Spatial and temporal resolution in cryo-electron microscopy—A scope for nano-chemistry

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Abstract

Cryo-electron microscopy has evolved in an established approach to study the structure of bio-colloids. Recent developments in instrumentation and automation, often demanded by life sciences, made cryo-EM a general tool in colloid chemistry. Recently improved instrumentation for vitrification has resulted in reliable and reproducible preparation of specimen in water and other solvents. A dynamic approach, following processes in time is gaining importance and a time resolution up to 1 ms is expected to become general available. With improved instrumentation and automation 3D reconstructions by cryo-EM tomography are becoming routinely accessible. Tomography as such or in conjunction with time resolved microscopy is expected to give new insights in (macro)molecular assembly/disassembly mechanisms and thus become an essential tool in nano-chemistry.

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Understanding the reaction mechanisms of macromolecules and the large complexes they may form is one of the goals of nano-chemistry and structural biology. Cryo-electron microscopy, the low temperature observation of vitrified specimens, allows the direct observation of macromolecules and molecular complexes in their projection with a spatial resolution of ~1 nm. Recent technological advances in specimen preparation, microscope operation and data handling contributed to a high throughput of useful data making three dimensional reconstructions of specimen feasible. 3D analysis together with a dynamic (time resolved) approach is now coming into scope. This will be illustrated by data on (self) assembly and disassembly of (macro)molecules.

Some thirty years ago the group of Glaeser [1,2] pioneered cryo-electron microscopy, the study of frozen hydrated material at low temperature. In their attempts to obtain good specimen preparations use was made of Langmuir–Blodgett film to cover support films with adsorbed (hydrated) purple membrane [3]. The first images of chemically unmodified (‘unstained’) materials in their natural aqueous habitat were obscured by ice-crystal damage. Dubochet et al. [4] introduced plunge freezing and cryo-transfer methods in the eighties to obtain vitrification and thus avoid ice crystals. This became an established method in the nineties. Plunging a thin aqueous film, with a thickness of a few hundred nanometers or less, in a suitable coolant (e.g. liquid ethane) resulted in a vitrified specimen. Such a specimen has no visible ice crystals and a diffraction pattern of amorphous ice. Suspended material may turn up in the image as if a snapshot was being taken of (nano) jelly fishes swimming in an aquarium. By applying particle averaging methods (“single particle analysis”) on periodic objects cryo-EM has contributed to structural biology to complement X-ray crystallographic data. In spite of this tremendous progress, a few groups struggled with the preparation of ‘vulnerable’ specimens such as liposomes which are sensitive to temperature and salt concentration (among them the authors). Trinick’s group was also contending with the salt concentration problem [5]. Evaporation from a thin aqueous film was found to be a key issue in plunge freezing/vitrification. Evaporation has a dual effect on the specimen since it lowers the temperature of the specimen relative to the environmental temperature (dew point effect) and, at the same time, the salt concentration rises considerably. ‘Fast’ preparation of the specimen in open air cannot prevent thermal and omotic effects. To illustrate the rate and magnitude of these
effects let us consider a 100 nm thick film at ambient conditions; 20 °C and 40% relative humidity. Within 0.1 s the temperature will be 8 °C lower (approx. dew point) and at the temperature difference thus established evaporation will proceed at a rate of about 40 nm/s (more details can be found in Ref. [6]). Therefore Bellare et al. [7] described the controlled environmental vitrification system (CEVS), a temperature and humidity controlled chamber for the preparation of thin films. This approach has since been the ‘standard’ for the study of vulnerable specimens. The CEVS requires that the operator possess the training and skill necessary to blot away excess liquid from the specimen. The challenge of further automation was taken on by the University Maastricht and a prototype PC controlled vitrification instrument was built in 1997 which included an automatic blotting device and a year later an ultrasonic humidifier was added. This made equilibration at high humidity (>95%) faster thereby increasing the throughput of vitrified specimen.

Most of the cryo-EM work deals with aqueous suspensions but with a comparable conditioning some organic solvents can be used as well [8–10]. After a redesign in 2000 the now named ‘Vitrobot™’ was patented by the University Maastricht in 2001 and the patent licensed to FEI for worldwide distribution.

Since the introduction of the CEVS, some 15 years ago, the era of open air preparations for cryo-EM should have ended. However, open air preparation for cryo-EM, with its osmotic and thermal effects, is still in use although obviously outdated. Through further automation (PC process control, blotting, environmental conditions, etc.) a high throughput of good vitrified specimens is now routine and standard, available to the novice as well as the experienced. Based on the high throughput of vitrified specimens it is now appropriate to consider linkage of spatial (3D) and temporal (3D in time) investigations with specimen structure. The automation of microscope operation has contributed to the efficient collection of 3D data and this is accessible for application in colloid chemistry. It is expected that 3D data will, in the near future, be complemented by time-resolved microscopy. This involves the implementation of old and established concepts (and possibly some new) in automated vitrification and automated microscopy. The recent developments may add another chapter to the Snow White tale of cryo-EM, thus a reawakening of electron microscopy in the fields of chemistry.

