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Analysis of Complex, Beam-Sensitive Materials by Transmission Electron Microscopy and Associated Techniques.

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Abstract

We review the use of transmission electron microscopy (TEM) and associated techniques for the analysis of beam sensitive materials and complex, multiphase systems *in-situ* or close to their native state. We focus on materials prone to damage by radiolysis and explain that this process cannot be eliminated or switched off, requiring TEM analysis to be done within a dose budget to achieve an optimum dose-limited resolution. We highlight the importance of determining the damage sensitivity of a particular system in terms of characteristic changes that occur on irradiation under both an electron fluence and flux by presenting results from a series of molecular crystals. We discuss the choice of electron beam accelerating voltage and detectors for optimising resolution and outline the different strategies employed for low dose microscopy in relation to the damage processes in operation. In particular, we discuss the use of scanning TEM (STEM) techniques for maximising information content from high resolution imaging and spectroscopy of minerals and molecular crystals. We suggest how this understanding can then be carried forward for *in-situ* analysis of samples interacting with liquids and gases, provided any electron beam-induced alteration of a specimen is controlled or used to drive a chosen reaction. Finally, we demonstrate that cryo-TEM of nanoparticle samples snap frozen in vitreous ice can play a significant role in benchmarking dynamic processes at higher resolution.

Introduction

Complex materials often include a component of soft matter that is sensitive to structural or chemical alteration when imaged by electron microscopy. These electron beam sensitive components might be organic crystals, polymers, interfaces and hybrid organic-inorganic materials, even some inorganic materials such as hydrates and hydroxides, as well as multiphase solid/liquid and solid/gas systems (1, 2). Arguably materials that contain such components constitute the majority of systems of current interest across a wide range of scientific and engineering disciplines including chemistry and chemical engineering, engineering materials, food science, biology and increasingly physics and electronic engineering.

For the last ninety years transmission electron microscopy (TEM) and subsequently scanning electron microscopy (SEM), have been the premier tools for observing matter at

the micrometre, nanometre and even atomic scale. This is mainly because of the image resolution obtainable with fast/high energy electrons but also arises, in part, because the interaction between electrons and matter is so strong. However, as a result of the latter property, these techniques have suffered from having to analyse samples in a vacuum leading to specimen dehydration and, in the case of TEM, has necessitated the use of very thin samples that often require considerable sample preparation. Furthermore, much of the progress of both TEM and SEM has often relied on a highly simplified and imprecise accounting of the interaction of the electrons with the material under examination. In order to progress research into new material systems and to observe them *in-situ* in both their native state and under dynamic conditions, it is imperative we attempt to address this limitation.

For the purposes of this communication, we concentrate almost exclusively on TEM of thin specimens using both near parallel beam, conventional TEM (CTEM) and scanning TEM (STEM) with a focused electron probe. Furthermore, we define native-state analysis of such complex, beam sensitive materials and systems, as the condition that they remain largely unmodified by the specimen preparation used to bring them into the microscope and that the interaction with the electron beam is controlled such that there is minimal measurable alteration of structure and chemistry of the specimen during analysis. *In-situ* analysis is the dynamic observation of the reactions in or on a specimen contained within the microscope when stimulated by mechanical, thermal, electrical, optical or chemical means. *In-situ* analysis therefore poses a number of challenges: First the inherent beam sensitivity of both the starting components and the product following *in-situ* phase transformation and/or reaction must be quantified and secondly, for chemical reactions, the inherent beam sensitivity of the reactant liquids or gases, with which the material is interacting *in-situ* must be understood or accounted for.

Initially we discuss the origins of electron beam sensitivity of materials and ways to mitigate these effects. Electron beam-induced damage has been investigated intermittently over the years including key studies in (3-7). In fact electron irradiation has even been used as a surrogate for fast neutron damage in the study of materials for application in nuclear fission and fusion (early work is summarised by (8) and more recent works by (9-11)).

The electron dose experienced by a sample is the energy absorbed by the specimen during electron irradiation and is a function of both the incident energy of the accelerated electrons, electron fluence (the number of electrons per unit area) and fluence rate or flux (the number of electrons per unit area per second) in the TEM, as well as parameters associated with the specimen itself such as the thickness and chemical composition. The absorbed dose is strictly measured in Grays (note a Gray (Gy) is 1 Joule of energy deposited per kg of matter), and for 100 keV electrons exposed to a typical organic material an electron fluence of 1 electron/ $Å^2$ or ca. $1.6x10^{-3}$ C/cm^2 is then equivalent to a total absorbed dose of a few MGy (12). The effects of ionizing radiation on materials has been a major topic of study for many years particularly in relation to cancer radiotherapy, atmospheric science, remediation of waste-water, food preservation/treatment, sterilization of pharmaceuticals, and synthesis and nuclear energy production. Yet the issue is often side-stepped in electron microscopy investigations. A distinct difference with electron microscopy is that electron dose rates (and hence total electron doses) are many orders of magnitude higher than those generated by other common radiation sources. This is especially so for the focused electron probes of STEM where understanding of the implications of very high dose rates for dose efficiency is still developing (13, 14) and will be briefly discussed later in this work. Nevertheless , the effects of electron beam radiation are almost universally present in electron microscopy experiments, complicating their separate study. It is therefore unsurprising that the electron beam sensitivity of many materials is a major issue limiting their study.

We discuss a number of useful concepts for TEM imaging and/or chemical analysis within a TEM, including a *dose budget* for experiments and a *dose-limited resolution* (15). We also highlight the current state-of-the-art in dose control during imaging and spectroscopy including the use of scanning TEM (STEM) as opposed to conventional TEM (CTEM), compressed sensing methods and the cryo-preservation of samples. Finally, we also discuss dynamic studies of such complex materials and systems including the use of *in-situ* liquid and gas cell TEM holders, which have their own additional limitations due to dose, as well as the use of time-resolved cryo-methods to provide snapshots of dynamic systems.

Damage of Beam Sensitive Materials by Electrons

In general, inorganic materials such as metals and metal oxides are more electron beam stable whilst biological materials, inorganic-organic hybrid materials and organic materials (including polymers and small molecules in both crystalline and non-crystalline forms) are more easily damaged. The mechanisms by which a material is damaged by the electron beam can be categorised by the different types of electron scattering experienced: either, (i) elastic scattering arising from an electron-nucleus interaction which can lead to atomic displacement and electron-beam induced sputtering, or (ii) inelastic scattering arising from an electron-electron interaction which can lead to both electron excitation and heating in the sample, or (iii) a combination of both. Inelastic scattering also causes the production of low energy secondary electrons that can generate highly reactive free radicals and ions that can cause bond breakage, an overall process known as *radiolysis*. There are a number of comprehensive reviews of these mechanisms and, in the first instance, the reader is referred to an example that considers typical TEM accelerating voltages between 10 and 300 kV (16). In this work, we focus on how understanding of these principles can be applied for improved experimental design and execution.

