Wet-STEM Tomography: Principles, Potentialities and Limitations

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Abstract: The characterization of biological and inorganic materials by determining their three-dimensional structure in conditions closer to their native state is a major challenge of technological research. Environmental scanning electron microscopy (ESEM) provides access to the observation of hydrated samples in water environments. Here, we present a specific device for ESEM in the scanning transmission electron microscopy mode, allowing the acquisition of tilt-series suitable for tomographic reconstructions. The resolution which can be obtained with this device is first determined. Then, we demonstrate the feasibility of tomography on wet materials. The example studied here is hydrophilic mesoporous silica (MCM-41). Finally, the minimum thickness of water which can be detected is calculated from Monte Carlo simulations and compared with the resolution expected in the tomograms.

Key words: wet-STEM, tomography, environmental, Monte Carlo simulation

INTRODUCTION

Tomography has become a key characterization tool in materials science as well as in biology. The principle of tomography is based on the acquisition of a series of projection images at different tilt angles, and on the calculation of the three-dimensional (3D) reconstructed volume (tomogram) using dedicated algorithms. Several tomography techniques are available, using different types of radiations, depending on the observation scale. X-rays are currently used for the 0.5 μm–1 mm resolution level (Maire et al., 2001) and the 3D characterization of nanoscaled structures requires transmission electron microscopy (TEM) tomography (Koster et al., 2000) or an atom-probe approach (Blavette et al., 1993). At the mesoscopic scale, corresponding to a resolution level between 10 and 500 nm, scanning electron microscopy (SEM)-based techniques—such as focused ion beam (FIB) (Kubis et al., 2004) or serial block face SEM (Mancuso, 2012)—use a slice-and-view method to directly obtain slices of the materials volume. Performing electron tomography in the environmental scanning electron microscopy (ESEM), with a scanning transmission electron microscopy (STEM) detector placed under the tilted sample has also been proposed (Jornsanoh et al., 2011). At this stage, one can mention the specificity of ESEM, for which a partial pressure of gas is introduced in the sample chamber (Nelson, 1988). Whereas the gas molecules amplify the signal for secondary electron detection (Fletcher et al., 1997), they are used only to neutralize the excess charges when using a STEM detector. However, it has been shown that using water as the environment, with a partial pressure around 5 torr, and maintaining the sample temperature to 2°C, it was possible to observe wet samples inside the ESEM (Donald, 2003).

The observation of wet samples requires dedicated specimen holders or microscopes, either in TEM or SEM. Whereas the first system in TEM was composed of an open environmental chamber (Ruska, 1942), it seems that the use of sealed liquid cells has become predominant for TEM observations, since it allows good stability—no evaporation or condensation—and controlled thickness of the water film (de Jonge & Ross, 2011). STEM-in-TEM observations on sealed cells have for instance allowed the observation of live eukaryotic cells (Peckys & de Jonge, 2011) and investigation of the movement of nanoparticles in liquid (Ring & de Jonge, 2012). Yet, tilting the sample to perform tomographic acquisition would probably be hindered by the geometry of the sealed cell. Indeed, at increasing tilt angles, the electrons have to pass through an increasing thickness of SiN (walls of the sealed cell) in addition to the increasing thickness of the water film. The thick support, surrounding the electron transparent window, may also hide the region of interest when tilting the sealed cell. In ESEM, the presence of the gaseous environment and control of the sample temperature have also permitted imaging of nanoparticles in liquid with nanometer resolution, through STEM-in-SEM observations (Bogner et al., 2005). Because of the absence of a sealed cell, the thickness of the water film remains unknown and variations can lead to contrast inversions (Bogner, 2006). On the contrary, its main advantage lays in the fact that...
water condensation or evaporation can be tuned by varying the environmental pressure, which enables in situ hydration/dehydration experiments.

In this paper, we describe the development of a new device for electron tomography in the ESEM, which enables the acquisition of image series on wet samples. After a brief description of the device, the best achievable resolution will be determined on dry volumes. Then, the specificities of a 3D characterization of hydrated samples will be discussed. In particular, the influence of the size and stability of water on parameters such as the number of projections or choice of the reconstruction algorithm will be discussed. Finally, Monte Carlo simulations will be used to estimate the minimum quantity of water which can be detected.

