1 Past, Present, and Future Electron Microscopy of Liquid Specimens

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1.1 Introduction

This is an exciting era for electron microscopy. Over the last several years, the resolution of electron microscopes has increased dramatically through the successful implementation of aberration correction. Solving the decades-old lens aberration problem now allows astonishingly detailed and quantitative analysis of atomic level structures, as well as the ability to use lower voltage electrons for imaging while maintaining high resolution. At the same time, improvement in electron detectors has transformed the applications of electron microscopy. Images can be obtained with fewer electrons, reducing damage to the sample; higher speed imaging of dynamic processes is now possible; and more efficient collection of signals such as X-rays improves chemical analysis at the nanoscale. In this book, we describe an advance that is smaller in scale, but that is also transforming electron microscopy and its applications to materials science, life science, and beyond: the ability to image samples that contain liquids, particularly water. Electron microscopy of structures and processes in liquids is important over broad areas of science and technology. The changes that occur inside batteries during operation, the attachment of atoms during the self-assembly of nanocrystals, and the structures of biological materials in their native, liquid state are areas in which microscopy is essential. This wide applicability has driven a wave of interest in developing ways to perform electron microscopy on liquid samples. And as the technique of liquid cell electron microscopy has become more widespread, it promises exciting possibilities for solving grand challenges in materials, geology, biology, physics of fluids, and many other fields.

The difficulty of imaging liquids was recognized from the start of the development of electron microscopy. And somewhat like aberration correction (although on a vastly less complex scale), the solution was understood early on but its implementation required modern advances. The key issue is how to separate a liquid, in particular a high vapor pressure liquid such as water, from the vacuum in the electron microscope, while still obtaining reasonable images. In transmission electron microscopy (TEM), the image quality is generally reduced as the sample thickness increases, so this implies that the liquid must be in the form of a thin layer, in the range of nanometers to a few

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micrometers. This goal was not achieved until modern microfabrication techniques could be used to build thin windows of silicon nitride with a controlled, sub-micrometer separation, between which the liquid could be confined. This "closed" liquid cell was rapidly developed to include electrodes and flow capabilities, and interfaced to the microscope with dedicated sample holders. Similarly, in scanning electron microscopy (SEM), the availability of thin and robust windows allowed closed cells to be developed for imaging liquids, complementing environmental SEM (ESEM), which is based on sample chambers capable of accommodating water vapor and cooling the sample.

The first TEM experiments to make use of modern liquid cell designs addressed questions in electrochemical deposition, nanomaterials synthesis, diffusion in liquids, and the structure of biological materials; liquid cell SEM enabled imaging of biological structures without traditional preparation procedures. The results demonstrated the ability of liquid cell imaging to probe areas that had traditionally been inaccessible to electron microscopy, and in doing so to achieve useful and unique information [1]. As interest in the technique increased, the quality and functionality of TEM and SEM closed liquid cells improved, and open cell TEM was devised for examination of electrochemical and other processes. These developments in turn expanded interest still further. Since the equipment for liquid cell TEM and SEM is not too expensive to purchase or can be made relatively easily in one's own laboratory, and is compatible with existing microscopes, programs were started by research groups around the world. Thus the impact of liquid cell electron microscopy has increased rapidly [2], in terms of both the scientific areas of applicability and the types of measurements that are possible. Materials and processes examined now include corrosion, biomolecular structure, battery operation, beam-induced nanofabrication, bubble dynamics, radiation effects, and biomineralization. The rich capabilities of modern microscopes have been applied to liquid cell experiments, including elemental analysis through energy loss or X-ray signals, aberration correction, and high speed image recording. Correlative techniques are starting to produce exciting information in which electron microscopy is combined with light microscopy, synchrotrons, or other probes. Liquid cell microscopy has the potential to extend into new areas, to adopt advances in instrumentation even more fully, and perhaps even solve "grand challenge" problems.

1.2 The Rapidly Developing Liquid Cell Microscopy Technique

1.2.1 Liquids in the TEM

The early pioneers of transmission electron microscopy were very interested in imaging hydrated samples and water itself for applications in both materials and life science [3, 4]. Remarkable progress was made, given the challenges of observing even solid materials with the microscopes of the time. Two techniques were developed for getting water into the electron microscope while still maintaining a good enough vacuum to operate the electron source. These have evolved directly into the techniques that are in use today.

