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EELS Characterization of Radiolytic Products in Frozen Samples

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Abstract

Electron energy loss spectroscopy (EELS) was used to obtain information about the radiation chemistry of frozen aqueous specimens in the electron microscope by observing the hydrogen and oxygen K-edges. Measurements on frozen solutions of 30% hydrogen peroxide revealed the presence of molecular oxygen identified by a distinct 531-eV peak at the O K-edge even for electron doses below 100 e/nm². The molecular oxygen content of irradiated H₂O₂ solution was determined by least squares fitting of O K-edge reference spectra from water and gas-phase oxygen. It was found that the fraction of molecular oxygen to water oxygen was in the range 0.03 to 0.05. EELS from pure frozen water showed no features attributable to molecular oxygen or molecular hydrogen (K edge at ~13 eV) even at high electron doses above 10⁵ e/nm². Spectra from frozen sucrose and protein solutions and their mixtures, however, did show evolution of a molecular hydrogen peak at ~13 eV for doses above 10⁵ e/nm², consistent with previous measurements and indicative of hydrogen bubble formation. Molecular oxygen was not observed in any of the frozen solutions of organic compounds indicating that oxygen is not a major product of free radical decay, in contrast to molecular hydrogen formation.

Keywords

Electron energy loss spectroscopy; EELS; radiation damage; biological applications; molecular oxygen; frozen samples; cryo-electron microscopy; electron tomography

1. Introduction

Understanding radiation damage in frozen aqueous specimens is of considerable interest in the fields of structural biology, materials science, atmospheric chemistry, and astrochemistry. In particular in cryo-electron microscopy, now a well established technique in structural biology (Dubochet et al., 1982a; Dubochet et al., 1982b; Frank, 2006; Glaeser, 2007), the major limiting factors in obtaining high resolution images is susceptibility of the specimen to radiation damage in the electron beam (Talmon, 1984; Dubochet et al., 1988; Henderson, 1995; Blanc et al., 1998; Borek et al., 2010). Cellular material typically contains about 80% water and 20% organic molecules and macromolecular assemblies, and these components undergo numerous types of damage when the specimen is irradiated in the electron microscope (Isaacson, 1977; Dubochet et al., 1988). Moreover, the aqueous environment in the cell enhances the sensitivity of biological molecules to damage due to

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reactions involving free radicals such as the hydroxyl species. (Symons, 1982b). At high enough dose radiolysis eventually leads to visible alterations in specimen morphology such as the formation of bubbles containing molecular hydrogen (Leapman and Sun, 1995). Such damage phenomena not only limit cryo-electron microscopy of isolated macromolecular assemblies (Frank, 2006; Glaeser, 2007) but also cryo-electron tomography (McEwen et al., 1995; Hsieh et al., 2002; Grunewald et al., 2003; Subramaniam and Milne, 2004), as well as quantitative mapping of water based on valence electron energy loss spectroscopy (Sun et al., 1993; Sun et al., 1995; Sousa et al., 2006; Yakovlev et al., 2010).

In this paper we show how electron energy loss spectroscopy (EELS) can complement electron microscopy and diffraction for helping to elucidate radiation damage mechanisms in frozen aqueous solutions (Misra and Egerton, 1984; Egerton, 1996). Characterization of the effects of the radiation at a molecular level can be accomplished by observing the chemical products of radiolysis (Egerton, 1980; Laffon et al., 2006). It is thus possible to observe morphological changes, such as the formation of gaseous bubbles, and at the same time detect chemical changes in the water and organic components (Myneni et al., 2002). Because water plays such an important role in radiation damage of hydrated specimens, it is particularly useful to observe the core edges of hydrogen and oxygen. Here we have analyzed EELS from vitreous ice, vitreous hydrogen peroxide (H_2O_2), and frozen aqueous solutions of sucrose and protein.

Although the detailed mechanisms of radiation damage are highly complex (Symons, 1982b; Symons, 1982a; Laffon et al., 2006) with a multitude of reactions and byproducts, it is also useful to determine the presence or absence of the small molecules, hydrogen and oxygen, for different specimen compositions and irradiation conditions. Knowledge of the byproducts of radiolysis can potentially provide insight into how frozen hydrated specimens are degraded in the electron beam.

