

Protein Oxidative Modifications During Electrospray Ionization: Solution Phase Electrochemistry or Corona Discharge-Induced Radical Attack?

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The exposure of solution-phase proteins to reactive oxygen species (ROS) causes oxidative modifications, giving rise to the formation of covalent +16 Da adducts. Electrospray ionization (ESI) mass spectrometry (MS) is the most widely used method for monitoring the extent of these modifications. Unfortunately, protein oxidation can also take place as an experimental artifact during ESI, such that it may be difficult to assess the actual level of oxidation in bulk solution. Previous work has demonstrated that ESI-induced oxidation is highly prevalent when operating at strongly elevated capillary voltage V_0 (e.g., +8 kV) and with oxygen nebulizer gas in the presence of a clearly visible corona discharge. Protein oxidation under these conditions is commonly attributed to OH radicals generated in the plasma of the discharge. On the other hand, charge balancing oxidation reactions are known to take place at the metal/liquid interface of the emitter. Previous studies have not systematically explored whether such electrochemical processes could be responsible for the formation of oxidative +16 Da adducts instead of (or in combination with) plasma-generated ROS. Using hemoglobin as a model system, this work illustrates the occurrence of extensive protein oxidation even under typical operating conditions (e.g., $V_0 = 3.5$ kV, N_2 nebulizer gas). Surprisingly, measurements of the current flowing in the ESI circuit demonstrate that a weak corona discharge persists for these relatively gentle settings. On the basis of comparative experiments with nebulizer gases of different dielectric strength, it is concluded that ROS generated under discharge conditions are solely responsible for ESI-induced protein oxidation. This result is corroborated through off-line electrolysis experiments designed to mimic the electrochemical processes taking place during ESI. Our findings highlight the necessity of using easily oxidizable internal standards in biophysical or biomedical ESI-MS studies where knowledge of protein oxidation in bulk solution is desired. Strategies for eliminating ESI-induced oxidation artifacts are discussed.

Oxidative modifications of proteins represent a focal point of research. In vivo oxidation has been linked to disease mechanisms

and aging.^{1,2} The oxidation of amino acid side chains can be induced by exposure to hydroxyl radicals ($\cdot OH$) and other reactive oxygen species (ROS). Many laboratories employ oxidative labeling in vitro for mapping protein structures and interactions in “footprinting” experiments. Studies of this kind provide information on tertiary structure and binding interfaces because the reactivity of each residue is modulated by its solvent accessibility.^{3–13} The types of covalent modifications generated during oxidative labeling in vitro are largely identical to those induced under conditions of oxidative stress in living organisms.^{14–16} Electrospray ionization (ESI) mass spectrometry (MS)¹⁷ represents the most widely used analytical tool for monitoring protein oxidation. ROS exposure usually culminates in the formation of covalent +16 Da adducts, but side products exhibiting other mass shifts may be formed as well.¹⁸ The exact location of modification sites within a protein can be determined by tryptic peptide mapping in combination with liquid chromatography (LC) and ESI-MS/MS.^{3–12,19}

A potential problem with these ESI-MS-based studies is the fact that oxidation can also occur during the ESI process itself.

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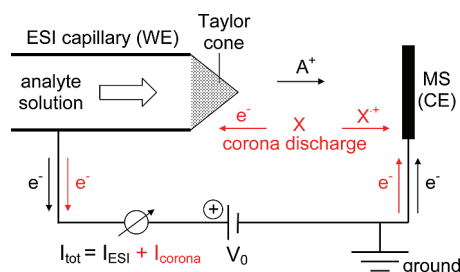


Figure 1. Schematic depiction of an ESI source in positive ion mode (black). Positively charged solvent droplets and analyte ions (A^+) are ejected from the tip of the Taylor cone. Flow directions of charge carriers are indicated by thin arrows. Shown in red are the additional current contributions under corona discharge conditions, where neutral gas molecules X break down to form X^+ and free electrons. MS, mass spectrometer; CE, counter electrode; WE, working electrode; V_0 , capillary voltage.

As a result, it may be difficult to assess how well the observed ions reflect the actual oxidation levels in bulk solution.²⁰ Two fundamentally different oxidation mechanisms can be envisioned, namely (i) processes linked to electron transfer at the metal/liquid interface of the ESI emitter, or (ii) reactions induced by ROS generated in a corona discharge. It is useful to briefly review the operation of an ESI source, with emphasis on these two factors. We will restrict our discussion to the commonly used positive ion mode.

An ESI source represents a controlled-current electrochemical flow cell (black elements in Figure 1).^{21–23} Analyte solution is infused into a stainless steel capillary that is held at high positive potential V_0 versus ground. The capillary exit is separated from the mass spectrometer by an atmospheric pressure gap. Positively charged solvent droplets are emitted from the tip of a Taylor cone. Solvent evaporation and droplet fission culminate in the formation of gas phase analyte ions (denoted as A^+ in Figure 1). These species then migrate toward the mass spectrometer, the potential of which is at ground.²⁴ The emission of positive charge from the Taylor cone necessitates the occurrence of charge balancing oxidation processes at the metal/liquid interface, for example, oxidation of the solvent, the analyte, or the capillary material.^{25–28} The electrons released in this way constitute the current I_{ESI} that flows through a wire to the high voltage power supply, and ultimately to the mass spectrometer where charge recombination takes place. In this series circuit the ESI capillary represents the working electrode (anode), and the mass spectrometer constitutes the counter electrode (cathode).^{24,29}