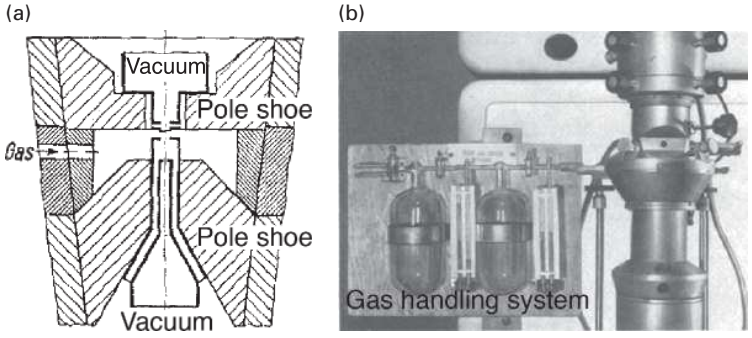

Figure 1.1. Transmission electron microscope with differentially pumped environmental chamber for imaging specimens at higher pressures. (a) The specimen in a gaseous environment is separated from the high vacuum of the electron column via pump-limiting apertures. The apertures are placed on the pole pieces (shoes) of the objective lens. (b) Photograph of electron microscope with gas handling system. From Ref. 5 with kind permission from Springer Science+Business Media.

One approach [5] is to use differential pumping to control the pressure at the sample region. Figure 1.1 shows how a relatively high pressure at the sample is maintained by placing pressure-limiting apertures on the pole pieces of the objective lens, enabling imaging in ambient air at up to 0.3 bar. Although not discussed in the original paper, this pressure is sufficient for water droplets to condense at room temperature. It would thus be fair to say that the first system for imaging specimens beneath a water layer was constructed in 1942! This "open cell" approach became highly successful in environmental SEM, as discussed below. The same open cell principle is now used in modern *in situ* TEMs to study gas phase reactions at moderate pressures – for example, imaging the growth of carbon nanotubes in a reactive gas atmosphere localized around the sample by differential pumping [6]. For reactions involving liquid water, the TEM community appeared less interested, perhaps because the geometry of the condensing water droplets was not controlled and the maximum pressure was limited. Nevertheless, open cell TEM continued to advance in diverse areas [3, 7], especially recently, when open cells using droplets of ionic liquids have helped visualize materials transformations during Li-ion battery operation [8–10]. The open cell TEM method and applications are described in more detail in Chapter 3.

The second approach is known as "closed cell" electron microscopy [11]. By enclosing the liquid between two electron-transparent windows, the limitation on maximum pressure imposed by the open cell could be circumvented. The earliest closed cells [11] made use of thin nitrocellulose films produced by floating a solution containing colloidal cotton on a mercury surface. The films were transferred to a perforated platinum support frame by raising the frame upward through the surface of the mercury. These windows were thick and provided poor resolution, and it was difficult to control the window separation, but interest continued over the next two decades. In the early 1960s, an environmental chamber [12, 13] consisting of two thin foils separated by a few micrometers (Figure 1.2a) enabled electron microscopy of condensed water

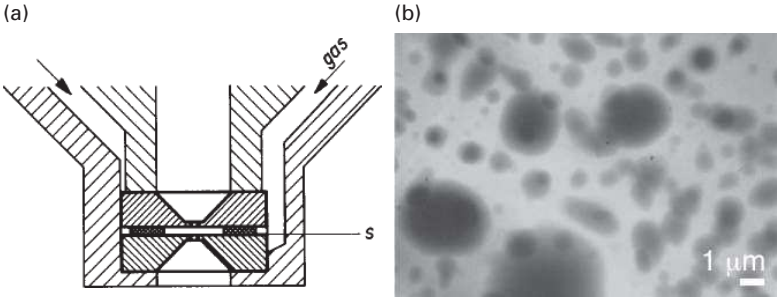

Figure 1.2. Environmental chamber closed with thin windows. (a) Two aperture holders support thin films and are placed face to face in the upper pole shoe of the objective lens. A thin film between the aperture holders (s) serves as a spacer of about 5 μm thickness. (b) Electron micrograph of water droplets condensed on the support film at a pressure of 0.13 bar. From Ref. 12 with kind permission from Springer Science+Business Media.