### 1. 3D specimen reconstruction

For three-dimensional reconstruction of vitrified specimens, two approaches are used: single particle analysis and tomography. Images of identical particles (purified proteins, viruses) are the starting point of single particle analysis. Every image of a particle is considered as one 2D projection of a 3D object and in an iterative procedure these projections are classified with regard to their spatial orientation and eventually grouped in preferential orientations [11]. Noisy images from a group of specimen orientations are added up to improve the signal to noise ratio and the summed images are back projected into a three-dimensional model. This three dimensional model is the starting point of an iterative refinement procedure; the provisional model is used for a more accurate determination of projection angles and positions of particles in the original data set. The ultimate resolution in the reconstruction (better than 1 nm is feasible) depends on the noise level in the individual images and the number of individual images (often between a few hundred and thousands) used for the reconstruction. This ‘single particle approach’ can therefore only be applied when large numbers of identical particles are available in various orientations. This is a privilege for life scientists but may turn into a burden when extensive purification is required. If particles are not (strictly) identical, which is often the case; tomography is presently the best method for obtaining optimal 3D structural information from a single element in a heterogeneous population. By tilting the specimen in the microscope column, angular projections are obtained from each tilt angle. These images are back projected to obtain a 3D model. Ideally projections should be obtained over a full turn (360°) of the specimen with small angular increments eventually over multiple tilt axes [12]. Tilting of cryo specimens is mechanically restricted (−70° to +70° is currently feasible). As a consequence, the 3D reconstruction has a missing wedge. Usually some 40° is missing when tilting over a single axis or by tilting over two perpendicular axes the missing volume takes the form of a missing cone/pyramid. The resolution of the reconstructed 3D form depends on the accuracy with which the projection angles of consecutive images can be determined. An important factor in 3D reconstruction procedures is the structural damage that may result from electron beam irradiation during image collection. Beam induced damage is inherent to electron specimen interactions. Lowering the specimen temperature, by working at ‘cryo’ conditions, slows down the rate of beam induced changes. For tomography it is essential that the tolerable electron dose is efficiently used over the whole tilt range. Each image should have enough information (e.g. electrons per pixel) to be useful in the reconstruction. Thus it is often the beam damage that sets the limit to the resolution of the 3D reconstruction as ‘beam economy’ dictates the magnification and number of tilt angles that can be used for the reconstruction. To prevent spillage, low dose electrons routines should be used. This implies that focusing and image tracking are done on an area just outside the image area. The low dose feature is therefore embedded in automated microscopy to facilitate the efficient use of image electrons.

Cryo-EM combined with tomography is likely to become an important approach in colloid science and combinatorial chemistry as it allows 3D analysis of molecular assemblies. This is illustrated in Figs. 2. The images are from doxorubicin, an anticancer drug, loaded into liposomes and imaged by cryo-EM. A still from a tomographic reconstruction is presented in Fig. 1a (jpeg movie available as supporting information) while a standard cryo-EM image of a similar preparation is presented in Fig. 1b. By using functionalized DSPE-PEG2000 protein-ligands can be attached for targeting (see Fig. 1c; Annexin V coated vesicles loaded with doxorubicin). The liquid crystalline doxorubicin core has a periodicity of 2.7 nm and this is
resolved in the tomogram as well as in the cryo-EM image. This resolution is approaching the practical resolution of 2 nm that is generally assumed for cryo-EM tomography [12]. The preliminary data presented illustrate the potential value of cryo-EM tomography for the study of the architecture of macromolecular assemblies at the nanometer scale. It is expected that soon more tomograms are coming available to further support this idea.

Three dimensional reconstructions are not restricted to molecules in suspension but can also be applied to interfacial phenomena. The principle of collecting Langmuir films on a specimen grid for cryo-EM is rather established [3,13]. The addition of 3D information by tomography opens an (additional?) exciting dimension that is currently explored by the authors.

