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Chapter 1

General introduction
Contents

1.1 Introduction .............................................................................................................. 9
1.2 Transmission Electron Microscopes ................................................................. 10
1.3 Electron scattering and radiation damage ...................................................... 12
  1.3.1 Imaging versus diffraction ........................................................................ 14
1.4 Organic molecules ............................................................................................ 15
  1.4.1 Small and big organic compounds ....................................................... 16
  1.4.2 Proteins ................................................................................................. 17
  1.4.3 Single particle imaging .......................................................................... 18
1.5 Crystal diffraction .............................................................................................. 18
  1.5.1 2D crystals by TEM .............................................................................. 18
  1.5.2 3D crystals by TEM .............................................................................. 19
  1.5.3 XFEL another new technique ............................................................... 19
1.6 Thesis outline ..................................................................................................... 19

Chapter image: this is a very nice example of a diffraction pattern; the almost perfect symmetry reminds of stars, the spots as well as the shape (Fig 1.4)
Chapter 1

1.1 Introduction

Electron Diffraction is used for structure determination since early last century. The first years it was mainly used to study metals, alloys, etc. The different techniques which have been developed for either imaging or diffraction are numerous, for example: alloy determination, consistencies of metals, glasses and polymers. (Williams and Carter, 2009). However, to study samples of organic biological origin soaking them in heavy metals, replacing the water (dark staining) was the only way to image them; by using heavy metals the contrast could significantly be improved. This method is still being used a lot, to study tissues in biology. It is, however, almost impossible to use heavy metal soaking to determine the structure of smaller things like proteins, or to achieve a resolution better than 12-20Å. Fifty years ago, a new technique was developed for Electron Microscopy (EM) which involved cryo-freezing the sample to -180 °C by plunge freezing (Taylor and Glaeser, 1974; Klug, 1979). This made it possible to study biological and organic samples embedded in amorphous ice for a longer time and at higher energies. Because of evolving technologies and the introduction of direct electron detectors like the Gatan K2 (Gatan, 2015) and the FEI Falcon 2 cameras (FEI 2015), resolutions of up to 2.2Å are now reported (Bartesaghi et al., 2015).
1.2 Transmission Electron Microscopes

A big variety of transmission electron microscopes (TEMs) is available (for example: HRTEMs, STEMs, HVEMs and cryo-TEMs), while all are slightly different, they are based on the same basic TEM principle: Using electrons which are transmitted and scattered through the sample and can then visualized by different electron detection systems.

Currently two different techniques exist to generate electrons in TEM with an Electron Gun (EG): thermionic and field emission guns. Thermionic sources consist of either tungsten filaments or lanthanum hexaboride LaB$_6$ crystals. The latter is now more common than the former. The Field Emission Gun (FEG) is getting more common as a source of electrons in TEM, especially at high end machines. The beam can be smaller, more monochromatic and more coherent than the older thermionic EGs. This improves the Signal to Noise (S/N) ratio and spatial resolution.

The illumination area of the sample is determined by one or more condenser apertures and lenses. The condenser apertures limit the amount of electrons which move through the illumination system towards the sample. The condenser lenses are also responsible for the exposure area on the sample, by changing to a converged, parallel or divergent beam. In a parallel beam condition the condenser aperture determines the total exposed area on the sample.

The beam passes through the objective aperture, the sample, the objective-, diffraction-, intermediate and projector lens as well as the selected area aperture. After which it enters the camera chamber.

On older TEMs the image is usually formed on the fluorescent screen which can be observed through a lead-glass window. On more modern TEMs this fluorescent screen is no longer visible from the outside and is integrated in the TEM with a (CCD) camera. Below the fluorescent screen is usually room for one or multiple detectors and/or imaging systems. Originally these detection systems consisted either of film, image plate or CCD cameras. (Figure 1.1 shows the common lay-out of a TEM).

