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Quantitative nanoscale water mapping in frozen-hydrated skin by low-loss electron energy-loss spectroscopy

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ABSTRACT

Spatially resolved low-loss electron energy-loss spectroscopy (EELS) is a powerful method to quantitatively determine the water distribution in frozen-hydrated biological materials at high spatial resolution. However, hydrated tissue, particularly its hydrophilic protein-rich component, is very sensitive to electron radiation. This sensitivity has traditionally limited the achievable spatial resolution because of the relatively high noise associated with low-dose data acquisition. We show that the damage caused by high-dose data acquisition affects the accuracy of a multiple-least-squares (MLS) compositional analysis because of inaccuracies in the reference spectrum used to represent the protein. Higher spatial resolution combined with more accurate compositional analysis can be achieved if a reference spectrum is used that better represents the electron-beam-damaged protein component under frozen-hydrated conditions rather than one separately collected from dry protein under low-dose conditions. We thus introduce a method to extract the best-fitting protein reference spectrum from an experimental spectrum dataset. This method can be used when the MLS-fitting problem is sufficiently constrained so that the only unknown is the reference spectrum for the protein component. We apply this approach to map the distribution of water in cryo-sections obtained from frozen-hydrated tissue of porcine skin. The raw spectral data were collected at doses up to 10^5 e/nm^2 despite the fact that observable damage begins at doses as low as 10^3 e/nm^2. The resulting spatial resolution of 10 nm is 5–10 times better than that in previous studies of frozen-hydrated tissue and is sufficient to resolve sub-cellular water fluctuations as well as the inter-cellular lipid-rich regions of skin where water-mediated processes are believed to play a significant role in the phenotype of keratinocytes in the stratum corneum.

1. Introduction

Biological materials display considerable heterogeneity in their water content at both cellular and sub-cellular length scales [1–3]. Although optical methods allow one to probe the water distribution spectroscopically [3], biological heterogeneity at sub-cellular length scales requires the development of experimental measurement methods with higher spatial resolution. One approach to study the nanoscale water distribution in tissue involves the imaging of freeze-dried cryo-sections using transmission electron microscopy (TEM) [4,5]. Freeze drying, however, causes deformation and nonuniform sample shrinkage [6]. In addition, the precision with which the water content can be determined by this method is limited [7].

An alternate approach to analyze the water distribution in tissue was developed by Leapman and coworkers [8] who used spatially resolved, low-loss, electron energy-loss spectroscopy (EELS) to map the spatial distribution of water in frozen-hydrated tissue from rat liver. Making the assumption that each experimental spectrum collected from the specimen could be modeled as a linear combination of reference spectra representing the individual pure components of the specimen – water and protein – the mass fraction of each component at each pixel in the spectrum image was determined using multiple least squares (MLS) analysis [8,9]. Leapman and coworkers achieved 80 nm spatial resolution with an estimated precision of a few percent.

The main factor limiting the spatial resolution of low-loss EELS imaging of soft materials, in general, and of hydrated biological materials, in particular, is their sensitivity to the incident energetic electrons in the transmission electron microscope [10]. Electrons with energies on the order of 10–300 keV can damage thin TEM specimens of soft materials over doses ranging from $\sim 10^1$ to 10^5 e/nm^2, depending on the material [11]. Chemical changes manifest themselves in low-loss EELS spectra [12,13] and such changes are often detectible before significant mass loss occurs.

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To avoid electron damage during TEM imaging of soft materials, the incident dose is typically limited to an amount below which the assumption is made that the specimen suffers negligible damage. Often, this limit is referred as the critical dose, D_c, at which some characteristic – e.g. a spectroscopic feature or a Bragg diffraction peak – has fallen to $1/e$ of its value at very low dose. However, such dose-limited imaging introduces a fundamental tradeoff between the achievable spatial resolution and the accuracy of the composition measurement. Decreasing the pixel size while remaining within the dose-limit constraint increases the noise, introduces random error, and thus decreases the precision with which a compositional measurement can be made. Random error can be reduced by averaging spectra from adjacent pixels prior to MLS fitting, but this is equivalent to collecting a single lower-noise spectrum from a larger pixel.

When a specimen is damaged during data acquisition, MLS analysis can lead to relatively poor fitting of reference spectra to experimental spectra. This leads to systematic errors in the compositional analysis, because the separately collected reference spectra are not necessarily those corresponding to the components of the radiation-damaged specimen. The analysis could be made more accurate while simultaneously achieving higher spatial resolution if more appropriate reference spectra were used. The issue then becomes one of how to identify the best reference spectra. In principle, these can be extracted from the experimental dataset itself. Multivariate statistical analysis (MSA) is one approach that has been used to extract the principal components from a wide variety of spectroscopic and image datasets [14–16]. Particularly in the case of energy-loss spectra from multicomponent systems, however, the principal components identified by an unconstrained multivariate analysis often bear little resemblance to the energy-loss spectra one can collect from relevant pure materials. Several applications of MSA to EELS data have thus centered on core-loss data where the abrupt onset of core-loss edges can be clearly identified [17–19], and there is recent work involving low-loss data analysis by the MSA approach [20,21].

