THE USE OF ELECTRON ENERGY LOSS SPECTROSCOPY FOR STUDYING MEMBRANE ARCHITECTURE: A PRELIMINARY REPORT *

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Some initial measurements of interest with respect to the use of energy loss spectroscopy (ELS) in the electron microscope are reported. We have obtained energy loss spectra in the region less than 15 eV energy loss for three components of biological components; lecithin, cholesterol and spectrin. Some indications of the effect of electron beam damage on the cholesterol spectrum are shown. In addition, energy loss pictures of erythrocyte membrane fragments are demonstrated for various energy losses less than 40 eV (taken with 0.75 eV width energy window) and it is shown that an individual ferritin molecule can be identified on the basis of its iron M_{II-III} excitation line. From the results of the preliminary data we have discussed the possibilities of ELS for studies of biological membranes.

1. Introduction

Since the inception of the electron microscope, there has been a continuous development of new ways to utilize a beam of electrons to yield more information about specimens. One now has the choice of using transmitted electrons, unscattered, elastically or inelastically scattered electrons, X-ray, backscattered or secondary electrons, or photons produced by the primary beam (e.g., 1). These signals result from various physical interactions of the specimen with the beam and hence a variety of different information about the specimen can be assembled by analyzing these signals. Particularly intriguing are the signals that can be used most advantageously to deduce the chemical composition of the specimen. In this paper, the use of transmitted electrons which have lost energy in traversing the specimen is considered to study the architecture of human erythrocyte membranes.

Electrons that are inelastically scattered by a specimen, can be analyzed with a magnetic or electrostatic spectrometer which separates out the no-loss from the energy loss electrons and disperses the inelastically scattered (energy loss) electrons such that the intensity at each value of energy loss may be measured (e.g., 2). A typical plot of intensity of the inelastically scattered electrons versus amount of energy lost (energy loss spectrum) is shown in fig. 1 for the nucleic acid base adenine [3]. This example illustrates the two distinct regions of spectral peaks that are useful for compositional analysis. First, the low-lying energy loss region, 0–10 eV, has peaks in the spectrum due mainly to $\pi \rightarrow \pi^*$ transitions within the molecules of the specimen. This region corresponds to optical absorption in

![Fig. 1. Characteristic electron energy loss spectrum of 25 keV electrons transmitted through an approximately 500 Å thick sublimed film of the nucleic acid base adenine (C_{5}N_{5}H_{5}) supported on a 30 Å thick carbon substrate. The spectrum was obtained with a total incident electron dose less than 10^{-4} C/cm^{2} (from ref. [3]).](image-url)
the ultraviolet region. Since this electron excitation involves the outer valence electrons, different compounds will exhibit different spectra in this region. Thus, a form of molecular identification using this information may be possible. The second region of interest in the spectrum is that beyond 50 eV energy loss. Here, sharp peaks with tails falling off approximately as the fourth power of the energy loss are caused by inner shell excitations [3-4]. Since these events do not involve outer shell molecular electrons but, instead, electrons closer to the atomic nucleus, the characteristic magnitude and excitation energy depends upon the type of atom rather than type of molecule. (There is fine structure on these peaks which is molecule dependent [3-6], but we will not consider this in this paper.) By cataloging the peaks in this high energy loss region, the number and kinds of atoms in the specimen may be analyzed.

Successful microanalysis via the energy loss technique entails that several basic aspects be investigated. We are interested in applying energy loss spectroscopy to analyze the human red blood cell membrane, so this will be used as an example. Since the total energy loss spectrum from an erythrocyte membrane is too complicated to be interpreted directly at this time, one approach is to "dissect" the membrane and record the spectrum of the components (carbohydrates, phospholipids, cholesterol and proteins). For quantitative analysis, absolute cross sections should be measured or reliable standards should be fabricated. Furthermore, it is known that energy loss spectra change as a function of dose since the beam causes destruction and mass loss [7,8]. This effect must also be investigated so that changes and limitations imposed by beam dose are defined for each component. Once the energy loss characteristics of the components are known, the intact membrane can then be studied. The low energy loss region (<10 eV) may be useful in distinguishing the constituents since each major component has a different spectrum (see fig. 2). The higher loss region (>50 eV) may be used to analyze the atomic composition. Since phosphorus is present in the phospholipids but not in the other components, detection of this element can localize this component. It may be possible to distinguish other components on the basis of the percentage of elements present, since these also vary among components. By using one or more of these techniques, it should be possible to form a micrograph

![Characteristics of electron energy loss spectra (in the region less than 12 eV energy loss) of approximately 25 keV electrons transmitted through thin films of various components associated with biological membranes. The spectra were obtained with total incident doses less than 2 x 10^4 C/cm^2. For clarity, the spectra have been displaced in the vertical direction.](image)

that maps the two-dimensional distribution of major compounds of non-crystalline membranes at a resolution of the order of 20-50 Å.