Recently direct electron detectors have been developed like the Gatan K2 (Gatan, 2015) and the Falcon 2 (FEI, 2015), which have, for imaging, a much higher throughput and S/N ratio than CCD cameras (McMullan et al., 2014). Because of the way they are designed these cameras are easily damaged when used during diffraction experiments. To prevent damage to these sensitive
cameras, hard coded restrictions are in place, to make sure they are not used under such locally intense beam conditions. Because of this, they are not suitable replacements for either image plate, film or CCD cameras, when used for diffraction experiments. An example of a detector which can handle these more extreme condition is of the Medipix family (Chapter 2). The full energy of the impacting electron is captured in the sensitive layer and the underlaying electronics are thereby protected from damage. Also the ability to discriminate between high energy electrons and particles with a lower energy (for example the abundant 5-10 keV X-rays) make it possible to detect Bragg spots with intensities of only a couple of electrons above the electron scatter noise.

Figure 1.1: Typical transmission electron microscope schematic. Gun system, apertures and lens systems. (Source wikipedia)
1.3 Electron scattering and radiation damage

An electron is a negative charged particle with low mass, therefore it is easily influenced by other electrons and/or the positive nucleus of an atom. These Coulomb interactions are well described in literature and understood. These interactions cause the electron to scatter, either elastic, inelastic or dynamically. (Wang, 1995; Cheng, 2013; Williams and Carter, 2009)

Elastically scattered electrons are usually coherent, which means that they stay in phase with each other. Elastic scattering usually occurs at low angles of about 1-10°, where at higher angles they become more and more incoherent. Inelastic scattering is in almost all cases incoherent and scattering usually occurs under < 1°. Dynamical scattering happens when an elastically scattering electron scatters multiple times in the sample. The thicker the sample the more often this can occur, this makes it almost impossible to determine where the electron scattered the first time. (Fig. 1.2). Lastly there are the electrons which are unaffected when transmitted through the sample (end up in the central beam in diffraction mode). As soon as the electrons have been transmitted through the sample the electrons are no longer in phase final which results is in a non uniform distribution.

Table 1.1: Energy deposited in biological/organic specimens per useful scattering event. (Henderson, 1995)

<table>
<thead>
<tr>
<th></th>
<th>Electrons</th>
<th>X-rays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200-300 keV</td>
<td>1.5Å</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Ratio (inelastic/elastic)</td>
<td></td>
<td>~5000</td>
</tr>
<tr>
<td>Mechanism of radiation damage</td>
<td>Secondary e⁻ emission</td>
<td>Photoelectric e⁻ emission</td>
</tr>
<tr>
<td>Energy deposited per inelastic event (eV)</td>
<td>20</td>
<td>8000</td>
</tr>
<tr>
<td>Energy deposited per elastic event</td>
<td>60</td>
<td>80'000</td>
</tr>
<tr>
<td>Energy deposited relative to electrons</td>
<td>(inelastic)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(elastic)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10000</td>
</tr>
</tbody>
</table>
Figure 1.2: Scattering in crystals. Top: kinematic 'single' diffraction (blue) coincides with dynamic diffraction (red). Bottom: fraction of scattered electrons in protein crystals at 300keV
1.3.1 Imaging versus diffraction

In imaging the spatial distribution of the electrons scattering is used; this can be observed as contrast in the image. This contrast can be enhanced when using the objective aperture. When doing diffraction experiments the angular electron scattering distribution is used. In imaging the unaffected electrons are interfering with the information the scattering electrons carry by adding shot noise, whereas for diffraction all these non-information carrying electrons are contained in the central peak (direct beam). Therefore, in diffraction, images are considered to be less ‘noisy’, because there is no shot noise in added to the information carrying signal.

Inelastic scattering leads to beam damage of the sample. This damage is called radiolysis: the interaction of the electron transmitted from the EG with usually another electron in the sample (e.g. ionisation). Radiolysis breaks the chemical bonds between atoms within a molecule. Two more types of damage also exist: (i) Sample heating by phonons. This is a major source of damage in organic materials (hence cryo-cooling). (ii) Knock-on damage, where the atom gets displaced in the lattice. Because this effect is more rare than the other two the knock-on effect is much less a factor when working with beam sensitive materials like organic compounds, proteins or zeolites.

