Macromolecular structure determination by electron microscopy: new advances and recent results
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Electron microscopy is undergoing a mini-renaissance, as a number of biological systems are yielding to higher resolution analysis as a result of advances in instrumentation, specimen preparation and image-processing technology. The atomic structure of tubulin has now been solved, crucial elements of secondary structure have recently been revealed in several membrane proteins (rhodopsin, gap junctions, aquaporin, and Ca\(^{2+}\) and H\(^{+}\) ATPases) and in a virus particle, and macromolecular complexes are being seen in increasingly fine detail. This growth has been enhanced further by the ability to combine structures of macromolecular complexes derived by electron microscopy with X-ray structures of their components, in order to reconstruct molecular machines and large multiprotein complexes in immense detail.

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Abbreviation
CCD charged-couple device

Introduction
In the past, electron microscopy has been used primarily in the exploration of macromolecular architecture and shapes. Shapes that spike curiosity and offer insights into the workings of biological macromolecules, but in the end leave one wanting for higher resolution. Fortunately, electron microscopy is truly beginning to satisfy the ultimate need to see structures and learn about mechanisms in atomic terms. The past year has yielded a number of results, ranging from the determination of the novel viral fold of the hepatitis B core protein to the complete polypeptide chain tracing of tubulin. Even though 1 Å structures of biological macromolecules are not around the corner, structure determinations around 20 Å are ubiquitous, those in the range 6–9 Å are rapidly multiplying and those near 3 Å resolution are becoming more common. The now widespread use of cryomeans, in which specimens are embedded in amorphous ice and imaged at low temperature with low electron doses, has underpinned much of the new research. Moreover, the direct imaging of freeze-trapped conformations is beginning to provide insight into biological mechanisms that cannot be obtained by any other means. In this short review, we attempt to convey some of the general principles and problems associated with current electron microscopy and highlight some of the findings of biological interest that have emerged as a result of the recent technological advances. Recent in-depth treatments of particular problems and issues discussed are also given elsewhere [1–4].

The sample – where it all begins
Samples that are suitable for examination by electron microscopy can be divided into three principal categories: single particles; one-dimensional crystals (usually with helical symmetry); and two-dimensional crystals. Because of their distinct properties and, hence, approaches by which they are analyzed, we also subdivide one-dimensional crystals into filaments and tubular crystals (or tubes). Each of these types of sample have advantages and disadvantages for structural analysis, which are discussed below and summarized in Table 1. As with any biophysical technique, the careful preparation of the sample to be studied is of paramount importance to success. Furthermore, there are a number of problems that all samples encounter to a greater or lesser degree, such as radiation damage and beam-induced movement due to charging and/or heating. Radiation damage and movement are themselves the major obstacles to any high resolution analysis by electron microscopy and methods for minimizing them are continually being sought and developed.

Single particles
Single-particle analysis is the most straightforward method, but only in so far as sample preparation is concerned. As long as the specimen is well dispersed in solution, it can usually be embedded in thin ice and imaged directly over holes in the carbon support film — the usual method for obtaining optimum structural preservation and signal-to-noise ratios. As single-particle analysis in general makes minimal demands on sample preparation, the majority of analyses are performed on such samples. The determination of an accurate three-dimensional structure from differently oriented particles necessitates the combination of data from many images. This entails sophisticated procedures to sort out good from bad particles, to obtain precise alignments relative to a reference and to evaluate the quality of the resulting map. The various computational methods that have been devised for these purposes are beyond the scope of this review, but for recent discussions, see, for example, [3–6].

The major limitations of single-particle analysis are due to the inherent flexibility of many macromolecules and the heterogeneity of the sample. These factors present difficulties for both the selection of particles and the ability to achieve a high accuracy of alignment from weak signals. The limitations depend to some extent upon the shape of
Table 1

Comparison of electron microscopy methods for macromolecular structure determination*

