h following preparation to minimize oxidation of the stock. A hexaammineruthenium(III) chloride stock solution of 1 mM was made by adding 30.91 mg of hexaammineruthenium(III) chloride to 1 ml of HPLC-grade water. From this solution, 100 μl was added to 900 μl of HPLC-grade water to result in 0.01 M Ru(III). From the 0.01 M Ru(III) solution, 100 μl was added to 900 μl of HPLC-grade water to result in 0.001 M $Ru(NH_3)_6^{3+}$. This solution was used within 1 h of preparation. A working redox solution (10^{-2} M $Ru(NH_3)_6^{2+}$/10^{-4} M $Ru(NH_3)_6^{3+}$ in HPLC-grade water) was made by adding 15 μl of 0.1 M Ru(II) and 15 μl of 0.001 M Ru(III) to 120 μl of HPLC-grade water to result in 150 μl of 10^{-2} M Ru(II)/10^{-4} M Ru(III). The solution was vortexed gently for a few seconds such that the redox solution was mixed completely.

To measure the initial potential, 150 μl of freshly prepared hexaammineruthenium(II/III) chloride redox solution in a 0.7-ml vial was measured. Then, 1.5 μl of formulation buffer was added, and the potential was measured to correct the initial potential of the redox solution for any change in potential due to excipients. The volume of FB added for correction may differ among proteins due to different concentrations of the proteins under study; however, the volume of FB added equals the volume of protein added at a next step.

The following steps were then conducted. First, 100 μg of unknown sample was added and the potential was measured. Second, 100 μg of standard 1 (STD 1) was added and the potential was measured. Third, 100 μg of standard 2 (STD 2) was added and the potential was measured. An indicator electrode was regenerated between the measurements by dipping it for 1–2 min into 0.1 M H_2SO_4 solution followed by rinsing with HPLC-grade water. STD 1 and STD 2 are 100 and 200 μg of the reference standard of given monoclonal antibody protein with percentage oxidation predetermined by mass spectrometry (peptide mapping analysis).

[1] Abbreviations used: ET, electron transfer; OCP, open circuit potential; FB, formulation buffer; STD, standard; RSD, relative standard deviation.