**Materials and Methods**

The wet-STEM tomography device, shown in Figure 1, was designed according to the literature (Gauthier et al., 2006; Jornsanoh et al., 2011). The device consists of four main parts: (a) a tilting system ensured by a rotating piezoelectric system, (b) a two-translation piezoelectric system to position the area of interest at the eucentric position and keep it in the field of view while tilting, (c) a sample holder, which can be either a cylindrical tip or tweezers, and (d) a detection system. A Peltier stage, not shown in Figure 1 but schematically represented by a dashed rectangle, is placed vertically between the piezoelectric elements and the sample. The cold transfer by conduction is ensured from the Peltier stage to the sample holder by slight friction. A thermocouple is inserted in the Peltier stage. The sample temperature is then controlled through the controller of the FEI conventional Peltier stage.

The wet-STEM tomographic device was placed in a XL-30 FEG ESEM from FEI, operating at 30 kV. The probe current was estimated to be equal to 240 nA for a 8 nm probe size (spot size 3). A gaseous secondary electron detector (GSED) was placed to perform the first observations, then a backscattered electron detector placed under the sample (Fig. 1d, detection system) was used for the STEM observations. As the presence of gas in the specimen chamber results in a broadening of the incident electron beam, known as the skirt effect, a pressure limiting aperture cone was placed on the objective lens to reduce the loss of resolution due to the skirt effect by decreasing the distance the incident electrons have to travel in the gaseous environment (Danilatós, 1993).

A 1 μL aqueous suspension of gold/polysiloxane nanoparticles (Martini et al., 2010) was directly deposited onto a 300-mesh copper TEM grid covered with a holey carbon film. The pumping step, sample temperature, and water pressure were optimized to prevent evaporation of the suspension droplet in the microscope. Then, the water pressure was decreased until the droplet was thin enough to obtain STEM images. Powders of Mobil crystalline materials (MCM-41; Trewyn et al., 2007) were directly deposited on a 300-mesh copper TEM grid covered with a holey carbon film. At the beginning of the experiment, the MCM-41 grains were in the dry state. Then, the water pressure was slowly increased so that water condensed onto the MCM-41 grains and the holey carbon film. Each time, the working distance was set to 11 mm and the sample-to-detector distance was 10 mm. With such a configuration, the electrons collected were those which had been scattered by the sample with angles ranging between 14 and 40°. 1,290 × 968 images were acquired in the STEM mode at different tilts, the tilt angles ranging between 14 and 40°. Monte Carlo simulations were performed with the software Hurricane® from SAMx. A scheme of the geometry covering

Figure 1. Device for wet-STEM tomography in the ESEM, composed of (a) a rotation and (b) two translation piezoelectric elements; (c) a sample holder and (d) a STEM detector placed below the sample. The dashed lines represent the position of the Peltier stage.
the copper grid was considered thin enough and was neglected in the simulations. Moreover, as the MCM-41 grains are hydrophilic, it was assumed that water preferentially condensed onto MCM-41 and not onto the carbon film. As a consequence, a single homogeneous water layer (H$_2$O, density 1 g/cm$^3$) was placed on the top of the MCM-41 film. The paths of 100,000 electrons were calculated. The collection angles were chosen to range between 14 and 40°, in accordance with the experimental geometry. Thus, the number of collected electrons refers to the number of electrons which passed through the water-MCM-41 computation box and which scattered with angles ranging between 14 and 40°.

RESULTS AND DISCUSSION

Best Achievable Resolution

Figure 3a displays an image of gold/polysiloxane nanoparticles. Such nanoparticles exhibit a gold core of about 5 nm (Martini et al., 2010), which will be used to probe the resolution of the tomographic device. A typical image obtained in bright field TEM is shown in Figure 3b. Initially, in a suspension in water, their morphology is not greatly affected by water evaporation. In this section, we will thus probe the intrinsic resolution of STEM in SEM coupled with the mechanical stability of the tomographic device.