2. Time resolved cryo-EM

Registering time-related changes in a specimen is a challenge for (cryo-electron) microscopy. If changes are in the order of minutes or longer, multiple samples can be drawn from the reaction mixture and studied in a time sequence. Classical examples are vesicle interactions with detergent (including bile salts) [14,15] and influenza–liposome fusion [16]. The time allotted for mixing and sampling is a threshold for the time resolution setting the sampling limit to some 3 s (e.g. when using the Vitrobot). Lower threshold times are attained when a reactant is applied during the processing of the sample grid. Spraying micro droplets on the grid has been proposed by the groups of Berriman and Unwin [17] and White et al. [18]. The latter group has described a sophisticated procedure, whereby a high tension source is applied between the spray nozzle and the grid so that electrostatic attraction directs the micro droplets towards the grid. A time resolution in the range of seconds up to micro seconds could be achieved with this setup [19], but safety hazards have hampered general acceptance. An alternative for liquid application is the application of dry (or dried) reactants to a wet specimen. This can be done by blotting away excess liquid from the specimen using a filter paper impregnated with reactant. This is illustrated by the cryo-EM image of Fig. 2 (see legend for details). The image shows the reassembly products that appearing when 100 nm vesicles of Soy-PC/DDAB are blotted with filter paper impregnated with SDS (air dried after impregnation with an SDS solution). Because of its negative charge SDS has an immediate effect on the positively charged lipid bilayer. The result is an immediate disassembly of large vesicles and the appearance of threadlike micelles as well as disc like micelles, which can be observed in coexistence with small vesicles. The small vesicles which dominate the image after longer incubation times (3–10 s) are probably formed by the assembly of disc like micelles into thermodynamic “equilibrium” vesicles (‘entropically stabilized’ for a limited time period). Some four disc-like micelles are needed to supply material for one of these small vesicles. Incomplete vesicles in the form of cup-shaped structures can be found and are tentatively interpreted as intermediates in the process of vesicle (re)assembly. In a
different lipid system a temperature change may also induce formation of ‘entropically stabilized’ vesicles and images are presented for comparison (Fig. 2).

Time resolution over shorter intervals can be attained by using events triggered by light [20]. Light may have a two-fold effect: on one hand it may heat up the specimen [21,22] on the other hand it may initiate a chemical reaction (e.g. UV photopolymerization, light induced structural alteration [23], liberation of caged compounds, etc.). Flash photolysis of caged compounds has been used by several groups [23,24]. Custom build instrumentation was used by these groups but the complexity of the instrumentation and its handling has hampered a general spreading of this elegant principle: using an UV(-flash) two induce the exposition of two or more compounds to mutual interaction. A number of caged compounds is available for such applications (also see Ref. [25] for principles and examples), which may allow pH changes, exposition of Ca, ATP, GTP, etc. over timed intervals. A minimum time interval in the order of a millisecond should be attainable. The threshold is set by the decomposition rate of caged compounds ($t_{1/2} \leq 1$ ms) and by the possibility to expose a sample to UV when it is moving to the coolant (entry speed about 2 m/s). Exposing the sample to UV light should result in the chemical as desired but not in a temperature rise. An excessive temperature rise should be prevented by using just enough UV intensity in combination with “reflecting” materials for UV light. For time-resolved microscopy in the seconds to millisecond range the combination of UV (flash) light and caged compounds will be further developed (e.g. PC control of key events). When proven versatile the UV option is likely to become part of the toolkit of cryo-electron microscopy.

3. Conclusion

The prospect of cryo-electron microscopy is to become a key technology in nano-chemistry. The ability to produce ‘through-vision’ 3D data at high resolution and document time related changes of (macro) molecules is fuelling this prospect. A survey is given of current trends aiming at the increase of sample throughput combined with an increase of the output of high quality data. Emphasis is put on careful specimen handling and sophisticated experimentation (e.g. Langmuir films, time resolved experiments) in the time span just prior to vitrification of a specimen. Colloid chemistry may benefit from the developments in cryo-electron microscopy; especially when automated specimen preparation, with all the options to document space and time related changes, is further integrated with automated microscopy. The pace of realization of these developments depends not only on the curiosity and excitement of scientists but even more on the impact of nano-(colloid) chemistry to society.

Fig. 2. Vesicle assembly from disc-like micelles induced by temperature and by detergent action followed in time as documented by cryo-EM. DMPC vesicles containing 6 mol % of DMPE-PEG 2000 were made by extrusion at 60 °C (ca. 100 nm diameter) and cooled to 30 °C to fall apart into disc-like micelles (diameter about 30 nm). Upon heating to 50 °C, these discs reassemble into small vesicles (diameter 25–30 nm) with a few larger ones. Detergent disassembly and reassembly of vesicles followed in time. Vesicles prepared by sonication from SoyPC (10 mM)/DDAB (2 mM) and blotted with filter paper impregnated with SDS. Sample vitrified 1 s after blotting thread-like and disc-like micelles can be observed together with small vesicles and tubes. Micelles are dominating at shorter incubation times, vesicles and tubes at longer incubation times.
References