2. Experimental methods

Thin films of frozen vitreous water were prepared by pipetting drops of deionized water onto bare 600-mesh copper EM grids. The grids were then blotted with filter paper immediately prior to freezing in liquid ethane at -175°C using a plunge freezing machine (KF80, Leica), using standard methods (Adrian et al., 1984). The films of frozen 30% H_2O_2 , 20% bovine serum albumin (BSA), and a mixture of 10% BSA + 10% sucrose were prepared in the same way.

Frozen specimens were cryotransferred (77 K) into transmission electron microscope (Tecnai30, FEI, Hillsboro, OR) operating at a beam voltage of 300 kV, equipped with a Tridiem post-column imaging filter (Gatan Inc., Pleasanton, CA) and a 2048×2048 pixel Ultrascan cooled CCD detector. Electron energy loss spectra were obtained with dispersions of 0.2 eV/channel and 0.3 eV/channel using a 40- μm objective aperture. The energy resolution, as defined by the full width at half maximum of the zero-loss peak, was approximately 1.5 eV. The resolution was limited by the spectra being recorded from large fields of view using low electron-optical magnifications, which increased the size of the projector cross-over, and the image size. The incident electron dose used to obtain the spectra was varied from ~ 20 to 10^6 e/nm² by changing the exposure time and by adjusting the beam diameter. Some of the acquired data were analyzed by fitting reference spectra using a multiple least squares function included in the Digital Micrograph software package (Gatan, Inc., Pleasanton, CA). The specimen relative thickness t/λ varied from .5 to 1.5.

3. Results and discussion

Electron energy loss spectra were first recorded from thin frozen layers of aqueous solution of 30% hydrogen peroxide, which readily dissociates into water and molecular oxygen. Low loss spectra at a low dose of 100 e/nm² and at a high dose of 10⁵ e/nm² only shows features that are seen in EELS of pure frozen water, i.e., an exciton edge at 9 eV and a broad plasmon peak at 20 eV. No bubbling is observed in these frozen H₂O₂ solutions, and none of these spectra showed evidence for a peak at 13 eV, which would have signified the formation of molecular hydrogen (H₂). However, the oxygen K-edge in Figure 1b does reveal a sharp feature at ~531 eV, which is present both at a low dose (40 e/nm²) as well as at high dose (5×10⁵ e/nm²). This 531-eV peak is consistent with published gas phase spectra from the Gatan EELS Atlas in Figure 1c recorded from air containing a mixture of molecular oxygen (O₂) and molecular nitrogen (N₂) (Ahn et al., 1983). We see evidence for the 531-eV peak even for a dose of just a few e/nm² (data not shown), suggesting an extremely low damage dose for dissociation of H₂O₂. The 531-eV peak that we see in our EELS data from H₂O₂ is also consistent with the feature attributed to molecular oxygen in the x-ray absorption spectrum obtained by Laffon et al. (2006). The radiolysis of H₂O₂ can be represented by a reaction:

It seems likely that most of the oxygen is formed at a low dose but remains trapped in the specimen, which then behaves in a similar way as pure frozen water. At high dose the specimen gradually thins due to ejection of atoms from the surface layers.

We have evaluated the molecular oxygen content of the irradiated hydrogen peroxide solution by fitting the O K-edge EELS to the reference spectra from water and molecular oxygen using multiple least square fitting, assuming that all the H₂O₂ has dissociated. These reference spectra were normalized by the integrated intensity over a 50-eV wide window starting at the O K-edge threshold energy. It was found that the fraction of molecular oxygen to water oxygen was in the range 0.03 to 0.05. If the H₂O₂ solution remained at full strength (30%) throughout the preparation procedure, the mass fraction of molecular oxygen to water oxygen would be 0.185. This suggests that about 70% of the H₂O₂ solution dissociates during the specimen preparation prior to plunge freezing, which is not surprising given the tendency of H₂O₂ to dissociate under ambient conditions.