Figure 2. Geometry used for Monte Carlo simulations. Electron Beam 1 nm; Collection angles between 14 and 40°. The dimensions of the MCM41 box have been chosen to limit the number of electrons escaping the sides of the box.

Figure 3. a: Typical image of gold/polysiloxane nanoparticles in suspension in water, at tilt angle equal to 0° (acceleration voltage 30 kV, probe size 3; magnification 100,000; H$_2$O pressure 5.6 torr; temperature 2°C; pixel size 0.9 nm); (b) TEM bright field image of the nanoparticles, from (Martini et al., 2010); (c) central section 3D view of the reconstructed volume. The gold cores have been segmented and are represented in red. d: Results of the FSC analysis. Resolution ≈1 nm (0.5 threshold), actually limited by the probe size.
As the STEM detector collects scattered transmitted electrons, the gold core appears brighter than the polysiloxane shell. The gold core diameter is measured to be equal to 8 nm, which is the diameter of the probe size used for the observations. Care was taken during the pumping procedure to keep the water pressure well above the dew point. Moreover, during the first observations, the water pressure was slowly decreased from 8 to 5.6 torr, to reach a steady state. Despite this strict protocol, the thickness of the water film cannot be measured and because of high surface tensions, the nanoparticles may be in the dry state. Nevertheless, the nanoparticle gold core is a good probe of the resolution which can be obtained best with our tomographic device.

The tomogram, calculated with a SIRT algorithm from a projection series of 41 images, with a tilt step of 2° (tilt range ±40°), is presented in Figure 3c. For better clarity, the gold cores have been segmented and are highlighted in red on the tomogram displayed in Figure 3c. The polysiloxane shells seem quite flat, which is undoubtedly an artifact of irradiation damage. Indeed, it has been shown that polysiloxane molecules undergo crosslinking (Si-O-Si chains) under irradiation. This has previously been shown to induce a collapse of the pores in open cell foams (Huang et al., 2002). When comparing the images at a tilt angle of zero before and after the acquisition of half the tilt series, the polysiloxane shells have indeed spread out. Nevertheless, this did not affect the position of the gold cores, which have been successfully reconstructed.

The resolution in the tomogram has been determined by FSC (Harauz & Van Heel, 1986) by comparing the tomograms calculated from even and odd projections, see Figure 3d. The criterion chosen is 0.5 since the other criteria (σ, half-bit) cannot apply in this case by lack of intersection. With the 0.5 criterion, the FSC resolution in the full
tomogram is estimated to be equal to 1 nm. However, it has to be mentioned that the curve does not yield a meaningful result since it does not drop to zero at high frequencies, most probably because of vibrations arising from water vibrations in the Peltier stage, leading to horizontal noise in the images. Nevertheless, the 0.5-FSC resolution is close to the pixel size (0.9 nm), but is actually less than the experimental probe size (8 nm) and thus has no physical meaning. As a consequence, it is concluded that the resolution is not limited by the wet-STEM tomography device capabilities, but by experimental conditions such as the probe size.

Specificities of the Acquisition of Tilt Series on Samples Containing Water

Figure 4 displays raw images of MCM41, a silica mesoporous material at two different hydration states. MCM41 is an electron-resistant material. Due to its hydrophilic behavior, water will preferentially condense onto MCM41 rather than onto the carbon film. This experiment will therefore be used to prove the concept of wet-STEM tomography, i.e. tomography on wet materials.

