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The study of nanostructured liquids by cryogenic-temperature electron microscopy — A status report

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ABSTRACT

Cryogenic-temperature transmission electron microscopy (cryo-TEM) is now a standard tool in the study of complex liquids, i.e., liquid systems with aggregates or building blocks on the nanometric scale. Methodologies have been developed to help capture the nanostructure of liquid systems, while preserving their original state at a given concentration and temperature. Cryo-TEM is now widely used to study synthetic, biological, and medical systems. Originally developed for aqueous systems, it has been also applied successfully in the study of non-aqueous systems, even in unusual solvents, such as strong acids. Recent developments in high-resolution scanning electron microscopy (HR-SEM) have made it an ideal tool for the study of nanoparticles and colloids in viscous systems or in systems containing large objects, three hundred nanometers and larger, in which small (nanometric) features are to be imaged, e.g., hydrogels or biological cells. Such system cannot be studied by cryo-TEM. Liquid nanostructured systems can now be studied by cryogenic-temperature scanning electron microscopy (cryo-SEM), using much-improved cryogenic specimen holders and transfer systems, even without conductive coating. In recent years we have developed a novel specimen preparation methodology for cryo-SEM specimens that preserves the original nanostructure of labile complex liquids at specified composition and temperature, quite similarly to what has been done in cryo-TEM. Here I describe briefly the state-of-the-technology of cryo-TEM and cryo-SEM, and demonstrate various variants of the methodology that allow the study of a wide range of nanostructured (soft matter) systems, taking advantage also of the combination of cryo-TEM and cryo-SEM.

1. Introduction

Quite often the tool of choice for high-resolution direct imaging of any material system is an electron microscope (EM), either a transmission electron microscope (TEM), or a scanning electron microscope (SEM). To image with an EM a specimen containing highly volatile components, such as water or many oils, steps should be taken to make the specimen compatible with the high vacuum in the EM, a process called 'fixation'. Chemical fixation, such as staining with a salt or an acid of a heavy metal, followed by drying the specimen, may be used only if the specimen is stable enough to undergo such harsh treatment, while preserving its nanostructure. The fixation method of choice is not chemical, but physical, i.e., fast cooling of the liquid, high-vapor-pressure specimen to cryogenic temperatures, at which it does not flow, and its vapor pressure is much lower than the pressure in the EM column, namely, less than 10^{-6} mbar (10^{-4} Pa). Ideally the liquid components should not freeze, but vitrify, i.e., become a very high-viscosity, low-vapor-pressure material, namely, a glass. Cryogenic electron microscopy was originally developed by biologists or biophysicists, who attempted to examine biological system at conditions as close as possible to their native state. Among the pioneers in that area were Patrick Echlin, who developed an early methodology of cryogenic-temperature SEM (cryo-SEM) [1], and Robert Glaeser, who worked on cryogenic-temperature TEM (cryo-TEM) [2]. An important breakthrough was the demonstration of successful vitrification (as opposed to freezing) of cryo-TEM specimen by Jacques Dubochet and his colleagues [3]. As commercial manufacturers introduced cryo-specimen holders, it gradually became easier to perform cryo-TEM. Cryo-SEM took the same path, but has never been as widely used as cryo-TEM.

Fairly early in the development of cryo-EM, it became apparent that the presence of water in the specimen, in the form of ice, either crystalline or vitreous (supercooled water), enhances electron-beam radiation-damage considerably, making imaging of those specimens quite problematic [4]. It has turned out that the very rapid free-radical reaction that follows irradiation of a cryo-EM specimen with an electron beam takes place at the interface between organic matter and water [5]. When water is mixed with organics, e.g., sucrose, radiation damage becomes very rapid. Before long it became apparent that to collect high-resolution data from cryo-EM specimens one needs to expose the specimen to no more than about 10 $e^{-}/Å^2$, which is 10^4 to 10^5 less electrons than most electron microscopy specimens experience before an image is recorded.

Many of the biological cryo-EM specimens are fairly stable with respect to temperature and concentration changes (within a certain

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range). Of course, all biological systems are water-based systems. The situation is different with more general specimens in the realm of physical chemistry. While water-based systems are very important in that area, many are complex systems of several components, including water, which form a spectrum of nanostructures, very sensitive to concentration and temperature changes. It is obvious that the nanostructure of such systems cannot be preserved when an alien compound (a stain or a fixative) is added. Obviously, fast cooling is the preferred method of preparation for such systems. To ascertain that the nanostructure in the vitrified specimen reflects that of the bulk system, preparation of the cryo-TEM or cryo-SEM specimen has to be carried out in an environment where temperature is fixed at a desired value, and evaporation of the volatiles from the specimen is prevented by proper saturation of the atmosphere around the prepared specimen. This calls for a proper sample preparation chamber. The first successful one was the 'Controlled Environment Vitrification System' (CEVS), designed and introduced by Bellare et al. [6]; it is still in use in various versions and modifications in many laboratories worldwide. Commercial apparatuses based on the same general idea, but designed for automated specimen preparation are now available. The CEVS was originally developed for the preparation of thin cryo-TEM specimens, as are the commercial units.