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample Preparation</th>
<th>Data Collection</th>
<th>Analysis/Processing</th>
<th>Resolution attained (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single particles</td>
<td>No crystallization</td>
<td>Weak amplitude signal</td>
<td>Alignment ambiguity</td>
<td>~7</td>
</tr>
<tr>
<td>Filaments/fibres</td>
<td>Heterogeneity</td>
<td>No diffraction amplitudes</td>
<td>Particle flexibility</td>
<td></td>
</tr>
<tr>
<td>Filaments/fibres</td>
<td>No crystallization</td>
<td>Weak amplitude signal</td>
<td>2D distortion correction</td>
<td>~9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No diffraction amplitudes</td>
<td>Phase restrictions</td>
<td></td>
</tr>
<tr>
<td>2D crystals</td>
<td>Crystallization</td>
<td>Diffraction amplitudes</td>
<td>2D distortion correction</td>
<td>~3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Many tilts required</td>
<td>Crystallographic methods</td>
<td></td>
</tr>
<tr>
<td>Tubular crystals</td>
<td>Crystallization</td>
<td>No diffraction amplitudes</td>
<td>3D distortion correction</td>
<td>~8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isotropic data</td>
<td>Phase restrictions</td>
<td></td>
</tr>
</tbody>
</table>

*Possible advantages are given in bold and disadvantages are in plain font. 2D, two-dimensional; 3D, three-dimensional.

the molecule in question and vary with particle size; larger particles giving a stronger signal but requiring greater precision in their alignment in order to achieve a given resolution. Nonetheless, this approach has continued to provide remarkable new insights into a range of complex molecules and will inevitably become even more powerful as computational methods are developed for combining more images with a greater accuracy. At the moment, the best resolution achieved is 7.4 Å for the hepatitis B core protein, by combining ~64,000 images, which is equivalent to almost 400,000 particles because of the icosahedral symmetry [71].

Filaments

Most filaments have helical symmetry and therefore have two advantages over single particles as objects for three-dimensional structure determination. First, the orientation and position of the molecules they are composed of are defined by the helical symmetry and are therefore less subject to the errors of alignment that one encounters with single particles. Second, the molecules are viewed from a complete set of equally sampled directions, ensuring that isotropic resolution is achieved and that there are no problems associated with them having a preferred orientation on the grid. As with single particles, improvements in computer processing [8,9] have led to the more accurate and rapid extraction of information from electron micrographs, enabling more filament segments to be averaged and hence more reliable maps, with resolutions of up to ~9 Å, being achieved [8,10].

Two-dimensional crystals

Two-dimensional crystals have been the most successful for obtaining high resolution structural information by electron microscopy. Methods for preparing and analyzing two-dimensional crystals of biological macromolecules were first reported for bacteriorhodopsin. Since this early study at 7 Å resolution, improvements in the sample preparation, the development of computer methods for correcting lattice distortions [11] and other technical advances have now led to a 3.0 Å structure of bacteriorhodopsin [12,13].

Electron microscopy of two-dimensional crystals has been applied primarily to membrane proteins, in which the
native membrane environment is naturally conducive to forming planar sheets. Crystallization methods have also been developed that exploit affinity tags or electrostatic interactions between proteins and lipids in order to initiate crystallization on a lipid surface [14]. These methods have been successfully applied to a range of soluble proteins; a recent example is the two-dimensional crystal formed by the histidine-tagged HupR response regulator protein on a nickel-chelating lipid [15].

A number of difficulties may arise in studies of two-dimensional crystals. For example, it may be difficult in the first place to obtain a high degree of order. A common problem is lack of uniform flatness, which results in poor statistics for high-tilt angle data, with a consequent loss of resolution in the direction normal to the crystal plane (typically the membrane plane). A primary advantage of large two-dimensional crystals is the ability to collect electron diffraction data. Methods for the extraction of phases from the images, which usually must undergo appropriate distortion corrections, have been developed [11], and once sets of diffraction and image data have been collected, they can be combined and manipulated in a similar fashion as in X-ray crystallography.

**Tubular crystals**

Biological molecules often crystallize as tubes with helical symmetry, rather than as two-dimensional crystals. For a given molecule, such tubes usually have the same surface lattice but a range of diameters, giving rise to a series of different helical families. Membrane proteins and also some soluble proteins form these crystals through protein–protein interactions or through specific associations involving lipids. Tubular crystals formed by membrane proteins in lipids are of particular interest, as they are amenable to combined structure/function studies in closer to physiological conditions than can be obtained with extracted proteins in the presence of detergent. The structures of the acetylcholine receptor at 9 Å resolution [16] and Ca^{2+} ATPase at 8 Å resolution [17] have been determined from such crystals.

Techniques for producing tubular crystals are becoming better understood [18]. Furthermore, the use of tube-forming lipids, in combination with ionic interactions or specific affinity tags, has produced tubular crystals of soluble protein complexes [19–21] and represents a promising future approach.