The interfacial potential drop $V_{\text{M/L}}$ at the metal liquid interface of the ESI capillary reflects the extent to which an electric double layer has formed, thereby determining whether oxidation of a given species at the electrode surface is thermodynamically possible.²⁵ Van Berkel et al. demonstrated the oxidation of substances with potentials up to almost 1.5 V versus SCE (saturated calomel electrode).³⁰ Computational studies³¹ and microelectrode measurements^{32–34} revealed that $V_{\text{M/L}}$ is not uniform along the capillary length, and that even higher potentials may be present at the emitter tip. Oxidation of the capillary material has a tendency to lower $V_{\text{M/L}}$,^{20,35} but this redox buffering effect is diminished by the formation of a protective oxide layer on the stainless steel surface.^{26,36–38} The conditions within the ESI capillary should therefore, in principle, allow the oxidation of a wide range of organic and biological species, including several amino acid side chains (e.g., Cys, Met, Trp, Tyr, His).³⁹ However, $V_{\text{M/L}}$ is not a fixed value but depends on a range of parameters such as the composition and flow rate v_f of the solution, as well as the applied voltage V_0 . Also, overpotentials and relatively unfavorable mass transfer in tubular emitters^{25,40} may limit the extent of oxidation for any given species. As a result, it is not always easy to predict the nature of the charge-balancing reactions within the ESI capillary.²⁵

The second aspect that has to be considered is the possible occurrence of a corona discharge in the ion source region. Most ESI sources employ a coaxial flow of nebulizer gas (not shown in Figure 1) to enhance solvent dispersion and evaporation. This gas fills the gap separating the positively charged emitter tip from the counter electrode. Dielectric breakdown of gas molecules X takes place once V_0 exceeds a critical value, generating a plasma that consists of radical cations and free electrons ($X \rightarrow X^+ + e^-$).^{41,42} The former migrate toward the mass spectrometer, and the latter toward the capillary tip (Figure 1, red elements). The onset of corona discharge is usually marked by a sharp increase in overall current I_{tot} ,^{20,43} which now represents the sum of the two contributions

$$I_{\text{tot}} = I_{\text{ESI}} + I_{\text{corona}} \quad (1)$$

I_{corona} is not associated with charge-balancing reactions at the metal/liquid interface. At very high V_0 it is easy to recognize the occurrence of a discharge by its light emission.⁴⁴ This

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regime is usually avoided because it leads to suboptimal ESI performance such as a gradual loss of signal intensity. Gas phase electrons liberated in the discharge are accelerated and induce the formation of OH radicals through inelastic collisions in the vicinity of the emitter tip ($\text{H}_2\text{O} + \text{e}^- \rightarrow \text{H}\cdot + \cdot\text{OH} + \text{e}^-$).⁴⁵ Hydroxyl radicals and other ROS produced in the plasma can induce various oxidation processes.^{44,46}

It is important to delineate the extent to which ESI-induced protein oxidation is linked to redox reactions at the metal/liquid interface, or to discharge-related processes. The answer to this question remains elusive, as previous studies have not examined this issue systematically from both directions. A correlation between the onset of corona discharge at elevated V_0 and the oxidation of Met, Trp, and Tyr was first noticed by Morand and co-workers.⁴⁴ In related work by Downard et al., an ESI source was operated at very high voltage ($V_0 = 8 \text{ kV}$) and with oxygen nebulizer gas for protein footprinting by "radical probe-MS".^{8,46} Analyte oxidation in those cases was attributed to corona-generated ROS. However, elevated values of V_0 that induce a discharge should also increase the rate of charge-balancing reactions and the interfacial potential drop $V_{\text{M/L}}$, thereby enhancing redox processes at the metal/liquid interface.²⁵ Notably, protein oxidation in electrochemical flow cells occurs at potentials comparable to those encountered during ESI, albeit under conditions that provide more efficient mass transfer, and with the possible involvement of secondary (indirect) processes.^{10,47,48} Bateman attributed protein oxidation in nanoESI-MS to purely electrochemical factors,⁴⁹ consistent with the conclusions of Nemes et al.⁵⁰ who used regular ESI in the $\mu\text{L min}^{-1}$ flow rate regime for the analysis of reserpine. Chen and Cook²⁰ tentatively ascribed methionine sulfoxide (MetO) formation at $V_0 = 4.5 \text{ kV}$ to corona discharge, but they noted that the exact oxidation mechanism remains unknown.

This work explores the mechanism of ESI-induced protein oxidation by focusing on bovine hemoglobin (Hb) as a model system. The native state of Hb represents a noncovalently bound $\alpha_2\beta_2$ tetramer.⁵¹ In previous work we found that the β subunit of this protein (βHb) undergoes spontaneous MetO formation during storage. Those oxidation events have major implications for subunit interactions, and for the assembly of the native tetramer.^{52,53} Note that MetO formation is unrelated to reversible oxygen binding under physiological conditions.⁵⁴ During the course of our experiments^{52,53} we noticed that βHb is also highly susceptible to ESI-induced oxidation. Interestingly, these modifications were found to occur even under fairly normal operating conditions, that is, they did not require extreme capillary voltage

and oxygen nebulizer gas as in radical probe-MS.^{8,46} This unexpected phenomenon prompted us to examine whether corona-induced ROS significantly contribute to protein oxidation under regular ESI conditions, or whether the effect is a manifestation of heterogeneous electrochemistry.