The most common way to prevent beam damage is to: (i) use techniques which make use of a low electron dose on the sample. (ii) coat the specimen with heavy metals; (iii) to cool the sample to very low temperatures. Low temperature does not only prevent beam damage but also prevents the sample from losing all the water content to the vacuum of the TEM when the sample contains water. (Protein crystals contain 35%-70% water). While the beam damage described above is a problem in EM, X-rays tend to damage the sample more per diffracted quantum than electrons (Henderson, 1995) (Table 1.1). Thus the interest in researching new EM methods for protein 3D protein crystallography and single particle imaging.

![Chemical structure perylene derivative](image-url)
1.4 Organic molecules

The size of organic molecules can vary from a few atoms to thousands of atoms in big structures like proteins. These molecules can be studied by different techniques like X-ray crystallography, SSNMR and single particle cryo-EM for big protein molecules and clusters. The importance of solving the 3D structure can vary by the type of molecule as well as its function. For example, the crystal structure information or packing is more important than solving the 3D structure for small organic compounds; because the crystal size and structure can say something about properties like solubility, which is very important for drugs. Secondly for proteins the packing itself is often not interesting but is essential for solving the 3D structure. Knowledge the 3D structure will help biochemists to understand the function and active sites of the protein, which may help to develop the drugs.

Figure 1.4: Diffraction pattern from perylene derivative.
1.4.1 Small and big organic compounds

There are many different techniques available to look at the packing of small organic crystals, for example: (i) single crystal X-ray diffraction and solid state NMR (SSNMR) for micrometer sized crystals. (ii) X-ray / electron powder diffraction for smaller crystals.

When these organic crystals are used in drugs or food additives, one of the most important characteristics is their solubility; this is directly linked to how the compounds are stacked in the crystal (Ozaki et al., 2014). While crystals can usually grow into bigger crystals, smaller crystals in the same sample can be a totally different crystal polymorph. For example, in powder diffraction it is possible that the signal of a low-abundant polymorph (< 5%) can be completely hidden within the brighter signal of the more abundant polymorph. (Chapter 6)

Because of technical advances in EM, many different methods have been developed for looking at 3D nano-crystals of organics with Cryo-TEM. Examples are: automated diffraction tomography (ADT) (Kolb et al., 2008; Kolb et al., 2007; Mugnaioli et al., 2009) and rotation electron diffraction (RED) (Wan et al., 2013; Yun et al., 2015; Zhang et al., 2010). These two techniques show solved crystal structures of beam sensitive compounds ab initio from 3D ED data acquired at low electron dose from cryo-cooled samples. However, there are multiple reasons to consider doing experiments at ambient temperature when possible. (i) Interference of ice crystals by ice formation during sample load; (ii) necessity of in-column cryo-plates to reduce contamination; (iii) possible change in crystal structure; (iv) reduced sample throughput, as loading/unloading samples can take up to 2 hours. There are examples of beam sensitive materials solved at ambient temperature by ED, but these structures were solved using the non–continuous ADT technique, e.g. Benzamides (Gorelik et al., 2012) and 9,9’-bianthracene-10-carbonitrile (CNBA) (Kolb et al., 2010).
In chapter 6 we discuss a method which overcomes these challenges by solving the structure of compounds \textit{ab initio} at ambient temperature conditions of sub-micrometer nano-crystals. We demonstrate this technique with two pharmaceutical organic compounds (carbamezopine and nicotinic acid). Data from XDS were prepared with XPREP (Bruker, 2004) for \textit{ab initio} structure solution using the program SHELXT; subsequent refinement of the given structure was done with SHELXL and ShelXle (Sheldrick, 2008; Hübschle et al., 2011). The eight parameter fitting for electron scattering factors from (Peng, 1999) was used for SHELXL. The carbamezopine structure was refined anisotropically unrestrained except for the RIGU restraint (Thorn et al., 2012). Nicotinic acid was refined isotropically without any restraints.