2. Experimental methods

2.1 Microscopes

Two scanning transmission electron microscopes equipped with cold field emission sources were employed in these studies. One instrument could form a probe as small as 100 Å in diameter and had a half angle beam divergence at the specimen of 0.625 mrad [9]. It was equipped with a magnetic spectrometer whose resolution was limited by the 0.25 eV energy spread of the electrons leaving the field emission source [10]. A rectangular aperture at the entrance of the spectrometer was used which subtended 12.5 mrad by 2.0 mrad half angle at the specimen. Spectra were obtained by sweeping the primary accelerating voltage so that the energy loss electrons were swept across a slit at the spectrometer exit.

The second instrument was operated in either of two modes. In one mode a 0.19 mrad half angle beam divergence at the specimen was used in conjunction
with a 1.1 mrad half angle aperture at the spectrometer entrance. In this mode, spot diameters as small as 400 Å could be formed. In the other mode, spot diameters as small as 2.4 Å diameter could be formed, but the incident beam half angle divergence was 15 mrad and the spectrometer entrance aperture half angle was about 25 mrad [10]. This instrument was equipped with an electrostatic spherical condenser spectrometer and the energy resolution was only 0.7 eV. The spectra were obtained by ramping the deflection voltages on the spectrometer so that the energy loss electrons were swept across a slit.

In both instruments, the electrons were detected either with a scintillator followed by a photomultiplier tube or with a surface barrier silicon solid state detector. A multichannel analyzer was used to record the data.

### 2.2. Specimen preparation

All samples were air dried onto thin (~25 Å) carbon films to a dry thickness of 300–600 Å. The only exception was the deposition of adenine by vacuum sublimation [3]. Lecithin (Sigma Chemical Co.) was sonicated in a 1 mg/ml aqueous solution before application to the grid, whereas cholesterol was dissolved in a 2:1 chloroform : methanol solution before use. Spectrin (bands 1 and 2) was isolated from human erythrocytes [12]. Ghost membranes were obtained as described by Nicolson [13].

### 3. Results

#### 3.1. Spectra of undamaged molecules

Spectrin, two membrane proteins (bands 1 and 2) comprising 20% of the erythrocyte membrane protein, cholesterol, and lecithin were chosen as representative major components to study as shown in table 1. The uncorrected low loss spectra for these three materials is shown in fig. 2. These graphs are given with a vertical scale of relative intensity since the thickness of each sample was not precisely known. The important peaks in these spectra are noted as follows. The spectrum of cholesterol has an energy loss peak at 6.7 eV and a larger one centered at 9.2 eV. The spectrum of spectrin has, at this resolution, a doublet with peaks at 6.6 and 7.4 eV and a shoulder at 9.4 eV. The lecithin spectrum has essentially no peaks in the 0–8 eV region and is essentially flat. At 8.43 eV there is a break at which energy loss intensity rises sharply and a shoulder is encountered at 10.2 eV. Since peaks in this region (0–10 eV) are due to π → π* transitions, these data may qualitatively be interpreted as follows. The peak in the spectrin is most probably due to the amide bond π → π* excitation which is known from U.V. data to occur at 185 nM (6.70 eV) [14]. Since proteins have one amide bond for each amino acid, a strong peak would be expected. Furthermore, any protein by this argument should have a peak at this point. Homoglobin does, in fact, also have a peak at approximately this value of energy loss [5,15]. There is a small shift of 0.2–0.4 eV between the optical ultraviolet absorption data and corresponding peaks in our studies, the electron inelastic scattering peaks showing slightly higher energy loss. This is expected from theoretical considerations of the difference between photon (transverse field) and electron (longitudinal field) interactions with the specimen (eg., 15). Also, additional absorption in this region is expected from the phenyl chromophores present in proteins. Cholesterol has an unsaturated carbon-carbon bond, and π → π* excitations would be expected. Ultraviolet absorption data [17] shows that cholesterol has an absorption peak at 6.5 eV. Lecithin, on the other hand, has a small content of π electrons since it is a predominantly saturated molecule and little energy loss in this region (0–8 eV) is predicted.

All of these spectra rise sharply after 10 eV because, this energy loss region corresponds to outer shell ionizations and σ → σ* excitations and would be seen in the spectrum of any organic compound. A representative shape of the energy loss spectrum for this region is shown in fig. 1 (adenine).