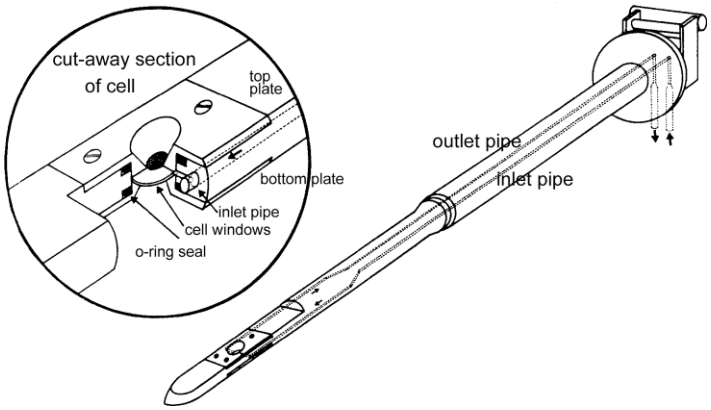

Figure 1.3. Early design of an environmental cell integrated in a side entry sample holder, with access pipes for gas flow from outside the electron microscope. Reprinted from Ref. 14, Copyright 1973, with permission from Elsevier.

droplets in a gas environment at 0.13 bar (Figure 1.2b). One could finally envisage imaging at atmospheric pressure, including samples enclosed in liquid, given foils of sufficient strength separated by spacers of sufficient narrowness. The next developments involved sample holders. The cell shown in Figure 1.2 is fixed in the microscope polepiece, making it difficult to modify the sample environment during imaging. Environmental cells integrated into side-entry sample rods allow simpler control, as shown in Figure 1.3, an early holder design that enables gas flow to and from the specimen [14]. With this type of equipment [4], the feasibility of imaging unstained biological materials such as bacteria in a wet environment was demonstrated, especially at higher accelerating voltages. Figure 1.4 shows a spatial resolution on the order of 100 nm achieved using ultra-high voltage TEM [4]. Similar systems developed for

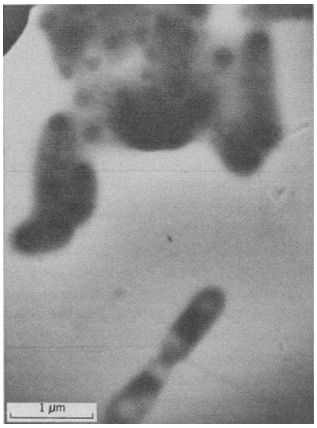

Figure 1.4. Coliform bacteria immersed in a layer of water in a closed chamber imaged with a 800 kV TEM. The environmental chamber was at room temperature with a water vapor pressure of 30 mbar. From Ref. 4. Reprinted with permission from AAAS.

standard voltage TEM were later used to inject liquid between windows for the study of a range of phenomena, including unstained bacteria [15], DNA [16], clays [17], catalytic reactions [18], and myosin head movement [19], described in more detail in Chapter 18.

These steps forward hinted at the opportunities possible with closed cell TEM, but the materials and technology available introduced severe practical difficulties. The windows were still thick and their separation, several micrometers, was large enough to reduce the image resolution to a value often not much better than that of light microscopy. Attempts to improve the resolution by filling the closed cell only partly with liquid resulted in poor control of the degree of water immersion of the sample. For biologists, the introduction of practical techniques to rapidly freeze biological specimens [20] superseded attempts to image in water with TEM. By rapidly freezing nanometer-scale biological objects, such as protein complexes and viruses, amorphous ice could be formed that preserved biological structure and prevented damage by ice crystals. Cryo-electron microscopy (cryo-EM) has become a key tool for structural biology [21–23], although the imaging of whole bacterial and eukaryotic cells, and of course dynamic processes, remain challenges.

The recent surge of interest in closed liquid cell TEM in both biology and materials science can be attributed to the use of modern microfabrication techniques to overcome the challenges of building closed liquid cells. Initially somewhat unreliable home-made cells that were sealed by hand with glue [24], modern closed liquid cells and their associated equipment have now developed to a point where they are readily available and simpler to use. Commercial systems can be purchased, and several groups have fabricated their own equipment [25–32]. The cells generally use windows made of silicon nitride supported on silicon chips. This highly practical solution, described further in Chapter 2, enables manufacture of strong windows of well-defined dimensions, strength, and electron transparency. Modern materials science also provides other

choices for the window material, most importantly graphene [33], whose advantage in terms of image resolution is discussed further in Chapter 19.