EXPERIMENTAL SECTION

Materials. Ammonium acetate and formic acid were purchased from Fluka (Buchs, Switzerland). H_2O was purified by distillation of reverse osmosis water. Bovine Hb was prepared from fresh hemolysate via standard procedures.^{53,55} 1.6 mM Hb stock solution was flash frozen in liquid nitrogen, and stored at -80°C in 10 mM ammonium acetate (pH 6.8). Acid-denatured Hb was prepared by diluting this stock solution to a protein concentration of 1 μM (as tetramer), that is, 2 μM αHb and 2 μM βHb , in 10 mM ammonium acetate and 2% (v/v) formic acid (pH 2). This solution was used for all experiments. Heme loss, subunit dissociation, as well as extensive unfolding occur under these acidic conditions for both subunits.^{52,53,56} Commercially available Hb was not used because of high levels of background oxidation.⁵³

Mass Spectrometry. Mass spectra were recorded on a Q-TOF Ultima API (Waters/Micromass, Manchester, UK) utilizing a standard Z-spray ESI source in positive ion mode. The commercial probe was modified to allow measurements of I_{tot} . For this purpose a Fluke 189 multimeter was isolated from ground and operated in series with the ESI power supply as indicated in Figure 1 (safety note: this arrangement may expose the operator to inadvertent electric shock). ESI capillaries were cut and polished in house from type 304 stainless steel hypodermic tubing (Small Parts, Miramar, FL) to a length of 18.5 cm (i.d. 140 μm , o.d. 235 μm). The probe tip was oriented 10 mm back and 3 mm lateral from the ion sampling cone. The cone voltage was held at 70 V, and the RF lens 1 was at 40 V for all acquisitions. Various capillary voltages were tested, up to $V_0 = 5 \text{ kV}$, which represents the highest possible value on our instrument. The Time of Flight (TOF) analyzer was calibrated with CsI. The nebulizer gas pressure was held at 100 psi, resulting in a flow of about 0.5 L min^{-1} . N_2 was purified in house with a Whatman nitrogen generator; O_2 (medical grade), Ar (ultra high purity 5.0) and SF_6 (99.9% purity) were purchased from Praxair (Mississauga, Ontario, Canada). Samples not requiring desalting were infused into the mass spectrometer via a syringe pump. These direct infusion measurements were carried out in the absence of an upstream ground to avoid the occurrence of external currents that might otherwise complicate data interpretation.^{25,26} Samples exposed to off-line electrolysis were analyzed by LC/ESI-MS using a 1525 μHPLC pump (Waters) and a Symmetry 300 C4 column ($2.1 \times 100 \text{ mm}$, Waters) at a flow rate of 100 $\mu\text{L min}^{-1}$ to reduce the extent of salt contamination introduced through the reference electrode (see below). The water and acetonitrile/propanol (50:50 v/v) mobile phase contained 0.1% v/v formic acid. The organic content was ramped from 0% to 90% within 6 min. All data were acquired and analyzed using MassLynx 4.0 software provided by the instrument manufacturer. Consistent with previous reports,⁵⁷ the subunit masses were found to be 15053 and 15954 Da for αHb and βHb , respectively.

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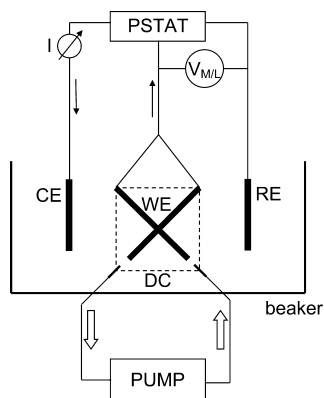


Figure 2. Schematic representation of the setup used for protein electrolysis. A peristaltic pump circulates the protein solution through a dialysis cassette (DC) which contains the working electrode (WE). Block arrows indicate the direction of liquid flow. The interfacial potential drop $V_{M/L}$ at WE is set relative to a saturated calomel reference electrode (RE) by a potentiostat (PSTAT), which ensures adequate electron flow (I) from WE to the Pt counter electrode (CE). No current flows between WE and RE, thereby allowing an accurate measurement of the interfacial potential drop at WE. DC and all three electrodes are submersed in electrolyte solution.