### Table 1

<table>
<thead>
<tr>
<th>Human red blood cell ghost membrane composition (by mass)</th>
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<tbody>
<tr>
<td><strong>Protein</strong></td>
</tr>
<tr>
<td><strong>Lipid</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Carbohydrates</strong></td>
</tr>
</tbody>
</table>

* from ref. [18].
The region <3 eV is not shown in these spectra since the unscattered peak, which is about two orders of magnitude greater than the low-lying energy loss peaks, falls of exponentially with energy and masks energy loss information in this region.

It is interesting to note the substantial differences in the spectra of these biomolecules in the 3–8 eV region. The fact that the peaks are of different magnitude and occur at different values of energy loss and that the phospholipid (lecithin) does not possess any distinct peaks in this region means that such characteristics might be used to distinguish these materials. A contrast mechanism based on these differences could be devised to map their distribution in a membrane.

The dose employed to obtain these spectra was $<2 \times 10^{-4} \text{C/cm}^2$ (0.12 e/A$^2$) and is, by comparison with other studies [7, 18], below the radiation damage level.

3.2. Spectra after damage

Another parameter that is necessary to measure is the effect of beam irradiation on the energy loss spectra. This is important to know since high spatial resolution studies that are free from statistical noise imply, at least for randomly ordered specimens, that the dose will be above the 0.1–1.0 e/A$^2$ level. Fig. 3 shows the low-lying energy loss spectrum of cholesterol ($<2 \times 10^{-4} \text{C/cm}^2$) along with the spectrum taken after a damaging dose (0.12 C/cm$^2 = 73$ e/A$^2$). The peak at 6.7 eV has been destroyed, whereas the peak at 9.2 eV has remained intact. This behavior demonstrates that even with “damaging” doses, unique spectral characteristics may remain and could still be used for compositional identification.

3.3. Energy window micrographs

Ultimately, energy loss spectroscopy could be used to analyze a specimen microscopically to determine the position of components. Ideally, the full spectrum should be recorded instantaneously from each point in the specimen (this would require some form of parallel detector array or optical multiplexing). Since we are not equipped at this time to record in parallel, only a particular energy loss can be measured at a time and insufficient data is produced to decipher a multiple component system. By combining measurements (i.e., images) taken using several different energy losses, however, analysis may proceed. A demonstration of the ability to record microscopic information obtained at specific values of low-lying energy loss is given in fig. 4. An erythrocyte membrane fragment is pictured which has several regions of thickness where the membrane has been folded over. The spectrometer slits were set to accept 3/4 eV (approximately the spectrometer resolution) and the energy loss value in the spectrum was selected by adjusting the voltage on the plates of the electrostatic spectrometer. The dose used to record each image was 1.6 e/A$^2$.

The image formed by the elastically scattered electrons presents a dark field view of the unstained membrane. Here, since thickness is roughly proportional to the signal obtained, whiter areas indicate thicker regions. The micrograph taken with the 3%4 eV energy window centered on 2 eV loss has a reversed contrast when compared to the elastic picture. The reason for this is that at 2 eV loss, the unscattered peak, although decaying exponentially, is still quite strong and swamps out energy loss data. Thus, we are effectively viewing an attenuated filtered bright field signal. The micrograph at 4 eV loss shows almost no contrast. If one refers back to the energy loss spectra (fig. 2), it is seen that each component studied has no peak and relatively low intensity at 4 eV loss. This accounts for the low signal and lack of contrast. At 6 eV loss, contrast is due both to differences in spectra of the components and mass thickness. Although a window at 6 eV includes a portion of the peaks in the spectrin and cholesterol spectra, the dose was not high enough in this
Fig. 4. Energy window micrographs of an erythrocyte membrane fragment obtained using 28 keV electrons. The micrographs were obtained using a 0.75 eV slit width centered about the energy loss shown in the figure. The full horizontal scale on all micrographs is 2.5 μ and the total dose per micrograph was $2.6 \times 10^{-5}$ C/cm². The micrograph in the upper left was obtained using the signal from an annular detector placed beneath the specimen (10) and represents a picture formed using mainly elastically scattered (no energy loss) electrons.
Table 2
Physical parameters for average erythrocyte membrane components

<table>
<thead>
<tr>
<th>Material</th>
<th>Average molecular weight</th>
<th>Density (g/cm³)</th>
<th>σ_{in}/D (X 10⁻³ A²)</th>
<th>λ_{in} (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>227</td>
<td>1.56</td>
<td>3.4</td>
<td>313</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>151</td>
<td>1.00</td>
<td>3.93</td>
<td>423</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>386</td>
<td>1.07</td>
<td>4.18</td>
<td>372</td>
</tr>
<tr>
<td>Protein *</td>
<td>35,560</td>
<td>1.37</td>
<td>3.52</td>
<td>344</td>
</tr>
</tbody>
</table>

† Calculated from values given in ref. [12].
‡† Calculated using the formula for inelastic scattering given in ref. [7].
* The membrane associated protein glyceraldehyde-3-phosphate dehydrogenase was chosen since its sequence is known.
** λ_{in} is the mean free path for total inelastic scattering. It allows us to compare the scattering from equal volumes of different materials.