The method has also been used to get an estimate of the packing of a perylene derivative crystal, a large antenna complex of 1427 g/mol. The crystals of this compound do not grow into consistent crystals of sufficient size to be analyzed by X-ray crystallography. TEM electron nano-diffraction allowed to estimate the unit cell with XDS, which than could be used to constrain the data obtained by SSNMR and X-ray powder diffraction (XRPD). This shows that a combination of these techniques can be combined. (Not discussed in this thesis. Added for completeness)

1.4.2 Proteins

There is a huge interest in the 3D structure of proteins. The structure can give information about the purpose but also malfunctions of the protein when certain DNA mutations inserted a different amino acid in the chain. Also the 3D structure makes it possible to design drugs or test compounds \textit{in silico}. Because of this interest in the 3D structure different methods and techniques have been developed not only in X-ray but also in EM. The main techniques for EM are described below.
1.4.3 Single particle imaging

Currently, single particle imaging of large protein complexes is evolving at a rapid pace. Recently a structure of β-galactosidase has been reported with a resolution up to 2.2Å (Bartesagh et al., 2015). One of the reasons is the quick advancement in resolution comes with the developments in semi conductor technology. This made it possible to design direct electron detectors like the Falcon 2 and the K2. Also the increase in computing power in combination with new algorithms make it possible to rapidly analyze big datasets which consist of thousands of images with hundreds of thousands particles in random orientations. However, also the improvements are made in the microscopes themselves with Cs aberration correctors (Batson et al., 2002), better coherent XFEG guns and phase plates (Marko and Danev, 2012).

1.5 Crystal diffraction

Another way of solving 3D structures of proteins is by crystallizing them. There are however some bottlenecks when it comes to solving protein 3D structures from crystals. The most important of these is that some proteins do not crystallize into the micro size crystal that is needed for X-ray diffraction systems. If it is too small the crystal will be destroyed before sufficient data can be acquired.

1.5.1 2D crystals by TEM

2D crystals are often used for membrane proteins and usually contain lipids and detergents. Techniques available from single particle imaging can be used since the crystals are made out of a single layer of proteins which are aligned all in the same orientation. However 2D crystals are prone to bending and cracking. Algorithms can “unbend” the crystal. The sample is usually rotated and a tomography series is taken of the crystal. Because of the high symmetry, diffraction patterns can be taken to help with the unbending and padding of the crystal. The crystal is usually vitrified or negatively stained. (Henderson and Unwin, 1975; Gil et al., 2006; Zeng et al., 2012; Raunser and Walz, 2009)
1.5.2 3D crystals by TEM

Large protein(s) (complexes) can be solved with single particle imaging and micrometer sized ‘thick’ crystals can be solved with X-ray diffraction; however, some proteins are just too small to be solved with single particle EM and other proteins do not grow into the large crystals needed for X-ray crystallography. These proteins form a target for electron nano-crystallography with TEM and X-ray Free Electron Laser (XFEL). Currently two approaches are developed for TEM: the microED method (Hattne et al., 2015) and our method of thin 3D electron nano-crystallography (Chapter 4, 5 and 6).

1.5.3 XFEL another new technique

X-ray Free Electron Lasers (XFELs) are also a valid instrument to study 3D nano-crystals. These can be used to collect datasets from crystals as small as 150 nm. Many crystals are needed to collect one full dataset since every crystal can only be measured (1-5 ml highly concentrated crystal solution is needed). With the intensities used in the XFEL the crystal gets vaporised (Lomb et al., 2011). After collection of the full dataset the pseudo randomly orientated diffraction datasets need to be analyzed and at least 10k images are needed for high resolution 3D reconstruction (comparable to single particle imaging). (Kirian et al., 2011)

Because it is really hard to get a good sample for XFEL and the running costs an interesting solution to check the sample quality is to diffract them on TEM before acquiring a dataset on the XFEL. The XFEL allows collecting data at room temperature and recently also previously unknown structures have been solved using XFEL data (Kang et al., 2015)

1.6 Thesis outline

Combining the phases acquired from images of protein 3D nano-crystals (Nederlof et al., 2013a) and diffraction data from electron nano-crystallography should make it possible to solve the structure of proteins. In this thesis I describe: (i) use of the special Timepix detector to get excellent diffraction data (Chapter 2, 4 & 6); (ii) growth of protein crystals (Chapter 3); (iii) acquiring diffraction datasets with electron microscopy (Chapter 4 & 6); (iv) improvement on the algorithm and filtering of images of 3D protein crystals (Chapter 5). As well as using these techniques for ab initio structure determination of organic compounds by direct methods without the use of cryo-TEM (Chapter 6).