The elastic scattering damage mechanism (known as knock-on) is dominant in conducting materials, often containing light elements, with strong primary bonds. Knock-on damage dominates for materials such as graphite and graphene, and is characterised by a threshold incident electron energy required to displace atoms within the specimen (5). Clearly this can be mitigated by lowering the energy of the incident electrons to be at or below this threshold and, in combination with spherical aberration correctors that retain image resolution at lower kV, has stimulated the field of low energy TEM (17, 18). Conversely, the predominant mechanism for damage of non-conducting materials containing light elements and weaker secondary bonds, such as molecular crystals, is radiolysis. Radiolytic bond breakage is a secondary mechanism that does not exhibit a distinct incident beam energy threshold, but does lead to a loss of order in crystals which can be observed through the fading of diffraction spots to amorphous rings in electron diffraction patterns (19, 20). A loss of molecular structure can be observed in electron energy loss spectroscopy (EELS) and, if mass loss occurs, there will be changes in chemical composition detectable by EELS and/or energy dispersive X-ray spectroscopy (EDX) (7). Mass loss of lighter atoms due to bond breakage and sputtering may also be seen as a change in mass-thickness contrast in images, whereby areas from which atoms have been removed appear more intense in the bright field (BF) image (7, 20).

The propensity for damage to the sample to be caused by the electron beam can be quantified by measuring the characteristic/critical electron fluence (C_F) for an image or spectral feature to significantly change, in units of $e^-/Å^2$ (7). Note the terms electron dose and electron fluence tend to be used interchangeably in the field of electron microscopy, although, as discussed, strictly dose is the energy absorbed by the sample per unit mass (in

units of Grays) (e.g. Garvie et al. (21)). The critical electron fluence (C_F) may be calculated by measuring the change in intensity of an electron scattering feature as a function of irradiation time under a given electron flux, such as an electron diffraction spot in the case of a crystalline material, and determining the total accumulated electron fluence at which the relative intensity of the feature decays to e^{-1} (ca. 37%) of its initial value during exposure. Electron diffraction monitors crystallinity and lattice periodicity, alternatively a C_F related to changes in bright field, dark field or phase contrast TEM images may be determined or, for analytical TEM, one related to changes in chemical composition or local chemistry measured by EDX or EEL spectroscopies; the latter may also be used to monitor changes in chemical bonding by tracking spectroscopic fine structure arising from the local electronic structure. These alternative approaches will yield differing sensitivities to the electron beam (i.e. differing values of the critical electron fluence), because the type and extent of observed damage varies from loss of atomic order, loss of material to changes in chemistry, and each of these will depend on the predominant damage mechanism responsible for these particular changes (22).

Fundamentally the value of the critical fluence depends on the material and a variety of experimental parameters (including fluence rate or flux, discussed below); in general, for irradiation energies of 80 - 300 kV, typical for TEM, biological materials have a C_F in the range of 1 - 15 $e^-/Å^2$, organic crystals 0.2 - 120 $e^-/Å^2$, zeolites and other hydrated or hydroxylated minerals 100 - 600 $e^-/Å^2$, and transition metal oxides > 10^7 $e^-/Å^2$ (3, 23-27).

Critical Fluences of Molecular Crystals

As an example, we focus on molecular crystalline materials and choose a model organic material theophylline, a metabolite of caffeine that can exist in one hydrated and seven anhydrous polymorphic forms (28). Anhydrous form II crystals of theophylline can be readily synthesised by recrystallisation from a solution in nitromethane and possess a thin platelet morphology. This thin platelet morphology causes particles to adopt a predictable <100> orientation, and samples tend to be electron transparent – so making them a good candidate to study.

The structural C_F of anhydrous form II crystals of theophylline has been investigated for a variety of microscope and sample conditions and is detailed in reference (29, 30)]. Figure 1a shows an example of a diffraction pattern of theophylline during prolonged irradiation over ca. 10 minutes at a beam energy of 200 keV and an electron flux of 0.098 $e^-/(Å^2s)$. Higher order Bragg reflections (corresponding to smaller lattice spacings) fade first as bond breakage and molecular rotation disrupts short range order, however general long-range molecular packing remains to higher electron fluences (3). Figure 1b shows the decrease in intensity of the {004} and {011} diffraction spots as a function of accumulated fluence from which the structural critical fluence can be determined as ca. 23 $e^-/Å^2$ (for the case of the {011} spot).

>Figure 1<

The decay of the diffraction intensity is due to damage of the chemical (i.e. the molecular unit) and/or crystal structure and, in most cases, it appears that both occur simultaneously. However, it has been demonstrated in phthalocyanines that the crystal structure is lost before the chemical structure while studies on tetracene show that the chemical structure is destroyed first (31). The form of the decay of the diffraction intensity as a function of increasing fluence is most often observed to be exponential, however sometimes variations are observed such as an initial plateau (as seen for the case of the {011} diffraction spot in

figure 1b) or even a rise in intensity (followed eventually by an exponential decay) as electron fluence increases. The latter may be attributed to a rapid loss of mass upon irradiation, reorientation of the crystal, or conformational changes occurring in the sample, producing a structure that is more stable to the electron beam (32-35). Reports of this type of initial plateau or rise in recorded diffracted intensities in X-ray crystallography have been attributed to initial changes in orientation or unit cell parameters throughout the sample volume (36). Similar observations have also been reported in cryo-EM where an initial rise in contrast at high spatial frequencies over a series of frames is attributed to initially rapid particle motion followed by a reduction in motion with increasing exposure time (37, 38). The existence of an initial plateau may also indicate that damage products need to reach a critical concentration before they can diffuse away and initiate further damage (39); the occurrence of a thermally-activated reverse reaction (or healing process) may prevent a proportion of the damage, allowing for the structure to stay intact until the cumulative damage is too great and the structure finally breaks down.