The observation scale, chosen as a function of the grain size, does not permit observation of the pore arrangement in the MCM41 grains. Indeed, with this magnification, 1 pixel represents 13 nm whereas the pore diameter is in the 5 nm range. In the wet state, a droplet of water condensed close to the MCM-41 grain (see the top of Fig. 4e). Moreover, when comparing Figures 3b and 3e, the smallest features on the wet MCM-41 grain seem to be blurred, and the contour of the grain is broadened. The disappearance of small features is clearly visible in Figure 4g, which represents variations in the grey level along a line in the images at tilt equal to 0° (Figs. 4b, 4e). The water pressure increase cannot lead to such a loss of resolution, since the skirt effect is limited by the use of a cone on the GSED located at the bottom of the objective lens. Therefore, the changes between the dry and wet states are attributed to the presence of water, as expected from the hydrophilic behavior of MCM-41. Collapse of the pore structure of MCM41 has previously been shown to occur when exposed to liquid water (Zhao et al., 1998) or even water vapor (Ribeiro Carrott et al., 1999). In our case, on the basis of images before and after the acquisition of the tilt series, it will be assumed, at our observation scale, that morphology of the MCM41 grain is not altered by water.

Attempts have been made to investigate the stability—in size and shape—of the water droplet during tilting. Whereas the droplet shape seems to be rather stable, irradiation damage and/or temperature instabilities induce undesirable evaporation of the water droplet (not displayed). The electron dose received by the sample was thus reduced by using a low-dose-like acquisition: the focus was set outside the region of interest, which was irradiated only during the acquisition of the tilt series. With an acquisition time of 3.36 ms per line, corresponding to 3.25 s per image, the number of projection series had to be reduced to 13 to minimize the water evaporation. Taking into account a pixel size of 13 nm, this corresponds to a dose received equal to 48 C/m²—or 300 e⁻/nm²—after acquisition of the last image. Moreover, in order to reduce artifacts in the tomogram (Weyland, 2002), the tilt range was kept as large as possible (±50°), while the tilt step was increased up to 10°. Despite such acquisition conditions, it can be seen on the top of Figure 5d (the last image of the tilt series) that the water droplet partially evaporated. Nevertheless, the tilt series was kept for tomographic reconstructions. Indeed, the hydrophilic behavior of MCM-41 makes the evaporation of water from the MCM-41 grain more difficult than the evaporation of water from the carbon film. It will thus be assumed that the MCM-41 is still in the wet state.

The tomogram of wet MCM-41, calculated with conventional algorithms, contains a lot of artifacts, as expected due to the large tilt step. Denoising algorithms such as BgART have been found to improve the reconstructed
Figure 6. (a) and (b) segmented reconstructed volume calculated from the projection series presented in Figure 4d–4f (tilt range ±50°, tilt step 10°), and using a Total Variation Minimization algorithm. Tilt angles similar to those of the projection series presented in Figure 4(c) YZ, (d) XY and (e) XZ orthoslices. The overall shape of the MCM41 grain and the water droplet are resolved in the tomogram.
volume (Messaoudi et al., 2013). BgART is an iterative reconstruction algorithm that removes the noise, considered as Gaussian white, in the background preserving the object signal. The reconstruction at step \( n \) is thresholded using \( \text{mean} + k \times \sigma \), where mean and \( \sigma \) are the average voxel value and standard deviation of the reconstruction, respectively, and \( k \) a user-defined factor describing the level of noise. Values below threshold are put to mean value and values above threshold are kept identical. An iteration of the reconstruction process is then applied. At the end, the background is uniform while the object keeps its intensity values. Figure 5 displays the tomogram obtained with the ART algorithm after BgART denoising. Unfortunately, the improvement of the signal-to-noise ratio does not compensate the fact that the tilt step is too large, as the XZ slice still contains a lot of artifacts.

Algorithms such as compressides sensing (Leary et al., 2012) or total variation minimization (TVM) (Goris et al., 2012, 2013) give more reliable tomograms from tilt series containing a limited number of projections. These algorithms are real-space iterative reconstruction techniques which assume that the object to be reconstructed has a sparse variation, meaning that only sharp gray value transitions in the reconstructed object are allowed. The TVM algorithm, used below, attempts to find a solution to the reconstruction problem by minimizing the total variation. A way to implement this constraint is by minimizing the norm of discrete gradient (i.e., total variation) of the reconstructed image and minimizing the projection distance simultaneously between the reconstructed object and original projections. Figure 6 shows the tomogram obtained by TVM. The overall shape of the MCM-41 grain, as well as the water droplet and the small features at the bottom of the MCM-41 grain in Figure 4e are resolved in the tomogram. The resolution in the tomogram may not be determined from FSC, because odd and even tilt series would contain very few images. Nevertheless, the smallest features in the tomogram are about 40 nm, which indicates that the resolution is \( \geq 40 \) nm.