Next we investigated the effect of irradiating pure frozen H₂O over a range of electron doses. The low loss EELS spectra in Figure 2a shows a water exciton at 9 eV (arrow) but there is no feature at 13 eV that would be indicative of H₂. Also, there was no evidence for bubbling even at high electron doses, which is consistent with earlier experimental results (Dubochet et al., 1988). EELS recorded at the O K-edge (Figure 2b) did not show the presence of a 531-eV molecular oxygen peak even at a dose of ~5×10⁵ e/nm² (dashed curve). Thus, neither H₂ nor O₂ are produced when pure frozen water is irradiated. This observation is consistent with the rapid recombination of free radicals formed from radiolysis of water:

Only radiolytic byproducts formed near the surface would be able to escape into the microscope vacuum, which would contribute to the thinning and eventual rupture of the specimen. Using x-ray absorption spectroscopy, Laffon et al. (2006) detect different byproducts, such as the OH radical and O₂, when frozen water is irradiated at liquid helium

temperatures (20 K). This phenomenon might be due to slower diffusion of intermediate radiolytic species and it would be interesting to determine if this behavior is also observed in the electron microscope for specimens cooled to 20K.

Having demonstrated the behavior of pure frozen H₂O as well as the release of O₂ from H₂O₂ under electron beam, we now characterize the radiolysis of frozen aqueous solutions of organic molecules. For these experiments we choose bovine serum albumin (BSA), which can be considered chemically as a generic protein, and sucrose, which can be considered chemically as a generic carbohydrate. These types of molecules account for a significant fraction of a cell's dry mass, so we expect to observe similar radiolytic reactions as the ones that occur in cells.

Electron energy loss spectra from aqueous solutions of 20% BSA and a mixture of 10% sucrose and 10% BSA are shown in Figures 3 and 4 respectively. In Figures 3a and 4a, peaks (arrow) are observed at approximately 7 eV, which are attributed to excitations of π -bonds in the organic compounds, such as peptide bonds and aromatic side groups. With the limited energy resolution of the present EELS measurements, these 7-eV peaks tend to overlap with the 9-eV water exciton peak, which makes that feature difficult to discern. At low electron dose the EELS data do not show evidence for hydrogen formation (solid curves), but at high dose peaks at 13 eV become clearly visible in the EELS from both the BSA and from the BSA/sucrose solutions (dashed curve). Generation of these 13-eV peaks was accompanied by bubbling of the specimen as observed by TEM, and eventually after a high enough beam exposure the bubbles grew and ruptured the specimen. These observations have been made previously and are attributed to the formation of molecular H₂, which accumulates in bubbles at pressures estimated to exceed 1,000 atmospheres (Leapman and Sun, 1995; Sousa et al., 2006). The formation of molecular H₂ was only observed in thicker specimens (~ 1 mean free path and greater), which is consistent with previous studies (Yakovlev et al., 2009).

There was no evidence for generation of O₂ by the observation of a 531-eV peak at the oxygen K-edge, either in the BSA or the BSA/sucrose solution, as indicated in Figures 3b and 4b, respectively. At very high doses of 10⁵ e/nm² alterations in the oxygen near-edge fine structure were also present in the dried as well as in the hydrated specimen (data not shown) (Fujii et al., 2003; Fujii and Yokoya, 2009), implying that some damage mechanisms occurred without involving radiolysis of water. The observation that molecular H₂ is formed in aqueous solutions of organic molecules but not in pure frozen water is consistent with hydroxyl radicals from radiolysis of water reacting rapidly with hydrogen-carbon bonds to produce H₂ before they can recombine with hydrogen radicals.

We have observed a similar behavior in radiation damage of frozen hydrated bacteria, where bubbling only occurs within the cells and not in the surrounding ice (data not shown).

4. Conclusions

Electron energy loss spectroscopy was used to study radiation damage in frozen hydrated aqueous specimens in the transmission electron microscope. By observing changes that occur in the low loss spectrum (5–25 eV) and the oxygen K-edge (525–545 eV) as a function of dose, we could determine whether molecular hydrogen (H₂) and oxygen (O₂) are produced. As expected from the lack of bubbling in pure frozen water, we found no

evidence for production of H₂ or O₂ when frozen H₂O was irradiated. One reason for this phenomenon could be that the radicals quickly recombine before the bubbles can nucleate. However, we easily observed O₂ production in frozen 30% hydrogen peroxide solution even at very low doses. In aqueous solutions of carbon-containing organic compounds, we observed the formation of bubbles, which could be attributed to release of H₂, but we failed to observe any O₂ production in those specimens at liquid nitrogen temperatures.