The micrograph to put the signal from the peak minus the background greater than twice the square root of the background. In other words, effects due to the peaks in the spectra were masked by the statistical variation of the signal. The micrograph taken with 20 eV loss electrons is more intense because of the large, broad peak in the spectra at this energy loss (fig. 1). The 35 eV micrograph comes from a region of the spectra where the large 20 eV peak is falling off about as the fourth power of the energy loss. Since the membrane components do not exhibit noticeable peaks in this region, contrast is determined simply by mass thickness and absolute cross section values. Calculated total inelastic cross sections for major membrane components are given in table 2 and on a weight basis are seen to vary less than 20%, whereas densities vary by 55%. To compare the scattering from equal volumes of material more easily, the inelastic mean free path, λ_{in}, has been calculated. The phospholipid, as expected from its low density, has a lower value of scattering per unit volume than the other components.

3.4. Microanalysis

As an additional test object for our energy loss studies, single ferritin molecules were examined (A,20). A beam of about 40 Å diameter was stopped on a ferritin molecule and counts were accumulated over twenty-six seconds while the spectrometer was repeatedly ramped to produce a spectrum. The results are shown in fig. 5. The onset of the iron M_{II,III} line occurs at 52.4 eV while the oxygen K shell excitation was found to be at 534 eV. The number of counts in the background for the iron M line was 10⁶ and the peak was 3000 counts above this level. This gives a peak/√background for the iron M line of 30, whereas for oxygen it is 13. These measurements demonstrate that such elements can be detected in this single molecule with a good signal/noise ratio (although the dose imparted to the molecule was quite high (>10³ C/cm²).

4. Discussion

In this section, we would like to investigate some of the immediate and future prospects of inelastic energy loss spectroscopy with the respect to doses required, damage and resolution that can be expected.
4.1. Resolution and detectibility

The ferritin data were collected to produce a peak well out of the background. However, a reasonable criterion for detection of a peak is to require the peak/√background (P/√B) to be equal to 2 (where the peak is the peak above the background). The measured signal from the iron atoms could therefore be reduced by a factor of 15. The maximum number of iron atoms in a ferritin molecule is about 5000 and assuming we were centered on a "full" molecule, the number of atoms that could be detected would be about 350 under the same conditions. If parallel detection were used, a factor of about the square root of the number of channels could be realized in the signal in the same counting time so that this number could be reduced by more than a factor of ten. (We used 128 channels to record the spectra). Looking at this in another way, we could record the ferritin spectra with 150 times less dose. Of course, another method of increasing detectibility is to increase the beam dose. This assumes that the mass is not lost or rearranged between resolution elements appreciably.

Since inner shell electron interactions relate to the atom rather than molecule, molecular damage is of little consequence and higher doses can be tolerated (provided that the material is not being "vaporized"). The above estimate of iron detectability assumes a constant background, that of carbon film and the apoferritin molecule.

Similar arguments may be applied to find the detectability limits of materials using the low energy loss peaks. For example, the 7 eV peak for spectrin has a measured value of 50 above the background of 500 (P/√B = 2.24). For a P/√B of 2, 400 counts would be required for the background. If a 1 eV window is used, then only 0.008 of the total inelastic scattering is collected (since by analogy to adenine, as shown in fig. 1, only about 8% of the inelastic signal is in the 0–10 eV region and a 1 eV window is roughly 10% of this value). From the calculated total inelastic cross section for typical protein (σ_in = 2.65 × 10^-3 Å^2/D at 35.5 keV) the σ_in for the molecule (molecular weight = 220,000) is 580 Å^2. Using the density (1.4 g/cm^3) and molecular weight, the molecule has a volume of 2.5 × 10^5 Å^3. We assume for this example that the molecule is 100 × 50 × 50 Å oriented with its long axis along the beam axis. The fraction of the beam that is scattered inelastically is given by

\[ N_i = \exp(-N_o t [1 - \exp(-N_o t)]) \]  