Using averaged measurements from a number of form II theophylline crystals (which averaged out any effect of sample thickness) at room temperature with samples supported on continuous carbon film substrate TEM grids, the critical fluence was determined to be ca. 26 $e^-/Å^2$ at 200 keV. Due to the inverse dependence of the inelastic cross-section on incident electron beam energy, the C_F increased to ca. 36 $e^-/Å^2$ at 300 keV and decreased to 11 $e^-/Å^2$ at 80 keV, as highlighted in Figure 1c (29). At least for continuous carbon substrates, specimen cooling was found to have relatively little impact on C_F, whilst specimen heating showed a slight adverse effect in that it decreased C_F, Figure 1c. More generally it would appear that proportionately, relative to room temperature, specimen cooling to liquid nitrogen temperatures has a larger effect the more beam-sensitive the specimen (40); the rationale here is that cooling may help limit the diffusion of secondary products generated during radiolysis (40). Overall, the largest value of C_F for theophylline form II at 200 keV (ca. 42 $e^-/Å^2$) was obtained using a graphene support substrate and cooling to liquid nitrogen temperatures (also shown in Figure 1c).

This methodology was extended to the analysis of 20 poorly soluble crystalline organic materials (Figure 2) that are generic active pharmaceutical ingredients (APIs) and were selected on the basis of a variation in their molecular chemical groups (40). A range of structural C_F values were obtained which did not appear to correlate with traditional or simple measures of sample stability, such as the melting point. As a result, a total of 19 different molecular descriptors that may have influenced C_F were used as input parameters for a Principal Component Analysis (PCA) which identified those which were statistically significant. The molecular descriptors which gave a positive correlation to C_F were related to the presence of aromatic and conjugated carbons suggesting that delocalization of electrons allows the energy deposited from the electron beam to be shared and dissipated more effectively, so decreasing the formation of damaging radicals (41-44). The molecular descriptors that exhibited a negative correlation to C_F were related to the number of rotatable bonds (which relates to the number of different structural configurations the molecules can undertake) and, more surprisingly, factors that related to the numbers of hydrogen bond donors and acceptors. The latter can be rationalised by the fact that when hydrogen bonding is present, the adjacent covalent bond is weakened making it more susceptible to radiolytic damage. An alternative explanation might be that, during radiolysis, the chemical groups involved in the hydrogen bond may readily form radicals (e.g. OH•) that can propagate and cause further damage. Potentially the presence of hydrogen bonding may also encourage the incorporation of ambient water during handling/processing.

Using the parameters identified as being significant, a multiple linear regression (MLR) model was generated to allow prediction of critical fluence for structural degradation of a molecular crystal based on the structure of the constituent molecule. The experimentally observed critical fluence versus the predicted value based on the model is shown in Figure 2; further details are discussed in reference (31).

>Figure 2<

Implications of Damage for the Measurement of Beam Sensitive Materials

The critical fluence gives a measure of the *fluence budget* (F_{Budget}) (a surrogate for the *dose budget*) available to a user during an experiment. This is the accumulated fluence required to locate (F_{Locate}), align (F_{Align}) and focus (F_{Focus}) the specimen region, prior to recording either an image, diffraction pattern, EDX or EELS spectrum or spectrum image from the specimen area of interest (which requires an additional accumulated fluence of F_{Signal}). Thus, the *total fluence budget* is given by:

$$F_{Budget} = F_{Locate} + F_{Align} + F_{Focus} + F_{Signal} \qquad \text{Equation (1)}$$

Beam blanking is often used between these separate experimental steps and the flux reduced so as to avoid unintended irradiation of the specimen. The concept of a dose budget has analogies with dose fractionation in cryo-TEM tomography (45).

Increasing the critical fluence of a beam sensitive material by appropriate choice of the sample itself, specimen preparation and the electron microscope operating conditions is obviously desirable, however this doesn't necessarily lead to an improvement in information obtained. Egerton (15) has introduced the concept of *dose-limited resolution (DLR)* for a polycrystalline or amorphous material:

$$DLR = \frac{SNR\sqrt{2}}{|C|\sqrt{DQE}\sqrt{FC_F/e}} \qquad \text{Equation (2)}$$

where SNR is the signal to noise ratio which must equal or exceed some chosen background value, typically > 3 - 5 times the standard deviation to satisfy the Rose criterion (46); DQE is the detector quantum efficiency which refers to the efficiency of a detector in converting incident electrons into an imaging signal (this may be modified by a modulated transfer function (MTF) which is a function of the spacing or spatial frequency that is being measured); F is the collection efficiency of incident electrons to detected electrons, which, for CTEM, depends on the sample thickness (t) and the elastic mean free path of electrons (λ_e) and is equal to $F = \exp(-t/\lambda_e)$; e is the elementary charge of an electron; C is the contrast which depends on C_0 (the initial contrast before electron beam exposure), the accumulated fluence and the critical fluence of the material (C_F) and may be given by $C = C_0\exp(-\text{Fluence}/C_F)$, assuming an exponential decay of contrast with increasing fluence.

Many of the factors in the DLR are inter-related in a complex fashion. For example, for a given specimen thickness, increasing the electron beam energy in order to lower the inelastic cross-section and hence increase the critical fluence, will conversely lead to a reduction in contrast and hence signal. There is a benefit to increasing sample thickness insofar as the larger number of scattering events in a thicker specimen results in more signal whereas the damage per unit volume remains the same, so the signal/damage ratio improves (15). The choice of beam energy, however, also depends on the collection efficiency, and higher electron beam energies (e.g. 300 kV) can offer improvements in DLR for thick samples for many imaging modes (15). Alteration of the electron beam energy will

however change the DQE of the detector that may be optimised for a particular electron energy range and future work should also consider this. The optimum resolution for thin (~ 10 nm) biological specimens has recently been experimentally determined by Peet et al. (47). They showed that the elastic cross-section is approximately two times greater at 100 keV than at 300 keV, whereas the radiation damage increased by only 1.57 times; hence 100 keV is recommended for measurements on thin samples, once a direct electron detector with optimal pixelation and performance at 100 kV becomes available.

Low dose TEM approaches for high resolution imaging

From the above discussion of DLR, besides the optimum choice of experimental variables (incident beam energy, detector etc.) and sample variables (specimen thickness, coating, cooling etc.), the primary task in low dose microscopy is to control the electron fluence incident on the sample to provide just enough fluence to collect sufficient scattered electrons to achieve contrast before significant structural alteration occurs.