Off-Line Electrolysis. Hb solution was electrolyzed using a three-electrode setup (Figure 2). Protein solution was enclosed in a $2.5 \times 3.5 \times 0.34$ cm³ Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) with a 7 kDa molecular weight cutoff. The solution was rapidly circulated through the cassette via a peristaltic pump at a flow rate of 15 mL min^{-1} to facilitate mass transfer. The total volume of protein solution in the system was 3 mL. The working electrode within the cassette consisted of two crossed stainless steel 304 wires with a diameter of 235 μm (Small Parts) with an exposed length of 3 cm. The orientation of these wires relative to the circulation inlet and outlet is indicated in Figure 2. The interfacial potential drop $V_{M/L}$ at the working electrode was measured with respect to a reference SCE. A Pt sheet with a total surface area of 12.5 cm², welded to a Pt wire, served as counter electrode. The dialysis cassette was submersed along with the three electrodes in a supporting electrolyte solution matching the solvent used for the protein (10 mM ammonium acetate in water, 2% v/v formic acid, pH 2). Applied potentials were controlled, and current responses recorded, with a Solartron model 1287 potentiostat (Farnborough Hampshire, U.K.). The entire electrochemical setup was placed in a grounded Faraday cage. LC/ESI-MS analysis of Hb after off-line electrolysis was performed as outlined above. Control experiments carried out at an extremely high value of $V_{M/L}$ (2.5 V vs SCE) resulted in almost complete protein oxidation (fraction unmodified⁵⁸ < 10%) within 3 min, demonstrating that mass transfer limitations become insignificant for $t > 3$ min (data not shown).

RESULTS AND DISCUSSION

Protein Oxidation under Normal Operating Conditions.

ESI-MS analysis of $1 \mu\text{M}$ Hb at a capillary voltage of $V_0 = 2.0$ kV, a solution flow rate of $v_f = 10 \mu\text{L min}^{-1}$, and with N₂ nebulizer gas results in the mass distribution depicted in Figure 3A. Signals for both αHb and βHb appear at their expected mass values, with no indication of protein oxidation. Minor sodium

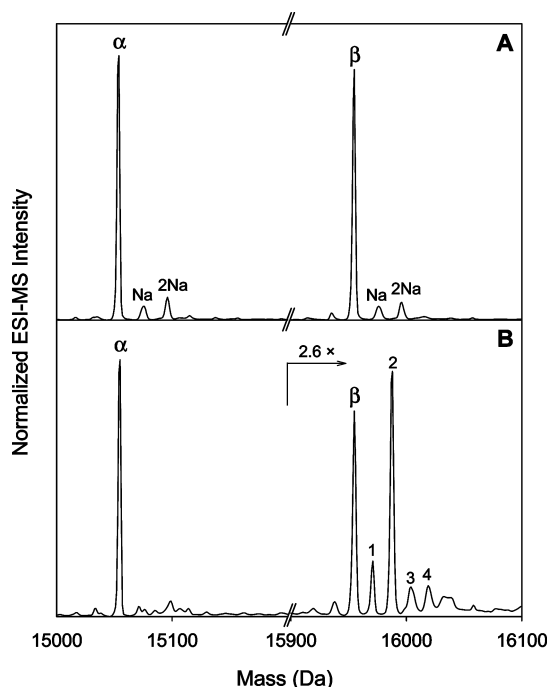


Figure 3. Deconvoluted ESI mass spectra of acid-denatured hemoglobin, with signals corresponding to both subunits (αHb and βHb). (A) $V_0 = 2.0$ kV, solution flow rate $10 \mu\text{L min}^{-1}$. (B) $V_0 = 3.5$ kV, solution flow rate $1 \mu\text{L min}^{-1}$. Minor satellite peaks in panel A represent sodium adducts. Selected peaks in panel B are labeled according to the number of incorporated oxygen atoms (1–4). Nitrogen was used as nebulizer gas for both spectra.

adducts of the type seen in Figure 3A are commonly encountered in ESI-MS.⁵⁹ An entirely different picture is obtained when analyzing the same sample at $V_0 = 3.5$ kV and $v_f = 1 \mu\text{L min}^{-1}$ (Figure 3B). Under these conditions βHb is heavily oxidized, with a mass distribution that is dominated by molecules that have incorporated two oxygen atoms (+32 Da). Additional oxidation products are observed as well, up to at least [$\beta\text{Hb} + 40$]. In contrast, αHb shows hardly any evidence of oxidation. This different behavior can be attributed to the higher number of easily oxidizable amino acids in βHb (1 Cys and 3 Met, vs no Cys and 1 Met for αHb).^{53,60} The data in Figure 3A prove that the base amount of oxidation in bulk solution is minimal for the protein samples employed here. Yet, a moderate change in ESI conditions (as in Figure 3B) results in extensive βHb oxidation. ESI-MS users unaware of this type of artifact might therefore be misled when assessing the properties of a protein sample.

For suppressing βHb oxidation it was not necessary to change both V_0 and v_f . Instead, data comparable to those in Figure 3A could be obtained by modifying just one of these parameters. Also, increasing the protein concentration by a factor of 10 at $V_0 = 3.5$ kV and $v_f = 1 \mu\text{L min}^{-1}$ had a similar effect (data not shown). The mass distributions in Figure 3 were highly reproducible over several months, and with capillaries that had been operated for different periods of time. A pronounced dependence of protein

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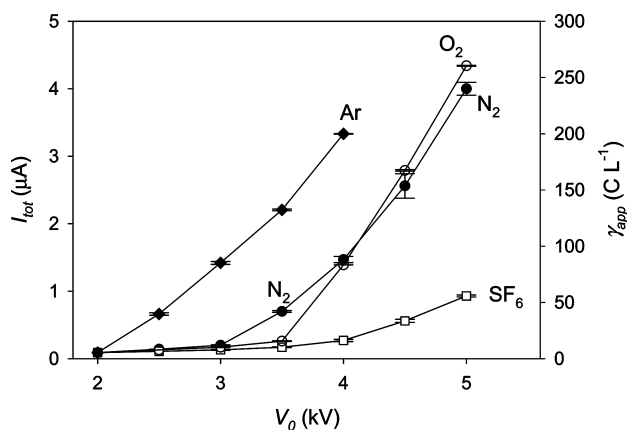