The main new aspect of this study is that molecular oxygen can be detected using EELS when frozen H₂O₂ solution is irradiated, but that no molecular oxygen was observed after irradiation of any of the frozen solutions of organic compounds, which indicates that oxygen is not a major product of free radical decay, in contrast to molecular hydrogen formation. These findings together with results of previous studies could help to elucidate mechanisms for radiation damage.

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Figure 1.

EELS obtained from frozen 30% solution of H_2O_2 . (a) Low loss EELS at dose of $\sim 100 \text{ e/nm}^2$ (bottom) and at dose of $\sim 10^5 \text{ e/nm}^2$ (top). There is no evidence for generation of molecular H_2 in either spectrum (K-edge at $\sim 13 \text{ eV}$); the peak at $\sim 9 \text{ eV}$ (arrow) corresponds to the ice exciton. (b) Oxygen K-edge at dose of $\sim 40 \text{ e/nm}^2$ (bottom) and at dose of $\sim 5 \times 10^5 \text{ e/nm}^2$ (top). For low and high doses there is evidence for production of molecular O_2 (arrow) at $\sim 531 \text{ eV}$, as shown in the reference spectrum (Gatan EELS Atlas) from O_2 gas in

(c). This result suggests that the damage dose for oxygen production in H_2O_2 is very low and persists at high doses.

Figure 2.

EELS obtained from pure frozen H₂O. (a) Low loss EELS at dose of ~ 40 e/nm² (bottom) and at dose of $\sim 5 \times 10^5$ e/nm² (top). There is no evidence, either at low dose or high dose, for production of molecular H₂ (K-edge at ~ 13 eV); peak at ~ 9 eV (arrow) corresponds to the ice exciton. (b) Oxygen K-edge at dose of ~ 40 e/nm² (bottom) and at dose of $\sim 5 \times 10^5$ e/nm² (top). Neither is there any evidence for production of molecular O₂ – see reference spectrum in Fig. 1c. Peak at ~ 540 eV corresponds to the near-edge fine structure from water.

Figure 3.

EELS from frozen solution of 20% bovine serum albumin (BSA). (a) Low-loss EELS at dose of $\sim 40 \text{ e/nm}^2$ (bottom) and at dose of $\sim 5 \times 10^5 \text{ e/nm}^2$ (top). At low doses there is no evidence for production of molecular hydrogen (H_2) – peak at $\sim 13 \text{ eV}$; peak at 7 eV (arrow) corresponds to excitations of π -bonds in the organic compounds. At high doses, molecular H_2 peak evolves at $\sim 13 \text{ eV}$ (arrow). (b) Oxygen K-edge at dose of $\sim 100 \text{ e/nm}^2$ (bottom) and at dose of $\sim 10^5 \text{ e/nm}^2$. There is no evidence for the production of molecular O_2 . Altered structure of the O K-edge at high dose was also observed in the dried specimen.

Figure 4.

EELS from frozen solution of 10% sucrose and 10% BSA. (a) Low-loss EELS at dose of $\sim 40 \text{ e/nm}^2$ (bottom) and at dose of $\sim 5 \times 10^5 \text{ e/nm}^2$ (top). As for the 20% BSA solution, there is no evidence for molecular H_2 production (K-edge at 13 eV) at low doses, but at high doses (arrow), the H_2 peak becomes visible. The 7-eV peak also disappears at high doses due to damage. (b) Oxygen K-edge at dose of $\sim 100 \text{ e/nm}^2$ (bottom) and at dose of $\sim 5 \times 10^5 \text{ e/nm}^2$ (top). Again, at the O K-edge, there is no evidence for the production of molecular O_2 ; a weak pre-edge peak in the low-dose EELS does not match with the 531-eV molecular O_2 peak, and is attributed to oxygen in sucrose. Similarly, the altered structure of the O K-edge at high dose does not fit with the 531-eV molecular O_2 peak and is explained by radiation damage of the sucrose.