The electron flux in CTEM can be controlled via adjustment of the extraction voltage of the electron field emission source, as well as by the condenser lens system and associated apertures so as to control the brightness and area illuminated. In contrast, for STEM the total electron fluence per image can be controlled via choice of: the magnification (and hence the specimen pixel size), the probe current (typically pA) using the illumination aperture and associated condenser optics or source extraction voltage, and alteration of the dwell time per pixel (typically microseconds). The electron flux and measured probe current in CTEM and STEM can be measured from the exposure time or fluorescent screen current reading which can be directly calibrated using a Faraday cup. In some S/TEM systems, continuous control of the probe or beam current is not possible due to the electron optical design. However, a monochromated electron source will permit continuous control of probe/beam current by various means depending on monochromator design, meaning that any chosen probe/beam current can be used without significant impact on the electron optical alignment lower down the column including, beam/probe size, or focus (48).

Previously high-resolution CTEM images of electron beam sensitive materials were generally recorded on photographic film, whilst more recently direct electron detectors have been used to obtain HRTEM images at low dose (26, 49-52). One example is by Zhang et. al. (52) where atomic resolution images of metal-organic frameworks (MOFs) were obtained by HRTEM using a total electron fluence of 5 $e^-/Å^2$ at 300 keV. The use of direct electron detectors increases the signal-to-noise ratio (SNR) and contrast in an image due to the high detector quantum efficiency (DQE) compared to conventional scintillator-based detectors and this is key to enabling high resolution CTEM at very low electron fluence. The capability for these detectors to operate in an electron counting mode, when operated at suitably low counts per pixel for charge localisation, also enables the elimination of spurious dark current which dominates CCD detectors at low beam currents incident on the scintillator. The counting capability of direct electron detectors also results in improvements in the detector MTF and, in turn, improvements in the DQE. Broadly, the available direct electron detectors may be classified as monolithic active pixel sensors (MAPS) or hybrid pixel array detectors (PAD) with characteristic differences in pixel size, number of pixels, and performance at different accelerating voltages. These have been reviewed in (53) and elsewhere. Furthermore, the rapid acquisition speed of some direct detectors allows any mechanical instability in a sample during the initial stages of damage to be identified and even corrected by post acquisition, cross-correlation of images.

S'ari et al. (54) investigated a model system of crocidolite (a form of asbestos which is moderately beam-sensitive) in order to determine the electron flux/fluence limits for low-dose, high resolution CTEM imaging for a particular scintillator-based, CMOS electron camera by testing a variety of electron flux and total electron fluence regimes. The results are shown in Figure 3 (a) - (i) and revealed that an electron flux of 10 $e^-/(Å^2s)$ and total fluence of 10 $e^-/Å^2$ provided sufficient contrast and SNR to resolve 0.30 nm lattice spacings at 300 kV. These parameters were then used to image an organic crystalline material, furosemide, which has a critical fluence of a little above 10 $e^-/Å^2$ at 300 kV (Figure 3 (j) - (o)).

>Figure3<

Low Dose STEM versus CTEM

In terms of maximising the fluence budget (Equation (1)) for actual signal measurement, a common option used in both low dose CTEM and STEM is to locate and align the specimen under study in a neighbouring (sacrificial) region of the sample and then move to a fresh, undamaged region of interest (ROI) for signal measurement. In terms of practical experimental considerations, in high resolution CTEM lattice imaging, which illuminates a specimen area in parallel, there is an additional requirement to refocus the image when moving from the sacrificial location/alignment area to a fresh unexposed area, which correspondingly uses up a proportion of the available fluence budget. However, in STEM one can significantly reduce F_{Focus} by focusing the Ronchigram (obtained using a static, focused STEM probe) in a small area directly adjacent to the ROI, so sacrificing only a small region of specimen and retaining a greater proportion of the fluence budget for actual signal measurement.

Unlike CTEM, STEM is a serial technique that collects signal pixel by pixel - making it more ideally suited to provide localised analytical information such as diffraction or chemical composition than CTEM. However, this serial nature means that STEM is relatively inefficient in terms of signal collection. The conditions for bright field STEM that still approximates to bright field phase contrast CTEM can be optimised in terms of signal collection by choosing the bright field detector collection semi-angle to be half of the probe convergence semi-angle (for the case of a non-aberration corrected STEM probe, and two thirds of the probe convergence semi-angle for an aberration corrected STEM Probe) (55). Notwithstanding that the DQE of STEM Photomultiplier Detectors, which, in some cases, can approach 1, is generally higher relative to CTEM cameras (typical DQEs of 0.2 and 0.96 for scintillator-CMOS and direct detection CCD cameras respectively) and, for STEM, in principle there is an absence of a modulation transfer function (MTF) as data are recorded independently pixel by pixel (although this may fail at faster scan speeds (56). Sader et al. quantitatively compared bright CTEM and bright field STEM for the case of a moderately beam sensitive hydrous phyllosilicate clay mineral, vermiculite and concluded that whilst absolute contrast levels were higher for STEM, signal to noise ratios were lower (57).

Collection efficiency and signal to noise ratio can be improved through recent developments in STEM, (integrated) differential phase contrast (DPC) using a segmented detector as well as focused (or defocused) probe ptychography with a pixelated detector, where the signal to noise ratio is able to exceed CTEM phase contrast images (58, 59). Both DPC and ptychography have shown promise for the study of beam sensitive materials and imaging of light element containing materials at atomic resolution is possible due to the efficiency of electron collection at low probe currents, although to date the dwell times per pixel required for suitable frame readouts on MAPS and PAD detectors have remained in the 1-3 ms range

(60-62). These dwell times are significantly longer than those possible with the scanning hardware and currently restrict the beam currents for low dose applications using pixelated direct electron detectors. A further area at the interface between crystallography and imaging is the use of scanning electron diffraction techniques, where a <5 nm diffraction-limited STEM probe combined with direct electron detectors can yield imaging of beam sensitive materials at estimated fluences of a few $e^-/Å^2$ at 200 keV (62).