**Numerical Determination Of The Minimum Water Layer Thickness**

In this section, we discuss the results of Monte Carlo simulations on a computation box containing a thin film of MCM-41 of varying thickness, eventually covered by a layer of water, also of varying thickness. Figure 7a displays the number of collected electrons as a function of the MCM-41 thickness. The curves for different water layer thicknesses all have the same overall shape, with a maximum when the MCM-41 thickness is equal to 2 \( \mu \text{m} \). For MCM-41 grains thinner than 1 \( \mu \text{m} \), the number of electrons increases since an increase in the sample thickness leads to an increase in the number of scattering events and thus the scattering angles. On the contrary for MCM-41 grains thicker than 2 \( \mu \text{m} \), the increase in the number of scattering events still leads to an increase in the scattering angles, which will exceed the maximum collection angle. Interestingly, at small MCM-41 thicknesses (below 1 \( \mu \text{m} \)), a slight shift of the curves is observed, see Figure 7b, which is the result of the increasing thicknesses—MCM-41 and water—the electrons have to pass through.

Based on the variations of the number of collected electrons, and considering that during the experiments the detector brightness and contrast are adjusted in the dry state and remain unchanged during water condensation, contrast can be defined as \( \frac{n_{\text{wet}} - n_{\text{dry}}}{n_{\text{dry}}} \) where \( n_{\text{wet}} \) and \( n_{\text{dry}} \) are the numbers of collected electrons with and without water, respectively.
Variations in the contrast are shown in Figure 8. When arbitrarily setting a contrast detection limit ≥5%, a maximum MCM-41 thickness can be defined for each water layer thickness. The values are summarized in Table 1.

Similar studies have been performed to determine the maximum sample thickness required to detect layers of water of 10, 20, 30, and 50 nm thick. The materials included in the simulations were SBA-15 (another mesoporous silica), carbon, dense silica, calcium carbonate, titanium oxide, and alumina. The results are presented in Table 1. Linear regressions have been investigated between the sample and the water thickness. The coefficients of determination greatly differ from one material to the other. Using a F-test for regression, it is possible to state whether the coefficients of determination are significantly different from 0 or not. In such a test, the critical value of the F distribution is equal to 18.5 when considering 4 points, and a level of significance of 95%, which corresponds to a critical determination coefficient of 0.9025. It can therefore be concluded that the coefficients of determination are significantly larger than zero for MCM-41, pure silica, and titanium oxide, only. When investigating linear relationships between the sample thicknesses and densities, for fixed water thicknesses and with a significance level of 95%, the coefficients of determination are far lower than the critical value, 0.5692. It is thus concluded that for a given water thickness, the maximum sample thickness enabling the detection of this water film thickness does not depend on sample density. This conclusion is confirmed by another statistical test, an analysis of variance of the sample thickness, with two factors—the sample density and the water thickness. With a level of confidence of 95%, it is found that the maximum sample thickness depends on the water thickness but not on the sample density.

Since the maximum sample thickness which permits detection of a given thickness of water does not depend on the sample density, the average sample thickness has been calculated for each water thickness, see Table 1. For each average sample thickness, it is possible to estimate the resolution which can be obtained during a tomography experiment. The Crowther’s criterion states that the lateral resolution \( d \) will be equal to \( \pi D/N \), where \( D \) is the sample thickness and \( N \) the number of projections (Crowther et al., 1970). According to the Crowther’s criterion, resolution in the tomograms has been estimated for each water thickness with sample thicknesses equal to the previously determined average sample thicknesses and tilt steps of 10°. Interestingly, for one sample thickness, the values found from the Crowther’s criterion are of the same orders of magnitude as the minimum water thicknesses. The slight differences observed could easily be explained by the fact that the Crowther’s criterion considers the resolution in 3D, whereas the sample thicknesses have been determined from Monte Carlo simulations in 2D. In conclusion, it is expected from Monte Carlo simulations that the thickness of the water film which can be detected during wet-STEM tomography experiments will not significantly depend on the sample density but will rather be determined from the tomography experimental conditions (sample thickness, tilt step).