Microfabrication brings another benefit to the fabrication of liquid cells for TEM: the integration of functionality into the cell. Liquid heating and cooling (Chapter 6), electrochemical reactions with customized electrode materials and geometries (Chapter 10), and patterned or modified silicon nitride surfaces (Chapter 17) enable a wider range of experiments. The sample holder has been developed in step with the liquid cell (Chapter 2). Its function extends beyond simply holding the cell securely. It carries the electrical connections between electrode or heater elements and their external controllers. It may also provide the vacuum seal by clamping the chips, and allow liquid flow via inlet and outlet tubes driven by a syringe pump. Integrated microfluidic systems for liquid flow [34] enable the possibility of replenishing or changing the solution chemistry rapidly while imaging.

One of the key ongoing developments in closed liquid cell TEM is its adaptation to the full range of TEM capabilities. Most liquid cell images are recorded using conventional bright field TEM or high angle annular dark field (HAADF) STEM imaging modes. Single images are recorded to minimize the dose for biological structures, while movies allow analysis of dynamic materials processes. Higher sensitivity detectors reduce the dose required (Chapter 8), or offer higher frame rates and hence better time resolution. Dark field and high resolution liquid cell TEM are available, and aberration-corrected imaging (Chapter 20) and ultrafast image acquisition (Chapter 22) are under development. Analytical microscopy is also making its mark on liquid cell experiments (Chapter 21), via both electron energy loss spectroscopy (EELS) [35, 36] and X-ray energy dispersive analysis (XEDS) [37, 38]. Improvements in liquid cell and holder designs, as well as higher performance microscopes and detectors, promise continuing development of liquid cell TEM.

1.2.2 Liquids in the SEM

For imaging liquids, SEM shares general features with TEM. Liquid droplets with a low vapor pressure, for example ionic liquids [39, 40], can be imaged without special precautions. For high vapor pressure liquids, particularly water, both open and closed cell concepts are useful and provide different types of information. Furthermore, innovative concepts for correlative light and electron microscopy (CLEM) have adapted the geometry of the microscope and sample to obtain data from the same sample area.

Figure 1.5 shows the first environmental system for a SEM, published in 1970 [41]. This was a closed cell system in which a supported 10 nm-thick carbon film sealed liquid inside an environmental chamber. We return to closed cell SEM below. In the following two decades an open chamber approach was also developed that enabled imaging at pressures high enough to permit liquid water [42, 43]. This concept, environmental SEM (ESEM), is based on a differential pumping aperture in the objective lens to maintain the sample chamber at an elevated pressure, similar to the open cell design described above for TEM [5].

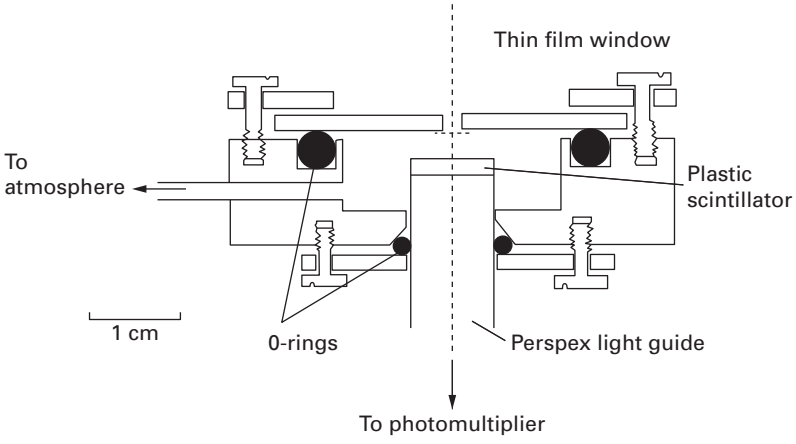

Figure 1.5. Environmental cell for a scanning electron microscope. From Ref. 41, © IOP Publishing. Reproduced with permission. All rights reserved.