There may also be further benefits possible to using STEM over CTEM in terms of the ultimate DLR achievable. Potentially the use of a fast scanned focused STEM probe reduces charging of a sample and hence lowers the mechanical stress and specimen movement observed (either due to reduced charging itself or from reduced mechanical stress induced as a result of localised damage) (19). Furthermore, electron beam heating of the sample can be minimal in STEM, for example, steady state modelling of heat generation by a beam incident on an amorphous carbon film balanced against heat loss due to radial conduction and radiation by the film indicates that a 5 nA electron probe of 1 nm diameter only produces a temperature rise of 1.4 K (7). Note however that beyond a certain probe current threshold (depending mainly on the thermal conductivity of the specimen) thermal heating can increase diffusion and hence result in greater damage of organic specimens (63). Furthermore, although electron beam heating of the sample is generally assumed to be minimal, it is possible that the focused, high current density probe in STEM could cause less heating than a parallel beam (although this is thought to be dependent upon the specific probe diameter and current). Note that it has been shown that beyond a certain dose rate, thermal heating can increase diffusion and hence result in greater damage (63). Furthermore, the intermittent illumination of a scanned, focused STEM probe may allow damage recovery or healing processes to operate. Recent studies by Vandenbusche et al. (64) using pulsed, laser-driven electron beams in CTEM imaging of an alkane crystal have indicated that controlled delivery of dose, both in terms of numbers of electrons per pulse and the time between pulses, can lead to a reduction in irreversible structural damage.

The significant differences in electron flux between STEM and CTEM could also lead to changes in damage for a given irradiation fluence of a particular material. A schematic is provided (Figure 4A) which postulates how specimen damage rate might correlate to electron dose rate (or electron flux) (13). If damage has a direct dose rate dependence (such that radiation sensitivity increases with increasing dose rate to give an above linear response in Figure 4A), then a high dose rate may cause an appreciable temperature rise or significant electrostatic charging. The former will increase radiolytic efficiency or even result in thermal damage (65), while the latter can cause specimen movement or rupture, ion migration and even ion emission or hole drilling (66, 67). If however radiation damage is time dependent or diffusion-limited (as can be the case for radiolytic damage of insulating materials such as polymers and organic crystals (13, 14)), the relationship between dose rate and damage rate will be sub-linear and the curve can plateau at higher dose rates. In the case of low dose CTEM, imaging is usually carried out using an electron flux of $<10\ e^-/(Å^2 s)$. Comparatively for STEM, imaging and analysis is carried out with a much higher localised probe flux on the order of $10^8\ e^-/(Å^2 s)$ for a 1.4 Å probe size and 60 pA probe current, even when taking into account beam broadening. Note here that we are referring to the probe flux and not the average flux per pixel, where the latter is commonly reported and can be orders of magnitude smaller than the localised probe flux. If the rate of damage of a specimen is indeed diffusion limited, then it may be possible to use a higher electron flux (as in STEM) to obtain a better signal to noise ratio for a given level of damage. This analysis assumes that the flux in low dose CTEM corresponds to the linear portion of the schematic in Figure 4A and the localised STEM probe flux relates to the region at which the dose rate

versus damage rate curve plateaus and hence the damage per unit fluence (i.e. the gradient of the curve) is lower.

Here, we also note that damage is in fact delocalised around the STEM probe (Figure 4B), typically over a radius of 3-5 nm for the low energy electronic excitations responsible for radiolysis, which may suggest that an appropriate choice of probe size, specimen pixel size (the distance between scanned points) and also scanning patterns may reduce the effects of damage (68). That said, we note that low energy EELS spectroscopic signals are also being generated from this larger delocalised volume which may present opportunities for chemical spectroscopy whilst reducing the information obtained in STEM imaging.

>Figure 4<

Thus, controlling or limiting diffusion may limit the movement of the secondary products of inelastic electron specimen interactions that are so damaging. As discussed previously, specimen cooling is perhaps *the* most well established approach that has been shown to improve sample stability under the electron beam, enabling higher contrast and so improved resolution to be obtained. This effect is particularly strong in vitreous ice formed by plunge freeze specimen preparation that is discussed in more detail later. Alternatively, as shown in figure 5, Hooley et al. (22) compared the radiolytic damage of calcium carbonate nanoparticles (a moderately sensitive, direct-damage inorganic material) by both CTEM and STEM and concluded that hydrocarbon contamination induced by the focused STEM probe was important in mitigating damage, as has been noted by others (69-71). The build-up of hydrocarbon contamination may protect the specimen in two ways: by improving the conduction of electrons, thus reducing the extent of radiolysis or electrostatic charging by suppressing charge build-up in a similar manner to intentionally deposited carbon coatings (67); or by preventing the migration of molecular fragments and facilitating their recombination, meaning that there may be some structural damage without significant chemical change (72). Hooley et al. used the benefits of contamination to enable low dose bright STEM lattice imaging of calcite nanoparticles at 0.05% of the observed damage threshold for pore formation in these particles (Figure 6).

>Figure 5<

>Figure 6<

Low dose STEM approaches

As mentioned above, low dose STEM involves use of the following: (i) fast scanning of the focused probe by decreasing the pixel dwell time (currently the lower limits are around 0.5 µs/pixel); (ii) lowering the probe current and; (iii) increasing the specimen pixel size (by using a lower magnification). Buban et al. (73) used a combination of all three strategies to produce low fluence, noisy images of a strontium titanate lattice that showed periodicity in the Fourier transformed image. Fourier filtering a low dose image for these periodicities can in principle return lattice information (although this is not ideal as it leads to a synthetic processed image), alternatively it is possible to average a stack of undamaged, noisy images to increase the overall signal to noise ratio.

Non-sequential/randomized pixel scanning has also been reported to reduce damage via lowering dose accumulation effects (74).An extension of this approach which is employed to obtain low-dose STEM images is the use of compressive sensing methods (73, 75) employ

the concept that data can be represented in a sparse form and by recording a sub-sampled image (using appropriate blanking of the beam), the missing data can then be recovered using mathematical algorithms (76, 77) fluence that the sample is exposed to. There is some debate as to how this approach compares with simply averaging low dose noisy data; however this approach may offer a benefit in cases where alteration of the optics (e.g. lowering the probe current) is itself problematic, as the compressed sensing methods can be implemented simply by using add-on hardware and/or software.

A further technique that has been used to obtain high-resolution, atomic lattice information at low electron fluence is the formation of scanning Moiré fringes (SMFs) in STEM (78-80). Scanning Moiré fringes arise from the interference between the atomic plane spacings in a crystal lattice and spacings in a similarly sized reference lattice, produced by the scanning of the electron beam. The spacing of the Moiré pattern that is generated depends on the magnitude of the spacings in the sample and reference lattice and their relative misorientation (81). Effectively the Moiré pattern produces a magnified image of the crystal lattice, including any imperfections, and allows lower magnification acquisitions and therefore larger areas to be imaged at a lower electron fluence than would normally be required. S'ari et al. (54, 79) have applied this to molecular crystalline materials and highlighted the ability to image, albeit indirectly, crystalline defects and surface strain, as shown in Figure 7.