CONCLUSIONS
We have demonstrated in this work the feasibility of tomography on wet samples observed using ESEM with a dedicated device. Mechanical vibrations and tilt positioning do not limit the resolution in the tomogram. Indeed, it has to be measured equal to a few nm on gold/polysiloxane core-shell nanoparticles—most probably in the dry state. Tilt series of MCM41 grains in the wet state were acquired and the experimental conditions were optimized to reduce water evaporation and irradiation damage. The choice of the reconstruction algorithm was discussed. Moreover, Monte Carlo simulations have shown that the minimum quantity of water which could be detected in tomograms did not depend on the materials composition but could realistically be estimated through

<table>
<thead>
<tr>
<th>Materials</th>
<th>Density (g/cm(^3))</th>
<th>10 nm H(_2)O</th>
<th>20 nm H(_2)O</th>
<th>30 nm H(_2)O</th>
<th>50 nm H(_2)O</th>
<th>Determination Coefficients</th>
</tr>
</thead>
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<tr>
<td>MCM-41</td>
<td>0.9</td>
<td>50</td>
<td>150</td>
<td>200</td>
<td>500</td>
<td>0.9657</td>
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<tr>
<td>SBA-15</td>
<td>1.19</td>
<td>240</td>
<td>–</td>
<td>230</td>
<td>230</td>
<td>0.75</td>
</tr>
<tr>
<td>Carbon</td>
<td>2.27</td>
<td>150</td>
<td>200</td>
<td>240</td>
<td>230</td>
<td>0.6349</td>
</tr>
<tr>
<td>Silica</td>
<td>2.65</td>
<td>220</td>
<td>290</td>
<td>340</td>
<td>400</td>
<td>0.9668</td>
</tr>
<tr>
<td>CaCO3</td>
<td>2.71</td>
<td>220</td>
<td>290</td>
<td>320</td>
<td>330</td>
<td>0.7475</td>
</tr>
<tr>
<td>TiO(_2)</td>
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<td>220</td>
<td>290</td>
<td>340</td>
<td>400</td>
<td>0.9568</td>
</tr>
<tr>
<td>Al(_2)O(_3)</td>
<td>4.23</td>
<td>220</td>
<td>250</td>
<td>260</td>
<td>260</td>
<td>0.6385</td>
</tr>
</tbody>
</table>

The determination coefficients are calculated when considering linear regressions of the sample thicknesses in function of either their densities or the water thickness. The resolution determined using the Crowther criterion is also reported, with the sample thickness equal to the average value for each water layer thickness. With a 95% confidence level it was found that the maximum sample thickness depends on the water thickness and not on sample density. For one sample thickness, the values found from the Crowther’s criterion are of the same orders of magnitude as the minimum water thicknesses.
Crowther’s criterion. The detection of smaller thicknesses of water therefore requires working with thinner samples and/or increasing the number of projection images, this increase being still limited by irradiation damage. This implies reconstructing the volume from a limited number of images and poor signal-to-noise ratios.

The 3D characterization of water-containing samples was possible before using X-ray tomography, with a resolution of about 1 μm. Higher resolutions could be obtained on frozen samples by TEM tomography on cryosections, of by scanning transmission X-Ray microscopy (STXM). Despite irradiation damage, wet-STEM tomography is complementary to those techniques. As far as the resolution is concerned, it is similar to STXM but images with good contrast can be acquired even on amorphous samples containing only light elements. Its main advantage probably lies in situ experiments such as hydration/dehydration become possible. For example, it should be possible to follow the 3D arrangement of latex particles during filmification, or the fabrication of gypsum or cement-based materials. Because of irradiation damage, the observation of live biological samples may be more difficult and require further developments to reduce the electron dose received by the sample.

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