Figure 4. Total current I_{tot} as a function of capillary voltage for different nebulizing gases, measured while electrospraying $1 \mu\text{M}$ Hb in 10 mM ammonium acetate with 2% (v/v) formic acid at $v_f = 1 \mu\text{L min}^{-1}$. Data points represent the average of 3–6 independent measurements, error bars indicate standard deviations. Also shown is the apparent electrolytic load γ_{app} (eq 4).

oxidation levels on capillary age, as reported in a previous study,²⁰ was not apparent in our measurements.

The observation of extensive oxidation under the conditions of Figure 3B is surprising, considering that the capillary voltage used (3.5 kV) is well within the typical operating range recommended by the instrument manufacturer. This value of V_0 resulted in excellent signal stability and intensity. No luminescence was observable by visual inspection of the emitter tip under ambient light. This scenario is quite different from previous radical probe-MS experiments where proteins were oxidized under much harsher conditions, by operating the ESI source at 8 kV with O_2 nebulizer gas in the presence of a clearly visible discharge.^{3,8,46}

Corona Discharge with Different Nebulizer Gases. The voltage required to induce corona discharge depends on the dielectric strength of the gas separating the electrodes.⁶¹ To eliminate experimental factors related to electrode geometry, the dielectric strength is usually reported relative to N_2 , which is arbitrarily assigned a value of unity. On this scale the reported values of Ar, O_2 , and SF_6 are 0.18 , 0.92 , and 2.6 , respectively.⁶¹

Measurements of the current I_{tot} versus ESI capillary voltage V_0 are depicted in Figure 4. These data were recorded while infusing Hb solution at a flow rate of $v_f = 1 \mu\text{L min}^{-1}$. The onset of discharge for N_2 is marked by a conspicuous “kink”⁴⁴ at $V_0 \approx 3 \text{ kV}$, followed by a steep rise in current up to $I_{\text{tot}} = 4.0 \mu\text{A}$ at $V_0 = 5.0 \text{ kV}$. Notably, light emission was not readily apparent up to at least 4 kV , demonstrating that visual inspection alone is not adequate for determining whether or not corona discharge occurs. Instead, voltage-current plots of the type displayed in Figure 4 are much better suited for this purpose. Discharge for Ar begins at a considerably lower voltage than for N_2 , around 2 kV . In contrast, strong suppression of corona discharge occurs for SF_6 , with I_{tot} values that are severalfold lower than for N_2 in the range of 3.5 to 5 kV . Nonetheless, a weak discharge appears to be present for SF_6 upward of around

$V_0 = 4 \text{ kV}$. These observations are in line with previous ESI-MS studies where SF_6 was used as nebulizer gas to suppress the extent of discharge.^{62–64} The behavior seen in Figure 4 for Ar, N_2 , and SF_6 is qualitatively consistent with the dielectric strength values quoted above. Interestingly, the voltage-current curve for O_2 resembles that for N_2 , except that the onset of discharge is shifted to about 3.5 kV , despite its slightly lower dielectric strength. This behavior can be attributed to the electron scavenging activity of molecular oxygen.^{45,65}

As a side note, it is pointed out that oxidation of all βHb at the metal/liquid interface would result in $I_{\text{tot}} = 12.8 \text{ nA}$ at the flow rate used. This estimate assumes that (i) four electrons are released from each βHb (corresponding to two $\text{Met} \rightarrow \text{MetO}$ conversions), (ii) mass transfer is not limiting, (iii) $I_{\text{corona}} = 0$, and (iv) βHb oxidation is the only charge-balancing redox reaction. The measured I_{tot} values are much larger than 12.8 nA , even for capillary voltages that do not result in corona discharge, for example $I_{\text{tot}} = 140 \text{ nA}$ with N_2 at 2.5 kV . This demonstrates that even extensive electrochemical protein oxidation could provide only a very minor contribution to the charge-balancing current I_{ESI} . Consistent with this notion is the observation of cyclic voltammograms that are indistinguishable when recorded with and without $1 \mu\text{M}$ Hb (data not shown). The major charge-balancing reaction under the conditions of our work is likely the oxidation of water ($2\text{H}_2\text{O} \rightarrow 4\text{H}^+ + \text{O}_2 + 4\text{e}^-$, $E^0 = 0.9 \text{ V}$ vs SCE at pH 2),⁶⁶ an assertion that is in line with earlier studies.^{26,67}

Dependence of ESI-Induced Oxidation on Nebulizer Gas and V_0 . ESI mass spectra of $1 \mu\text{M}$ Hb were recorded at $v_f = 1 \mu\text{L min}^{-1}$ for the various voltages and nebulizer gases examined in Figure 4. We will restrict our discussion to βHb because of its greater oxidation susceptibility. Representative data are depicted in Figure 5, highlighting that the extent of oxidation increases as the capillary voltage is raised. Compared to the behavior observed for N_2 (Figure 5A–D), oxidation for SF_6 is much less prevalent and remains virtually undetectable up to about 4.0 kV (Figure 5E–H). O_2 provides less oxidation than N_2 at 3.5 kV (Figure 5J), whereas the situation is reversed at 5.0 kV (Figure 5L). Of the four gases studied, only Ar yields significant oxidative modifications at 2.5 kV (Figure 5M), but no oxidation is observable at 2.0 kV (data not shown). Ar spectra acquired at $V_0 > 2.5 \text{ kV}$ are not included in Figure 5 because of their unacceptable S/N ratio, which might be the result of oxidative backbone cleavage.