>Figure 7<

Analytical spectroscopy in the TEM

Analytical spectroscopy of beam sensitive materials using EDX and EELS to determine chemical composition and bonding is significantly more challenging than imaging or diffraction. For CTEM, in general, spatially localised information is achieved by focusing the beam using the condenser lens system that, in turn, leads to a higher fluence on the specimen and the associated risk of damage. Such damage may change both the chemical composition (via preferential mass loss from the illuminated volume) and the chemical bonding and, as previously discussed, may in itself be used to monitor the progression of damage. Energy filtered CTEM is possible by selecting particular ranges of inelastically scattered, energy loss electrons, although, for thin samples, the inherent signals are generally much lower than for elastically scattered signals which provide the information in images and diffraction patterns (82). Hence, overall, STEM methods are more suited for analytical spectroscopic mapping (known generically as spectrum imaging).

EDX spectroscopy in the TEM is inherently low efficiency since X-rays are emitted isotropically from the sample over a solid angle of 4π steradians. However, the advent of larger solid angle EDX silicon drift detectors (SDDs) and placing several detectors around a specimen have improved signal collection efficiencies (typically up to 1 steradian) and, if specimen damage can be mitigated, it is possible to chemically map beam sensitive specimens at the scale of a few nanometres (see Figure 9 below).

EELS, whilst in principle a more efficient technique in terms of signal collection, generally suffers from the problem of a small analytical signal on a relatively large background which increases drastically with specimen thickness. Preliminary studies on model organic crystals of theophylline using low fluence diffraction mode in CTEM have indicated the potential for monitoring radiation damage by both low loss and higher energy core loss EELS spectroscopy on beam sensitive materials (83). Li and Egerton (43) have shown that the radiation induced changes to the conjugated organic compound coronene are identified by

diffraction at lower doses than changes in molecular structure are identified by EELS. Inorganic oxides however can show electron beam induced changes in EELS fine structure at doses where changes in structure are not identified by STEM imaging (84). Freeman et al. (85) have demonstrated the ability to accurately measure an electron beam induced change in Fe(II)/Fe(III) ratio in green rust: a redox-active, mixed-valence layered double hydroxide using low dose EELS. Using both anaerobic sample transfer procedures and also cryo-TEM methods, electron fluences above 40 ± 5 $e^-/Å^2$ were shown to induce in-situ oxidation of Fe(II) to Fe(III) in green rust platelets without a change in structure being identified by electron diffraction. The advent of direct detection EELS systems (based on the same principle as direct detection cameras for imaging) should enable low fluence EELS measurements to become more commonplace in the future (86). A further interesting development in recent years has been the improvement in electron monochromator design and hence the improvements in associated EELS energy resolution (down to ca. 10 meV) that has allowed the recording of vibrational losses induced by the electron beam. Such ultra-low energy excitations may be significantly delocalised (to the order of nanometres), particularly in non-sample-penetrating geometries, and Rez et al. (87) have demonstrated the ability to record a vibrational EEL signature (analogous to an infra-red spectrum) from an organic crystal of guanine with the beam aloof from the crystal at a distance of a few nanometres. As this aloof distance is greater than that required to cause electronic excitation and hence radiolysis, this makes the vibrational EELS measurement effectively damage free.

Liquid Cell TEM/STEM

Recent developments have led to the emergence of liquid cells for (S)TEM, which allows for *in-situ* characterisation of materials in contact with liquids (such as nanoparticles or thin cross-sections produced by focused ion beam (FIB)-SEM methods; figure 8). Dynamic processes such as nanoparticle growth (88), crystallisation (89, 90) and some biological processes (91) have been captured by this technology. Recently so-called 4D liquid cell TEM has been shown to enable single particle reconstruction of the 3D morphology of the iron storage protein ferritin in water (92) and even the structure of water itself has been probed by (vibrational) EELS of water encapsulated between two sheets of boron nitride (93).

All liquid cell designs involve a microfluidic device and incorporate a membrane that prevents evaporation of the liquid sample in the microscope vacuum. This membrane is most often fabricated from thin (typically 10-50 nm) silicon nitride that can be functionalised with surfactants or plasma-treated to provide either a positively or negatively charged surface, or (depending on the liquid and nanoparticle type) either a hydrophobic or hydrophilic surface. Functionalisation may assist nanoparticle attachment to the membrane that aids the imaging process. However, issues associated with the use of a membrane include: alterations to the particle dispersion arising as a result of the membrane attachment process itself, charging of the membrane under the electron beam, and secondary electron production in the dense, solid membrane which could result in localized or enhanced radiolytic damage to either the sample or the liquid close to the membrane. Initial shortcomings in the design of liquid cell holders meant that it was difficult or even impossible to undertake elemental analysis, but these have now been overcome and Lewis et al. (94) have successfully demonstrated imaging and EDX analysis of a multi-component nanoparticle suspension in a liquid cell.

Besides issues with the membrane, the main artefact in liquid cell TEM analysis arises from beam-induced effects that occur in the liquid suspension. For aqueous samples, the radiolysis of water leads to the production of hydrated electrons (e_h) and OH•, H• and H_2• radicals. This is a rapid process occurring within 10 ps of energy transfer between the incident electron and a water molecule. Additional reactions of these radiolysis products cause further damage products to form; H_2O_2, H_3O+ and H_2O• (12). These damage products can then diffuse in solution and undergo reactions with each other and any surrounding molecules or particles in the liquid (95). These effects can lead to beam-induced charging of both the membrane and the sample, gas bubble formation, pH changes (in the absence of competing processes, the increased level of H_3O+ may cause a decrease in the solution pH), increases in the ionic strength of the solution and even changes in chemistry of the material under investigation. Furthermore, mass transport of species produced by radiolysis outside the irradiated volume can also occur. These damage mechanisms can be advantageous, in principle, for investigating and driving dynamic processes (96). However, they are highly detrimental when aiming to accurately characterise a starting solution and any suspended products. A continuous slow flow rate of liquid can partially mitigate these radiolytic effects, but they can all severely affect the dispersion state and chemistry of a nanoparticulate suspension, potentially causing heterogeneous nucleation of particles on the membrane, particle agglomeration (including self-organisation of particles on the membrane), aggregation, etching and growth of nanoparticles, or even dissolution.