Open chamber ESEM proved highly successful and is widely used in industry and academia to image a variety of specimens in a vapor environment with a spatial resolution in the few nanometer range [44]. Some microscopes can handle pressures in the sample chamber of up to 4000 Pa. Typical operation for optimal resolution is at a sample temperature of 4 °C and a pressure of 814 Pa, so that the sample is maintained at equilibrium between water and vapor. By fine-tuning the pressure it is possible to remove most of the bulk water over the sample to eventually leave a film of water thin enough to allow imaging. A key feature of ESEM is the variety of signals that can be collected. The standard backscatter detector generates contrast at objects somewhat below the surface of the liquid. The gaseous secondary electron detector provides surface information. It is mounted above the sample and held at a positive potential with respect to the sample, so that secondary electrons are accelerated towards it and cause an avalanche of ionized gas amplifying the signal. This enhancement in detection efficiency is an important benefit of the water vapor present in ESEM [43]. The spatial resolution achieved with these two detectors is typically around 10 nm for thin liquid layers. It is also possible to use a detector beneath the sample to record electrons transmitted through the sample. This approach, known as wet-STEM [45, 46], requires a very thin liquid layer (especially given the low accelerating voltage, compared to TEM) but is capable of detecting nanoscale objects of a high density within a liquid layer via atomic number (Z) STEM contrast, with minimal sample preparation. Resolution of 3 nm has been demonstrated for gold nanoparticles in a water layer [47]. The large sample chamber volume in SEM allows sample tilting, such that 3D ESEM is feasible [48]. ESEM applications involving liquid layers include imaging of biological cells [47], nanoparticle movement [49] and wettability studies [50–53]. In Chapter 16 we discuss ESEM of whole biological cells under thin water layers. The reader is referred elsewhere [44] for a more detailed description of ESEM and its broad range of applications.

When the sample involves a thicker liquid layer or complications such as electrodes or liquid flow, closed cell SEM is expected to have distinct advantages over ESEM. The closed cell concept was first revived [54] by enclosing the liquid in a capsule sealed with a thin polymer membrane, and forming images using a backscatter detector. The window thickness used, ~100 nm, limited the spatial resolution to ~20 nm for heavy materials. On the introduction of super resolution fluorescence microscopy techniques [55] with a similar resolution range, biological research adopted these revolutionary optical methods. But closed cell SEM continued to evolve to improve resolution and enable correlative or *in situ* capabilities.

Several strategies have been developed to increase the resolution in closed cell SEM, as described in Chapter 4. Reducing the window thickness using silicon nitride or graphene [56–58] is important. It is even possible to make a pore in a capsule window a few micrometers in diameter to allow imaging of a liquid meniscus without any membrane present [59]. The meniscus is stable over a narrow range of pressure differential, temperature, and surface tension. Resolution is also increased by using TEM-style closed cells with STEM detection through the liquid and windows [28]. Graphene can even be used to coat and seal samples on a thick substrate without mounting it in a capsule [60].

Correlative microscopy has been enabled through several imaginative concepts based on electron-transparent windows. Such designs are discussed in Chapter 5. A closed cell with an electron-transparent window above and a glass slide below allows SEM imaging from above and light microscopy from below, using an optical lens within the SEM vacuum system [61, 62]. Alternatively, the liquid can be open to the atmosphere, but placed in a dish that contains a silicon nitride viewing window. The sample is imaged from below with an "inverted SEM" using backscatter contrast, and from above with light microscopy [63]. The use of an open cell culture dish makes it easy to combine biological techniques, such as incubation and fixing, with SEM observation. A related inverted SEM concept involves focusing the electron beam in a small spot on a phosphor layer on the viewing window, thus creating a nanoscale light source for high resolution correlative fluorescence microscopy [64]. Finally, it is also possible to mount a silicon nitride window directly at the exit of an SEM column [65] to image samples in air. Unlike the simultaneous collection of the electron and light images with inverted SEM, this microscope utilizes a precise stage to move the sample between SEM and light microscopes. We anticipate exciting developments in these and other concepts for liquid cell CLEM.

1.2.3 Limitations of Liquid Cell Electron Microscopy

A series of developments is beginning to address the two key challenges of TEM and SEM of liquids: image resolution and electron beam effects.

For imaging purposes in TEM, liquids behave like amorphous materials and do not show distinctive contrast. However, when a liquid surrounds an object or covers a surface, the resulting images will generally show a lower resolution than if the liquid were absent. There are two main reasons for this (Chapter 8): loss of information due to multiple scattering of the electrons in the thick liquid and window layers, and blurring