where \( N \) is the molecular density, \( t \) is the thickness, and \( \sigma_e \) the total elastic scattering cross section (328 Å^2 in this case). \( N_i \) is calculated to be 0.18 for the single molecule (assuming an incident beam of 50 Å × 50 Å cross-sectional area). Since in the 1 eV window only 0.008 of \( N_i \) is collected, we find that 1.4 × 10^-3 of the incident beam is collected. Thus, the incident beam would have to be 3.2 × 10^5 electrons into the 50 Å × 50 Å area to obtain a P/√B of 2 from a single spectrin molecule on a ~25 Å carbon film so that it would be detected using the 7 eV peak in its energy loss spectrum. This yields a dose of 128 electrons/Å^2. This value is easily attainable with the present instruments but is a relatively high dose with regard to electron beam damage effects and changes in the spectrum at this level must be considered.

4.2. Energy window pictures

One would like to scan over a specimen and, point by point, analyze the composition by making energy window maps. As with ultraviolet absorption spectroscopy, for example, a value at just one energy is not enough data to solve the composition of a multi-component system. By taking measurements at two or more energies (the number of energies measured must at least equal the number of components) the absolute composition of each material may be determined.

Several methods for collecting the requisite data are possible. The best method, mentioned earlier, would be to record the spectra in parallel. Such parallel recording devices are not yet perfected (particularly with regard to single electron counting). An alternative method is to record one micrograph at one energy and successive ones at different energies. Some problems associated with this are the fact that the incident beam intensity may change and measurements would have to be normalized. Also, specimen drift may occur. More difficult to correct for, however, would be any damage or contamination that occurs between frames. A method which circumvents some of the problems of separately recording pictures using different energy loss electrons is that of recording three adjacent energy losses for each point in the image (one above and below the peak of interest, and one at the peak). This
can be done time-sequentially so that, for example, every third pixel would correspond to the same energy loss. By recording the picture on magnetic tape, one could then replay the picture by subtracting the two background channels from the peak channel thus obtaining a picture in which the intensity was only proportional to the peak signal in the region of interest and not on the background. (Of course, the number of picture elements per picture would be three times less than would be obtained if only one energy window were used.)

5. Conclusion

In summary, microspectroscopy via energy loss analysis appears to be feasible even with presently available instrumentation. Although examination of a small portion of a spectrum implied a greatly reduced signal, and thus more of a problem with regard to radiation damage, many specimens will probably be suitable. The resolution of this technique is limited with the low-lying losses by the diffuseness of inelastic scattering (about 10 Å) [21], but will, practically speaking, depend on the damage to the specimen. Although we have only measured inner shell losses for iron in this paper, inner shell losses can be used to look for various atoms. In particular, in the membrane, several components uniquely contain certain elements. The phospholipids contain phosphorus (4% of the molecule) while this element is non-existent in the other components. In selecting an atom inner shell excitation to measure, one must take into account the background created by the tails of other atomic or molecular absorptions so that a reasonable $P/\sqrt{B}$ can be obtained. Not only can specific elements be used in identification, but the ratio of elements can also be used. For example, the ratio of oxygen to carbon for cholesterol is 0.48, whereas this ratio is 0.42 for proteins.

The low-lying energy loss peaks, in general, have higher scattering cross sections than the inner shell peaks, but damage rates also differ. The components of the membrane show substantial differences in their low loss peaks and it appears possible to resolve them with about 50 Å resolution using this information.

Since energy loss spectroscopy is a quite different method of study and several elements are very favorable with respect to detectability (single fluorine atoms should be detectable) [4], a whole new class of stains could be generated that would be identifiable by their energy loss characteristics. In fact, an energy window technique has recently been used by Joy and Maher [24] to detect large concentrations of fluorine in human platelets.

One point with which we have not dealt in this paper is the effect of the loss of material due to the high beam doses that may be necessary for recording energy loss spectra peaks. Previous studies have shown that significant mass loss can occur upon electron mean irradiation (at room temperature) of some amino acids and proteins with doses lower than 0.1 C/cm² [eq. (7)]. The total fractional mass which can be lost due to beam irradiation can be as large as 80%. Different materials exhibit different sensitivities to the electron beam, and there are indications that in some specific cases, the material left is suitable for analysis [eq. (22)]. Moreover, there are experiments which seem to indicate that mass loss is appreciably reduced if the specimen is kept at cryogenic temperatures [23]. Thus, although there may be beam-induced problems associated with ELS of biological materials, the problems may be surmountable.

Energy loss microspectroscopy is still in a primitive state, but it could have the potential of becoming a powerful technique as more laboratories become involved in its application.

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References

J. Hainfeld and M. Isaacson / Electron energy loss spectroscopy for studying membrane architecture