All of the events described above can have severe implications for studying dynamic processes (e.g. crystallisation or dissolution) within a dispersion or solution using liquid cell TEM, as any changes in pH, particle charge or concentration including supersaturation can severely modify behaviour. Abellan et al. (96) have discussed the factors influencing quantitative imaging in liquid cells and Schneider et al. (12) have produced a predictive model to calculate the radiolysis effects in water under a given set of microscope beam conditions. Typically dose rates have to be kept at or below MGy per second to avoid unwanted alteration (97), although consideration should be given to the effects of different imaging modes, i.e. global irradiation in CTEM versus local irradiation in STEM - generally observation by STEM appears to be advantageous relative to CTEM, possibly because intermittent scanning allows recombination of damage products in aqueous media. Electron beam-induced alteration of aqueous-based suspending media will occur even at relatively low electron fluence (<100 $e^-/Å^2$) (96). It has been established that under STEM conditions operating at an averaged flux per pixel/frame close to 140 $e^-/(Å^2 s)$, nanoparticle clusters can break apart and move out of the field of view in seconds due to alterations of their surface charge induced by radiolysis products generated in the suspending solution (98).

A potential option here is to measure the chemical kinetics (of, for example, a nucleation, growth or dissolution process) as a function of electron fluence and then extrapolate to ultra-low fluences. Alternatively, chemical scavengers can be used to mop-up reactive radical species created during radiolysis (96) or non-polar liquids can be employed which are less susceptible to radiolytic damage than polar liquids.

Apart from changes to local chemistry in the liquid cell (S)TEM arising from radiolysis, a further issue to consider is that associated with the volume confinement of the liquid which may potentially influence fluid mixing and alter chemical reaction pathways relative to macroscale environments. Indeed, the induction time, polymorph selection, shape evolution and composition for the crystallisation of calcium carbonate (99) and calcium sulphate (100) are known to differ significantly when in confinement (101, 102), possibly as a result of changes in ion flux from solution due to differences in diffusion and ion incorporation rates (103).

Similar microfluidic devices can be used to provide in-situ gaseous environments in the TEM allowing the study of gaseous-solid interactions up to gas pressures of typically a few bar (104), however here the damage issues are somewhat reduced relative to liquids due to the much decreased gas density. Alternatively, differential pumping around the sample can be used to allow direct gas injection into the column (so called true environmental TEM), however here pressures are limited to the order of mbar.

>Figure 8<

Cryogenic-CTEM/STEM

An alternative to liquid cell TEM is the preparation of frozen, hydrated suspensions of a material which are transferred and imaged in the TEM at temperatures of < -165ºC (referred to as cryogenic (cryo-)TEM). A sample is blotted onto a TEM grid and plunged rapidly into a cryogen, commonly liquid ethane, such that the suspension is captured in a thin layer of electron transparent, vitrified ice. Rapid vitrification of the liquid ensures the sample is captured in its native state, i.e. without re-dispersion or crystallization of the suspending solution or vacuum-induced drying of any hydrated surfaces. Time-resolved spraying (at a time resolution of tens of milliseconds) of dispersions onto TEM grids is also possible (105). Cryo-TEM (almost exclusively CTEM) is most commonly associated with the determination of three dimensional biomolecular structures at near-atomic resolution (106), either by single particle analysis (essentially viewing many different projections of identical objects) or by tomographic methods (i.e. tilting a single object over a range of projections), both of which have undergone significant advances in recent years. However, cryo-TEM has also been used to investigate liquid crystal structure (107) and the ordering of aggregates of gold nanoparticles within organic solvents (108).

There are significant benefits of cryo-TEM over liquid cell TEM. Firstly, the reduced temperature of vitreous ice relative to liquid water will lower the rates of some, if not all of the reactions involved in the electron beam-induced damage mechanisms (109, 110), therefore reducing the rate of radiation damage of both the suspending media and the sample. In addition, radiolysis of water is a diffusion-limited process (95), and the diffusion rates in an amorphous solid held below the glass transition temperature (-137ºC for water) are orders of magnitude slower than in the liquid (111). Reactive species (e.g. e_h, OH•, H•, H_2• H_2O_2, H_3O^+ and H_2O•) produced during radiolysis in vitreous ice at -165ºC will diffuse more slowly than in liquid water, reducing secondary damage to the vitreous ice (112). Furthermore, provided the suspending ice remains structurally intact, the native dispersion of the material should remain unaltered (there is always physical movement of a specimen in vitreous ice during the beginning of irradiation and further charge accumulation in the ice can cause unwanted specimen movement and loss of contrast at atomic lattice resolution, however recent work shows this can be minimised to only a few percent of information transfer if a conductive support is used (113, 114). The agglomeration of nanoparticles dispersed in cell culture media has been reported using this technique (115) and flattening of nanoparticle agglomerates by the blotting process, pre-plunge freezing can even be accounted for (116). Cryo-TEM also removes any compositional drying artefacts as well as preventing physical movement that occurs during conventional drop cast TEM sample preparation (117). A variation on this technique involves plunge-freezing a particulate dispersion followed by vacuum sublimation of the vitrified ice so as to study snapshots of dynamic behaviour (115) such as agglomeration and also nucleation and growth processes during crystallisation. The latter approach has been used to highlight nucleation processes which follow non-classical pathways (118).

Ilett et al. (119) undertook a direct comparison of cryo-TEM and cryo-STEM methods. Bubbling of the vitreous ice caused by the electron beam was shown to occur at far higher electron fluences in STEM (<2000 $e^-/Å^2$) compared to CTEM (<100 $e^-/Å^2$) resulting in unwanted movement of entrapped nanoparticles. Bubble visibility is dependent on both breaking of bonds in the water, the diffusion rate of hydrogen within the solid layer and the nucleation of a bubble and so is not itself a primary metric of radiation damage in the ice, however STEM did produce less movement of entrapped nanoparticles than CTEM (Figure 9a). They also demonstrated the possibility for STEM/EDX elemental mapping of samples in vitreous ice. This opens up the exciting possibility for studying the structure, chemistry and potentially dynamics of solid/liquid interfaces at high spatial resolution, Figure 9b.