A major obstacle to obtaining high resolution information from tubular crystals has been the presence of distortions, such as bending in and out of the plane of the image, and variations in both scale and twist. These distortions may be made more serious by the fact that tubes usually need to be imaged in ice over holes in the carbon support film, so that their helical symmetry is fully retained. Recently, a procedure has been developed that allows accurate determination and correction of distortions in all three dimensions by the independent alignment of short tube segments against a reference structure [22]. The application of this and similar approaches may soon enable atomic resolution to be achieved for this kind of sample.

**Technical and instrumental advances**

At the heart of any electron image analysis is the microscope. Although electrons cause less radiation damage than X-rays, the small sample size and the consequent much greater dose required per molecule means that radiation-induced damage is invariably a serious problem. Gyromethods of ice embedding in order to maintain molecules in a hydrated state [23] are well established and data collection at near liquid nitrogen temperatures has become routine. Several microscopes have also been developed that incorporate liquid helium-cooled stages [24,25]. The advantages seem clear, since the results suggest that the lower temperatures lead to a greater than twofold reduction in the damage at high resolution compared to that at liquid nitrogen temperatures [26]. The operation of these microscopes requires considerably more sophisticated instrumentation and there are additional practical difficulties associated with the poor conductivities of ice and carbon at very low temperatures. The improved image quality now obtained for a wide range of specimens suggests, however, that very low temperature microscopy will play an increasingly significant role in the future.

The field emission source is another technological development that has become widely implemented over the past two or three years. The improved coherence of the field emission source (both spatial and temporal) leads to substantial improvements in contrast transfer at high resolution, particularly when the image is strongly defocussed. Its advantage becomes most apparent in studies of ice-embedded particles, filaments and tubes, where strong defocussing is needed in order to produce sufficient contrast for accurate alignments to be made, while at the same time retaining the high resolution detail in the image. Almost all high resolution studies on these kinds of samples are now performed using microscopes equipped with field emission guns.

More extensive use has been made of the charged-couple device (CCD) camera in recent years, because of its large dynamic range and digital output. CCD cameras of up to 2k × 2k pixels have greatly enhanced the speed, accuracy of collection and the processing of electron diffraction data. Primary image data are still collected on film, however, as dynamic range is not as important as the film's high detective quantum efficiency for electrons, high resolution (<10 μm) and, hence, immense (~1 gigabyte) storage capacity. As with the field of X-ray crystallography, data processing has also been greatly enhanced by advances in computational speed and power. Thus, it is now possible to contemplate the analysis of orders of magnitude more particles than was possible 25 years ago, when the advantage would not have been so apparent anyway, since most studies then were only of molecular imprints made by negative stain.
Electron scattering differences between charged and neutral atoms and the observed effects on experimental coulombic potential maps. (a) Electron scattering factors ($|f(0)|$) for neutral and negatively charged oxygen versus resolution ($\AA$). The scattering factors differ at resolutions lower than 2.5 Å and show significant differences below 5 Å resolution. (b) Coulombic potential map in the region of Asp212 from bacteriorhodopsin, calculated using all data from 54 to 3 Å resolution. (c) Same as in b, but calculated using only the data from 7 to 3 Å resolution. The dramatic lack of density around Asp212 and Asp85 in (b) is proposed to arise from the negative scattering potential of negatively charged oxygen at low resolution. Adapted with permission from [13].

Theoretically, because of the substantial differences in the electron scattering factors of neutral and ionized atoms at resolutions below 5 Å (Figure 1a), electron microscopy has the potential to determine the charge state of atoms. This phenomenon has been observed with small organic molecules, and results obtained with the plant light-harvesting complex II [27] and, most recently, with bacteriorhodopsin [13] (Figure 1b,c) suggest that the charge state of atoms may have a significant effect on the observed coulombic potential maps. Although there is a need to establish the reliability of this method, these observations suggest that electron microscopy may be used in the future for the direct experimental determination of atomic charge states in macromolecules.

Some structural highlights

The special advantage of electron microscopic structure determination is its ability to probe areas that, for one reason or another, are currently inaccessible to the more commonly used technologies of NMR spectroscopy and X-ray diffraction. Single particles of large complexes, including filamentous structures and cellular organelles, fit into this category, as do some soluble particles, such as tubulin, which aggregate or are difficult to crystallize in three dimensions, as well as many membrane proteins, for which there is the additional opportunity of exploring them directly in their lipid bilayer environment. The past year has seen a number of important results arising from electron microscopy of a variety of sample types and we briefly describe several of these here.