We now return to the question whether protein oxidation during ESI is predominantly due to corona discharge-generated ROS, or whether the effect is linked to heterogeneous electron transfer at the metal/liquid interface. cursory inspection of the data in Figures 4, 5 immediately points to a correlation between discharge and oxidation, suggesting a causative relationship between the two factors. Still, alternative explanations cannot

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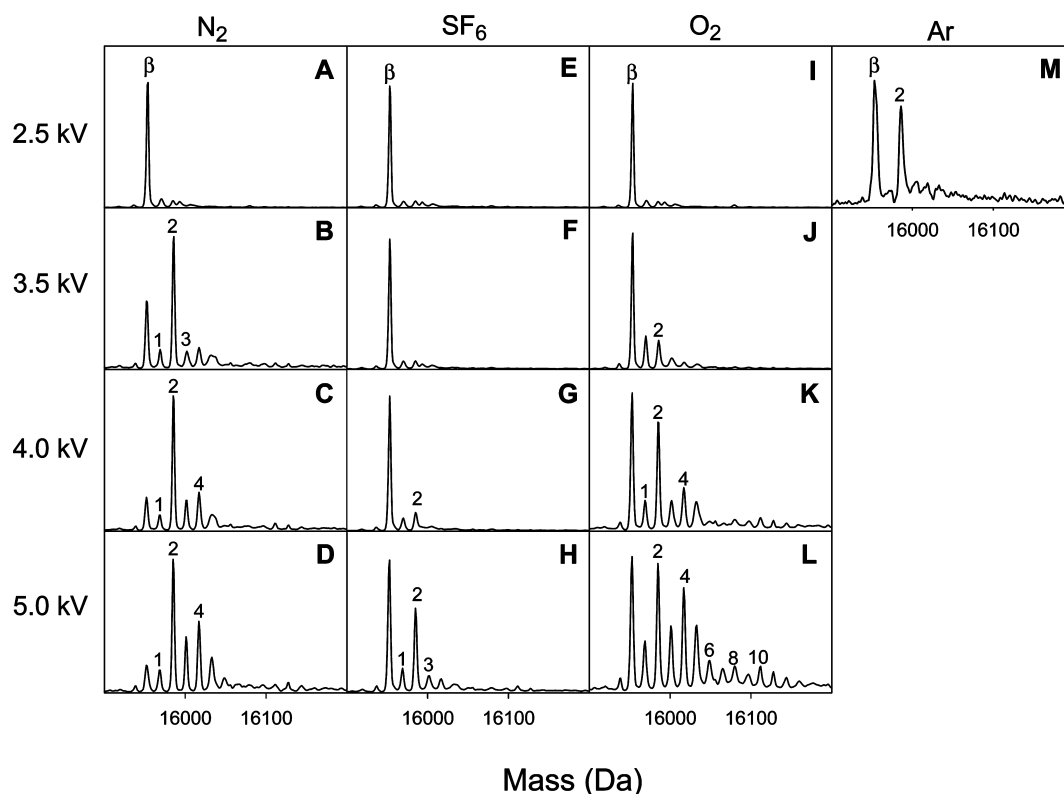


Figure 5. Deconvoluted ESI mass spectra of 1 μM βHb acquired as a function of capillary voltage (2.5–5.0 kV; indicated on the left-hand side) for different nebulizer gases (N_2 , SF_6 , O_2 , and Ar; indicated along the top) at a flow rate of 1 $\mu\text{L min}^{-1}$. Selected peaks are labeled according to the number of incorporated oxygen atoms (1–10). The charge state distributions of αHb and βHb maintained maxima around 15+ to 17+ for all the conditions used here, but the relative intensities of protein ions in low charge states decreased somewhat with increasing capillary voltage (data not shown).

be discounted without additional considerations. Specifically, an increase in V_0 applied to induce discharge might at the same time render the conditions for electrochemical protein oxidation more favorable, for example, by enhancing $V_{\text{M/L}}$ and I_{ESI} .²⁵ But can such a scenario be sufficient to account for a dramatic rise in βHb oxidation? This issue can be addressed by comparing spectra recorded with and without corona discharge under otherwise identical conditions. For example, a corona is absent with SF_6 at 3.5 kV (Figure 4), and no protein oxidation is observed under these conditions (Figure 5F). In contrast, repeating the measurement with N_2 under otherwise identical conditions induces significant oxidation (Figure 5B), as well as discharge (Figure 4). If we assume that the electrochemical conditions within the ESI capillary are largely the same in both experiments, it follows that charge-balancing redox reactions can *not* account for the observed βHb oxidation. Instead, the occurrence of oxidative modifications in Figure 5B must be attributed to the presence of the corona discharge. Analogous conclusions can be drawn by comparing other data sets, for example, N_2 and O_2 (Figure 5B/J), or N_2 and Ar (Figure 5A/M).

