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Problems associated with high incident beam voltage and probe current during biological x-ray microanalysis.

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X-ray microanalysis of nearly all biological specimens use a reductionist and analytic approach to combine the separate disciplines of chemistry and microscopy. Unfortunately it is necessary to use energetic electrons both to form the high resolution images and generate the emitted x-rays which are the basis of the in situ chemical identification and location. The technology is very invasive, particularly for biological samples which are poor electrical conductors, radiation sensitive and full of water. The improvements in specimen preparation and the algorithms used for analysis, now make it possible to measure very low concentrations of light elements at better than 10nm spatial resolution. This paper will consider ways to diminish the damage which high voltages and probe currents cause during sample examination and microanalysis.

Plant, animal and microbial samples have a very low electrical conductivity. For example, the conduction of silver and the noble metals are 10^9 times higher than either bone or wood. Biological materials invariably exhibit charging phenomena when irradiated with an electron beam. Charging effects the specimen in many ways. (1) It will cause changes in the primary beam landing energy, decrease the electron penetration depth, alter the spatial location of emitted x-rays and severely distort the appearance of the secondary electron image. Charge elimination may be achieved either by non-invasive procedures which make minor alterations to the microscope or invasive procedures which can severely alter the specimen.

Charge elimination is readily achieved by lowering the incident beam voltage and probe current to ensure that minimum energy is used to obtain the maximum information about the specimen. Every sample has it unique set of values which may be easily determined. Much is now understood about the general features, and advantages of low voltage scanning electron microscopy. The recent papers by Boyes (2) and Joy (3) show that voltages as low as 20eV can be used to obtain images in the scanning electron microscope. The minimum voltage needed to ensure quantitative x-ray biological microanalysis is 15 to 100 times higher. However, low voltage operation has additional advantages.

For all samples, there is a marked decrease in the size of the sample-beam interactive volume as the specimen density increases and the incident beam voltage decreases. One way around this apparent paradox is to only use a thin flat section of the biological material to ensure that a substantial part of the energy of the incoming electron beam passes through the specimen without scattering. Unfortunately, all sectioning procedures are invasive. Low temperature procedures have the least effect (4) while the use of chemicals will readily lead, either to translocation or complete loss of the elements. An alternative way to reveal the interior of a sample is to either make an impact fracture or microplan a broken surface with a sharp knife. Both processes produce a bulk sample with a clean, relatively smooth, surface. If the empirical formula of the specimen is known, the dimensions of the sample-beam interactive volume can be measured by using one of the different range equations. An example now follows.

Frozen hydrated tea plant epidermal cell wall are considered to have the following composition. Water (ice) 60% δ=978kg/m^3, structural carbohydrates 34% δ=300kg/m^3, protein 3% δ=1200kgm^3, and the principle combined elements Mg, Al, Si and Ca 3% δ=1800kg/m^3. The weight average density of the frozen hydrated material at 150K is 780kg/m^3. Using the Bethe Range equation Rb=70(E1.66/δ) where Rb is the penetration depth in μm, E is the incident beam energy in KeV and δ the density, the dimension of the interactive volume may be readily calculated for different beam voltages. For this particular specimen, the interactive volume is 18μm^3 at 10kV, 0.6μm^3 at 5kV and 0.006μm^3 at 2kV and there is a 9x10^4 decrease in the dimensions of the interactive volume between 10 and 1kV. The

diminished interactive volumes associated with reduced voltages, have to be balanced against the higher energies needed to generate sufficient x-ray in the sample in order to carry out quantitative analysis. For the type of sample discussed above, this may be achieved at 1.0keV for C, N and O, at 2-3keV for Na, Mg, Al and Si, and at 4-5keV for P, S and Cl. It has been found necessary to go to 8keV for K and Ca. It remains to be seen whether it will be possible to analyse K and Ca at 1.0keV by using the L lines for these two elements at 0.259 and 0.341 keV, respectively. Before undertaking any quantitative biological microanalysis it is important to first establish the minimum voltage and beam current which will give the smallest sample interactive volume while at the same time generating sufficient x-rays.

The charging of non-conductors is readily overcome by coating the specimen with a very thin layer (2-3nm) of a conductive material which must not contribute to the x-ray spectrum of the specimen being analysed. Tables 1 and 2 give an outline of some of the properties of seven materials which may be used.

Table 1. Physico-chemical properties of seven elements which may be used for coating for the x-ray microanalysis of non-conducting biological samples. Ev=evaporation, Sp=sputtering.

	Be	C	Al	Cr	Ti	V	Pd
Density ($Kgm^{3)}$	1800	2300	2700	7200	4500	6100	12024
Conduction (W.cm)	2.00	1.29	2.37	0.93	1.74	0.31	0.72
Resitivity (μohm.cm)	4.27	3500	2.83	13.0	5.50	18.2	10.0
Evaporation(0^{c})	1247	2727	1002	1177	1819	2161	2363
Coating method (Sp/Ev)	Sp	Ev	Sp/Ev	Sp/Ev	Sp/Ev	Ev	Sp/Ev
K series x-ray (KeV)	0.110	0.282	1.487	-	-	-	-
L series x-ray (KeV)	-	-	-	0.571	0.452	0.510	2.838

Table 2. Possible interference between the x-ray lines of coating materials and biological light elements (Z=6-20) using an energy dispersive spectrometer with a resolution of 130eV.

Element used for coating	Elements which will suffer interference
Beryllium $K\alpha_1$ line = 0.110 Kev.	No interference
Carbon $K\alpha_1$ line = 0.282 Kev.	Interference with K-$L\alpha_1$ (0.259) and Ca-$L\alpha_1$ (0.341)
Aluminium $K\alpha_1$ line = 1..487 Kev	Interference with Mg-$K\alpha_1$ (1.254) and Si-$K\alpha_1$ (1.740)
Titanium $L\alpha_1$ line = 0.452 KeV	Interference with N-$K\alpha_1$ (0.392) and O-$K\alpha_1$ (0.525)
Vanadium $L\alpha_1$ line = 0.511 Kev	Interference with O-$K\alpha_1$ (0.525)
Chromium $L\alpha_1$ line = 0.571 KeV	Interference with O-$K\alpha_1$ (0.525)
Palladium $L\alpha_1$ line = 2.838 KeV	Interference with Cl-$K\alpha_1$ (2.622)

All seven elements indicated above have both advantages and disadvantages and it is unlikely that a single element will provide an ideal material for all the elements of interest in the particular biological material being analysed. If it is necessary to apply a coating layer prior to carrying out x-ray microanalysis, it is important to first check whether any adverse interrelationships may exist between the elements being analysed and the elements being considered as a coating material.

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