The Sec61 oligomer is thought to be the protein-conducting channel that is responsible for transporting the nascent polypeptide chain through the membrane during translation by the ribosome. A recent single-particle analysis of the ribosome–Sec61 complex at 26 Å resolution revealed that Sec61 forms a ring-like structure at the base of the ribosome [28]. The 15 Å pore (narrowest point) made by the ring of the Sec61 oligomer is presumed to make a continuous conduit across the membrane for the nascent polypeptide chain. The pore of the Sec61 oligomer is in precise alignment with an opening in the large ribosomal subunit that represents the presumed nascent polypeptide tunnel. This study has given us the first structural evidence for the fact that Sec61 specifically interacts with the ribosome, forming a pore-like structure that would allow the nascent polypeptide to cross the endoplasmic reticulum membrane.

The structural analysis of the 14.6 MDa tricorn protease assembly, which is one of the largest homo-oligomeric enzyme complexes known, has been performed recently at 25 Å resolution [29]. This remarkable protease was shown to have icosahedral symmetry similar to many virus particles and thereby forms a large cage for selecting and sequestering potential substrates.
The past year has seen several electron microscopic studies of important proteins at resolutions ranging from 6 to 9 Å, sufficient to reveal elements of their secondary structure. The membrane-spanning α helices comprising rhodopsin [30], Ca²⁺ ATPase [17], H⁺ ATPase [31] and aquaporin [32–34] have been described in studies analyzing tubular or two-dimensional crystals of the proteins. The projection structure of the gap junction from isolated two-dimensional plaques [35] has given hints about its three-dimensional α-helical organization. Together, these studies have characterized four new folds for membrane proteins. In the same resolution range and serving as landmarks for single-particle analysis, are the visualization of secondary structure in the core protein of the hepatitis B virus [7,36] and the determination of the structure of the larger (600 Å diameter) papillomavirus capsid [37].

The attainment of high enough resolution such that a complete polypeptide tracing can be achieved is always an important advance. The atomic model of εβ tubulin at 3.7 Å resolution in the past year [38] was the third structure for which the complete polypeptide chain was traced based on electron microscopic data, following on from bacteriorhodopsin and plant light-harvesting complex II. The results of this study are critical to our understanding of motor protein–tubulin interactions and to our general understanding of the mechanisms of these molecular machines.

The merging of methods
As already mentioned, one area that is providing more and more insights into biological systems is work in which electron microscopic maps of large macromolecular complexes, obtained at moderate resolution, are combined with atomic structures of their component parts, thereby putting the atomic structures into their functional context. An example for which this approach promises to be especially enlightening is understanding the molecular basis of the movement of motor proteins on microtubules. A start has already been made [39] (Figure 2) and with the structure of tubulin solved, it is clear that there soon will be more to come. Another good example is the location and arrangement of tRNAs at the A, P and E sites within the ribosome in pretranslocational and post-translocational states [40]. Similar methods have been used to study viral cores [41] and conformational changes analyzed by electron microscopy at moderate resolution may also be combined with detailed X-ray maps of the static structures. The analysis by electron microscopy of GroEL, freeze trapped in an ATP-bound state [42] offers a recent example of the power of this method. The conformational changes observed were able to be tied in closely with details of the X-ray structure, yielding important information concerning the nature of the allosteric switching that underlies the chaperonin mechanism. All of these examples illustrate the power of combining high resolution X-ray crystallographic data with lower resolution electron microscopy data in order to gain additional insights into the workings of biological systems.

Conclusions and future prospects
Structure determination by electron microscopy is not as widespread as by X-ray crystallography. However, several technical advances made in the past few years are broadening the scope of this approach and helping to push it into the atomic resolution arena on a more permanent basis. Some of the most critical advances have come from improved instrumentation for data collection and methods of data analysis. In the end, however, it is through the continuing interaction of a small but rapidly growing community of researchers that new developments emerge. The exchange of ideas and information is an important factor in maintaining the rapid improvements being achieved. We are confident that these advances will continue and that macromolecular structure determination by electron microscopy will play an increasingly important role in the study of biological systems.
microscopy will play a much more prominent role in our quest for understanding biological systems at the atomic level.

References