>Figure 9<

Conclusions

The native state analysis of complex, beam-sensitive materials by transmission electron microscopy firstly requires an understanding and characterisation of the dominant electron beam-induced damage mechanism in the sample under study. In those materials for which radiolysis dominates, it is clear that beam-induced damage to a specimen cannot be eliminated or switched off and so analysis has to be done within a dose budget to achieve an optimum dose-limited resolution. Here thought should be given to the sample thickness, the mode of operation of the microscope (STEM versus CTEM), the detectors, as well as the microscope operating parameters such as accelerating voltage. This understanding can then be carried forward for *in-situ* analysis of samples interacting with liquids and gases provided the electron beam-induced alteration of these latter phases are controlled or used to drive a chosen reaction. Finally, we stress that cryo-TEM still has a significant place for benchmarking dynamic processes at higher resolution.

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Figure 1. (a) Diffraction series of theophylline form II along the <100> zone axis using an electron flux of 0.098 $e^-/(Å^2 s)$ and at 200 kV. (b) Plot of the logarithmic normalised spot intensity versus total electron fluence for the {004} and {011} diffraction spots. The CF is measured at 1/e of the initial spot intensity which in this case is approximately 11 $e^-/Å^2$ and 23 $e^-/Å^2$ for the {004} and {011} spots respectively. (c) Comparison of experimentally determined critical fluence values relative to standard conditions (SC: 200 kV, room temperature and continuous carbon support film) for theophylline form II arranged in order from lowest to highest (least stable to most stable). Values with units of kV refer to electron beam accelerating voltages, while values with units of K refer to sample temperatures. GF stands for graphene film substrate respectively.

Figure 2. Predicted critical fluence (CF) of 20 different pharmaceutical compounds, to calculate the predicted CF a multiple-linear regression equation was created using different chemical parameters that related to the chemical structures such as number of hydrogen bond donors/acceptors and number of conjugated carbons/non-conjugated carbons. The points shown in red are two of the compounds that were poorly predicted and the data point for tolnaftate is not shown due to the experimental CF being twice as large as the next highest compound. Adapted from (40).

Figure 3: HRTEM of a crocidolite needle (low magnification image in (c) shows the magnified area and SAED pattern), used as a model system to determine which electron flux and electron fluence regime would be best suited for clearly resolving lattice information at low dose. Images (a), (d) and (g) were captured using an electron flux of 1 $e^-/(Å^2s)$ and show the same area of the crocidolite crystal but are exposed for a total electron fluence of 1, 10 and 100 $e^-/Å^2$ respectively. Images (b), (e) and (h) were captured using an electron flux of 10 $e^-/(Å^2s)$ at a total electron fluence of 1, 10 and 100 $e^-/Å^2$ respectively. Finally, (f) and (i) were captured using an electron flux of 100 $e^-/(Å^2s)$ at a total fluence of 10 and 100 $e^-/Å^2$ respectively. Inset in images (a) - (i) is the corresponding FFT with visible lattice points highlighted. The bottom panel shows HRTEM of two different areas (j) and (m) from the same furosemide crystal acquired using an electron flux of 10 $e^-/(Å^2s)$ and total fluence of 10 $e^-/Å^2$. The corresponding FFTs are shown in (k) and (n) and filtered FFTs showing the lattice fringes more clearly are shown in (i) and (o). Adapted from (54).

Figure 4: (A) A schematic illustrating the differences in the relationship between damage and dose rates for different samples. It is postulated that diffusion limited processes correspond to a power law relationship with exponent <1 (blue line). For such samples high flux STEM would be preferential in reducing the damage rate. In comparison samples that exhibit a *direct* dose v damage relationship (green line) tend to exhibit more damage at higher dose rates and CTEM is predicted to be more advantageous. The schematic in (B) illustrates the concept of beam broadening and delocalised damage in STEM. The predicted minimum delocalised damage is typically a few nm, suggesting a pixel size > a few nm would be preferential to avoid over-sampling. Adapted from (13, 119).

Figure 5. Left Panel: a comparison between high resolution 300 kV CTEM (1-3) and STEM (4-9) imaging of calcite nanoparticles. STEM BF imaging was able to detect a lattice at higher fluences than was possible in CTEM. However, this was found to be due to the contamination deposited during STEM imaging, as when the contamination was removed (7-9), STEM imaging caused more severe specimen degradation

than CTEM, converting the specimen to calcium oxide at a lower fluence. Right Panel: Comparison of O:Ca atomic ratios extracted from time-resolved CTEM EDX spectra from both a contaminated and (cleaned) uncontaminated calcite nanoparticle specimen. Adapted from (22).

Figure 6. 300 keV bright field STEM image of a calcite nanoparticle showing contamination and also 1.6 Å calcite (122) crystal lattice measured with a 1 pA probe current and a fluence per pixel of 165 $e^-/Å^2$ which is 0.05% of the measured 300 kV damage threshold. Note 25% electron collection efficiency due to non-reciprocal conditions used.

Figure 7. (A) Schematic showing example moiré fringes generated from a scanning lattice (ds) and crystal lattice (dl) of a similar size being overlaid at different ds / dl ratios and rotations. (B) Example of a scanning moiré fringe image of furosemide using ds of 1.32 nm and dl of 1.50 nm (C) Magnified area from the area highlighted in (B) showing a defect within the furosemide crystal. Adapted from (79).

Figure 8. Schematic of typical liquid phase TEM holder (a) and cell (b). Silicon nitride windows are typically 200 µm x 50 µm x 50 nm. An example of using liquid phase TEM to follow the hydration of calcium sulfate in aqueous solution is shown in (c) and (d); bassanite rods in (c) transform to larger gypsum crystals (d).

Figure 9: A comparison between cryo-CTEM and cryo-STEM indicated far less bubbling was observed in STEM. CTEM images were captured using a definitive electron fluence and showed that at 100 $e^-/Å^2$ (A) little bubbling in the vitreous ice was observed, but after exposure to 400 $e^-/Å^2$ (B) significant bubbling in the ice occurred. In comparison HAADF STEM images taken using 300 $e^-/Å^2$ (C) and after exposing to a further 1000 $e^-/Å^2$ (D) indicated much less bubbling of the ice. The inset in (D) indicates more clearly the bubbling of the vitreous ice shown by the white arrow. Cryo-analytical STEM has been used to characterise a calcium phosphorus rich corona around $BaTiO_3$ nanoparticles suspended in cell culture media (E-F). STEM/EDX maps are shown in (G) for Ti Kα (blue), Ba Lα (red), P Kα (green), Ca Kα (yellow), Mg Kα (turquoise) and Na Kα (pink) and indicate Ca and P spatially resolve to the position of the coating. The EDX data was acquired using a total electron fluence of 1900 $e^-/Å^2$. Adapted from (119).