In summary, the discussion presented above supports a mechanism whereby ESI-induced protein oxidation is mediated solely by ROS that are generated in the plasma of the discharge. However, the analysis relies on the assumption that the electrochemical conditions inside the metal capillary are largely the same with and without discharge, as long as all other parameters (V_0 , solution composition, flow rate, etc.) do not change. Because

this assertion may not always be valid we sought to corroborate our conclusions based on an alternative strategy.

Protein Electrolysis in Bulk Solution. The electrolysis cell depicted in Figure 2 was used in an attempt to mimic electrochemical processes occurring at the metal/liquid interface of the ESI capillary. For describing the extent to which a solution has been exposed to oxidative conditions it is convenient to define an “electrolytic load” γ which describes how many Coulombs of electrons have been removed from a given volume, that is,

$$\gamma = (\text{electron charge extracted})/\text{volume} \quad (2)$$

In an ESI source this value is given by

$$\gamma = I_{\text{ESI}}/v_f \quad (3)$$

As noted above, the magnitude of I_{ESI} in the presence of a corona discharge cannot be determined directly, but only the value of I_{tot} which includes the contribution I_{corona} (eq 1). We thus define an “apparent” electrolytic load γ_{app} as

$$\gamma_{\text{app}} = I_{\text{tot}}/v_f \quad (4)$$

with $\gamma_{\text{app}} \geq \gamma$. The two parameters are equal only in the absence of corona discharge. Although the electrolytic cell depicted in Figure 2 uses the same solvent system and working electrode material as our ESI experiments, it is clear that such an off-line device does not perfectly reproduce the geometry, potential

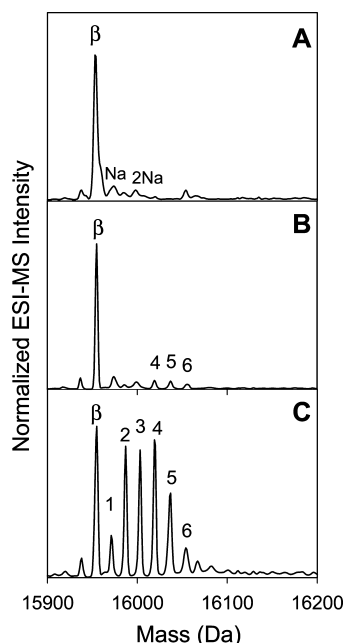


Figure 6. Deconvoluted ESI mass spectra of 1 μM βHb after electrolysis using the cell depicted in Figure 2. (A) no potential applied, circulation time $t = 1$ h, $\gamma = 0$ (control); (B) $V_{\text{M/L}} = 1.5$ V vs SCE, $t = 3.7$ min, $\gamma = 90$ C L $^{-1}$; (C) $V_{\text{M/L}} = 1.5$ V vs SCE, $t = 44$ min, $\gamma = 1000$ C L $^{-1}$.

distribution, and mass transfer properties of an actual ESI source. Nonetheless, relevant information can be obtained when comparing results for the two systems based on their electrolytic load.

Following off-line electrolysis of 1 μM Hb solution, mass distributions were measured by LC/ESI-MS under conditions that are free of artifactual in-source oxidation (Figure 6A). We will initially focus on off-line data obtained for a working electrode potential drop of $V_{\text{M/L}} = 1.5$ V versus SCE, which represents a typical value that might be encountered within an ESI capillary.³⁰ The electrolytic load γ of the solution in the cell can be controlled by adjusting the electrolysis time t . Off-line electrolysis does not result in discernible protein oxidation up to about $\gamma = 90$ C L $^{-1}$ ($t = 3.7$ min, Figure 6B). To put this into context, extensive oxidative modifications are observed for the ESI-MS data in Figure 5C which were recorded under corona discharge conditions with $\gamma_{\text{app}} \approx 90$ C L $^{-1}$ (the actual electrolytic load in the latter case is considerably below 90 C L $^{-1}$ because $\gamma_{\text{app}} > \gamma$). This comparison implies that electrochemical oxidation at the metal/liquid interface of the ESI capillary is *not* capable of accounting for the protein oxidation observed under the conditions of Figure 5C. In other words, protein oxidation observed in the ESI-MS measurement (Figure 5C) must be ascribed to the effects of ROS generated in the corona discharge of the ion source.

As noted above, water oxidation ($E^0 = 0.9$ V vs SCE at pH 2)⁶⁶ likely represents the major charge-balancing reaction within the ion source under the conditions of our work. The values of $V_{\text{M/L}}$ for the ESI-MS measurements in Figure 5 might therefore be expected to remain considerably below 1.5 V because of redox buffering.^{35,38} Cyclic voltammetry using our electrolysis system (Figure 2) showed the minimum $V_{\text{M/L}}$ required for water oxidation to be around 1.3 V versus SCE (data not shown).

This relatively high value can be attributed to an overpotential.³⁷ Nonetheless, off-line electrolysis experiments analogous to that in Figure 6B were repeated at lower $V_{\text{M/L}}$ to test if these conditions resulted in more extensive protein oxidation (e.g., $V_{\text{M/L}} = 1.0$ V, $t = 2$ h, $\gamma = 90$ C L $^{-1}$). Protein solutions treated in this way showed very little oxidation, yielding mass distributions comparable to that in Figure 6B (data not shown). These results demonstrate that our conclusions do not depend on the specific choice of $V_{\text{M/L}}$ during off-line electrolysis.