The problem of reference-spectrum extraction from an experimental dataset is solvable in the specific case of biological tissue, such as the porcine skin studied here, when the specimen can reasonably be approximated as a two-component system of water and protein, the latter being the most significant part of the non-aqueous component of most tissues. If the reference spectrum of one component in a two-component system is known and the average composition can be measured, then the system is sufficiently constrained so that the second reference spectrum can be extracted from an experimental dataset. In the case of the frozen-hydrated skin system, the spectrum of amorphous ice does not change significantly under electron doses where frozen-hydrated protein undergoes obvious radiation damage ($\sim 10^3$–10^5 e/nm^2). Thus, we extract from experimental datasets, the ones collected using doses where there is clear damage to the proteinaceous component of the skin, reference spectra for the protein under conditions most appropriate to a given experiment. Using this reference-extraction approach we are able to use incident electron doses $\sim$100 times greater than previous work, achieve higher spatial resolution, and maintain the accurate compositional analysis associated with low-dose low-resolution experiments.

2. Experimental procedure and analysis

2.1. Experiments

Fresh samples of porcine skin were frozen using a Leica EM PACT high-pressure freezing system. Sections approximately 60 nm thick were cut using a Leica Ultracut S cryo-ultramicrotome at -160 °C. These were collected on folding grids, sandwiched between carbon and formvar support films, and stored in liquid nitrogen.

Reference spectra were collected from the pure components. A specimen of vitreous ice was prepared by depositing an aliquot of deionized water (pH 5.5) on a copper support grid, blotting the excess, and then freezing the specimen using a Gatan cryo-plunge device with liquid propane. Reference spectra from both undamaged and damaged dry skin were collected from frozen-hydrated specimens warmed in the microscope to 20 °C for 20 min to enable sublimation and/or evaporation of the water. These freeze-dried skin specimens were subsequently cooled to -170 °C for data acquisition. Dry skin is composed of protein and lipids, and these are distributed nonuniformly both within and between cells. However, except in the inter-cellular region of skin, lipids are typically present at a much lower concentration than proteins. Furthermore, spectra from various lipids and proteins are quite similar. Following Sun et al. [22] we group these together, and except where explicitly noted, we simply refer to dried skin as protein.

Image and spectral data were collected using a Philips CM-20 TEM/STEM FEG electron microscope. The microscope was equipped with a Gatan 767 Enfina EELS spectrometer. It was operated at 200 keV with an energy resolution of 0.8 eV (FWHM of the zero-loss peak). Digital Micrograph software (Gatan) was used to control the microscope during spectral acquisition. To control the radiation dose and the incident probe size, the incident beam was broadened by defocusing the condenser lens. The defocus necessary to achieve a specific beam size was previously determined by scanning across magnesium oxide crystals [23,24].

After spectral data acquisition, a series of standard post-processing steps were followed. The zero-loss peak was centered, and the effects of plural scattering were removed using analytical tools within the Digital Micrograph software system. Subsequent data processing was performed with custom software written using Matlab release 14. The contributions to each spectrum from the carbon and formvar support films were removed using spectra collected independently from the support film with no skin present [8]. The relative contributions of skin and support film were determined based on a Beer's Law analysis of specimen thickness [25].

2.2. Multiple least squares analysis

Post-processed datasets were quantified using multiple least square (MLS) fitting [8,9]. MLS fitting works by minimizing the residual of fitting an experimental spectrum using a linear combination of reference spectra. The residual, e, can be expressed as

$$e = \sum_{i=1}^{ch} \frac{\left[S_i - \sum_{j=1}^{k} a_j R_{ji}\right]^2}{[\sigma(E_i)]^2} \tag{1}$$

where S_i is the intensity of the experimental spectrum in channel i, ch is the number of channels used for the fitting, R_{ji} is the intensity of the jth reference spectrum in channel i, k is the number of reference spectra, a_j is the fitting coefficient corresponding to the jth reference, and $[\sigma(E_i)]^2$ is the variance associated with the experimental spectrum in the ith channel. The variance amounts to a weighting factor that biases the fitting in favor of regions where the confidence in the experimental measurements is higher because the noise is less there. If the only source of noise is shot noise, the variance is equal to the intensity: $[\sigma(E_i)]^2 = S_i$ [9,26]. However, we have shown previously that the variance characteristic of our EELS experiment includes other sources of noise [24]. Among those important in the present experiments are readout noise and pixel-by-pixel uncertainties