While cryo-TEM is now a well-established methodology that can be applied in the study of a wide range of biological and synthetic systems, quite often it needs to be replaced or augmented by cryo-SEM. This is the case when the liquid system to be studied is either very viscous, and thus cannot be made into the thin liquid films that are vitrified into a cryo-TEM specimen, or when the system contains objects that are too large to fit in the thin cryo-TEM specimen, typically less than 300 nm thick, but where we look for nanometric details. SEM cryo-holders are also available from a number of manufacturers. The need for specimen preparation under controlled conditions exists, of course, also in the case of cryo-SEM. This need has been answered a couple of years ago by Issman and Talmon [7] who modified the CEVS to accommodate cryo-SEM specimen preparation under controlled temperature and surrounding air saturation. The same apparatus may be used also for the preparation of freeze-fracture replicas under controlled conditions [8].

When systems that are either entirely composed of non-aqueous components, or those where the continuous phase is non-aqueous, are to be examined by cryo-EM, special precautions must be taken. Many organics are either flammable, or their vapors form explosive mixtures with air, thus the CEVS should be placed in an efficient fume-hood. Note that the commercial systems mentioned above can be used only for specimen preparation of aqueous systems. For non-aqueous systems the cryogen of choice, liquid ethane at its freezing point, i.e., "liquid–solid ethane", LSE, cannot be used, as it is a good solvent for most organic liquids. Instead we use boiling liquid nitrogen, LN_2, a far less efficient coolant. However, the relatively low cooling rates achievable by LN_2, no more than 8000 K/s (as compared to 100,000 provided by LSE), are sufficient to vitrify most organic solvents, except for linear hydrocarbons [9]. In recent years we have extended cryo-EM of nonaqueous systems even to the extreme of systems based on superacids, such as oleum and chlorosulfonic acid (CSA) [10]. In that case water and any organic material should not come in contact with CSA, as water causes it to disintegrate into hydrochloric and sulfuric acids, and it oxidizes easily any organic material. Thus, when working with CSA the CEVS is continuously flushed with dry nitrogen gas, and blotting of the specimen, a necessary step in cryo-EM preparation is done with fiberglass, not paper, sheets. Fast cooling is achieved by plunging the specimen into LN_2, not LSE, which gives sufficient cooling rates to vitrify CSA [11].

The above paragraphs describe the main cryo-EM tools that are available nowadays for the study of liquid systems that contain nanostructures or aggregates of a few to a several hundred nanometers, quite often called 'complex liquids' or 'nanostructured liquids'. Many of those liquid systems are related to biology and medicine, many others are synthetic. Some are water-based, some are water-free, and others are made of both oil and water. Quite often those systems have important technical and economical applications. The nanostructure of such systems depends on the molecular structure of their components, and has a profound effect on their macroscopic properties. Direct imaging of complex liquids is essential to determine the building blocks of those systems. In some cases one could use both cryo-TEM and cryo-SEM on the same system. Agreement between the images obtained by the two methodologies may rule out specimen preparation and imaging artifacts. Sometimes, if larger objects are present, or when in a process, smaller aggregates evolve into larger ones, light microscopy, especially using differential interference contrast (Nomarski) optics, or polarized light between crossed polarizers, can also augment the EM data. Whenever possible, imaging should be complemented by other experimental techniques, such as X-ray or neutron scattering, NMR, or electrical conductivity measurements. Those could support suggested models based on imaging, and give accurate measurements to the suggested nanostructure.

Following this introduction I will survey several relatively recent applications of cryo-EM in the study of a number of diverse complex liquids. My aim is to demonstrate the strength of the methodology and the breadth of its application. I would also like to demonstrate how the methodology is fine-tuned to accommodate the specific nature of each studied system.

2. Microemulsions

The original incentive to develop cryo-EM was the study of biological systems, all of which contain water. Water is also present in many other systems of interest. And water has turned out to be a relatively easy substance to work with, as it is safe, it can be vitrified in SLE, and vitrified water gives reasonably good contrast relative to the aggregates that biological systems contain. It is true that water is easily radiolyzed by the electron beam, producing free radicals that attack organic material in the irradiated areas of the specimen, but using low-dose imaging techniques, one can record good images of such specimens with minimal damage. Extending cryo-EM (originally cryo-TEM only, and more recently cryo-SEM) to non-aqueous systems turned out to be quite challenging. Fortunately it is quite easy to vitrify organic solvents in liquid nitrogen (except for normal hydrocarbons), but it has been found out that those specimen are typically more electron beam sensitive than aqueous ones, although careful low-dose imaging does work for most specimens, with the one notable exception of DMSO (dimethylsulfoxide; Talmon, unpublished results). However, quite often contrast between the imaged aggregates and the organic solvent is rather low, and may be enhanced by electron beam selective etching in cryo-TEM [12], or by selective sublimation in cryo-SEM [13].

In my own research an on-going theme has been the study of microemulsions (MEs). While water continuous microemulsions, namely aqueous systems of oil-swollen micelles, can be imaged just like any other aqueous system [14], oil continuous or bicontinuous MEs pose a much more difficult challenge for cryo-EM. In addition, many MEs are very sensitive to relatively small changes of concentration and temperature, and thus much care should be taken during specimen preparation to keep those parameters constant at all times, until the specimen is vitrified. Quite often we study a system over a wide range of concentrations and temperatures. In that case a combination of cryo-TEM and cryo-SEM should be used. In some cases both methodologies can be used for a given system, and thus mutually support the validity of the results obtained by each. In other cases only one of those methodologies can be used in a given part of the phase diagram due to considerations of specimen preparation or imaging.

A good example of the application cryo-SEM to study a bicontinuous or an oil-continuous microemulsion is the study of ternary DDAB-water-octane microemulsions originally studied quite some time ago by Ninham, Evans and their coworkers [15]. Fig. 1a shows a cryo-SEM