Finally, it is pointed out that substantial βHb oxidation can be induced under off-line conditions by using a much larger electrolytic load, for example, $t = 44$ min at 1.5 V, corresponding to $\gamma = 1000$ C L $^{-1}$ (Figure 6C). These data confirm that electrochemical protein oxidation at a metal/liquid interface is possible in principle, thus reiterating that the question posed in the title of this work is not unreasonable. Nonetheless, our results clearly demonstrate that such an electrochemical mechanism cannot account for covalent oxygen adducts formed under typical ESI operating conditions.

CONCLUSIONS

The occurrence of protein oxidative modifications in radical probe-MS is well established. In those experiments, oxygen incorporation into amino acid side chains is induced by operating an ESI source at extremely high capillary voltage and with O₂ nebulizer gas in the presence of a clearly visible corona discharge.^{3,8,46} The current work demonstrates that considerable oxidation can take place even under standard operating conditions, that is, with nitrogen nebulizer gas and at much lower capillary voltage. The extent of oxidation is protein-dependent, for example, αHb is less susceptible than βHb , presumably because of the greater number of Met and Cys residues in the latter (Figure 3). To our knowledge, this study represents the first attempt to systematically explore the basis of ESI-induced protein oxidation. Corona discharge-induced hydroxyl radicals are commonly cited as the causative agent, but a possible role of heterogeneous electrochemistry has never been explicitly ruled out. Our results confirm that ROS formed in the plasma of a corona discharge are responsible for protein oxidation, even at low capillary voltage where the presence of a discharge can easily go undetected. This conclusion is based on measurements of I_{tot} in the ESI circuit (Figure 4), comparative ESI-MS experiments with nebulizer gases of different dielectric strength (Figure 5), and off-line electrochemical studies (Figure 6). The occurrence of charge-balancing oxidation reactions at the metal/liquid interface of the ESI capillary is undisputed.^{22,25,26} While those processes do not lead to the formation of covalent (+16 Da) modifications on proteins, they may be responsible for other effects such as the $\text{Fe}^{2+} \rightarrow \text{Fe}^{3+}$ oxidation of protein-bound heme groups.^{27,28}

Careful control of artifactual protein oxidation has considerable practical implications, particularly for studies that use oxidative labeling for exploring solvent accessibility^{3,4} and for experiments aimed at monitoring the level of oxidative damage in bulk solution.^{1,2} For example, the Hb model chosen for this work illustrates a possible quality control issue for medical blood supplies. One possible strategy for avoiding oxidation artifacts is to operate the ESI source without corona discharge by lowering the capillary voltage. However, very low values of V_0 will compromise sensitivity, and therefore this course of action is

not always advisable. Detecting the onset of discharge by measuring I_{tot} as a function of V_0 is simple, but unfortunately most commercial mass spectrometers are not equipped with an ammeter at the appropriate point in the ESI circuit (Figure 1). Our results demonstrate that a discharge can be present long before it is detectable by visual inspection of the emitter tip under ambient light conditions. The use of N_2 nebulizer gas for eliminating oxidation artifacts is not a viable strategy. In fact, because of its electron scavenging activity^{45,65} O_2 suppresses the onset of corona discharge, thereby reducing the extent of oxidation at moderate capillary voltage compared to N_2 (Figure 5B, J). Consistent with this finding is the fact that oxygen atoms incorporated into protein side chains originate from water, not from molecular oxygen.^{20,49} Suppression of corona discharge-induced oxidation by using SF_6 nebulizer gas is most effective, but associated with considerable cost. Argon and other noble gases have to be avoided because of their extremely low dielectric strength (Figure 4).

Corona discharge-induced oxidation can be strongly reduced by increasing the solution flow rate v_f and/or the protein concentration. This relationship may be rationalized based on the molar protein: ROS ratio,¹⁵ but the actual origin of such a "dilution strategy" is likely more complicated.⁵⁸ Previous work indicated that repolishing the ESI capillary tip after accumulation of an oxide layer can also suppress protein oxidation.²⁰ This effect appears to be instrument-dependent since we did not notice a pronounced dependence of capillary age on the Hb spectra. The addition of radical scavengers⁵⁸ or redox buffers²⁰ represents another possibility, although such a strategy may lead to nonspecific adduct formation or ion suppression. It appears that ESI-MS experiments aimed at

characterizing protein oxidation levels in bulk solution should always involve easily oxidizable test proteins as internal standards. Polypeptide chains containing multiple Met residues, such as βHb , are most suitable for this purpose. In future work, it will be interesting to explore the extent of analyte oxidation under nanoESI conditions.^{68,69} Because of the very low flow rates used in nanoESI-MS, analytes should be highly susceptible to corona-induced oxidation. The onset of a corona should be facilitated by the close proximity of the nanoESI capillary exit to the counter electrode, although the typically employed low values of V_0 might be expected to counteract extensive oxidation. Also, possible implications of our findings for the mechanism of analyte oxidation during desorption-ESI (DESI)-MS⁷⁰ will have to await further investigations.

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