Carbon Nanotubes as Diffraction Microscopy Mounts

Lisa Larrimore

February 1, 2006

Question for the A Exam from Professor Veit Elser

Consider the possibility of using carbon nanotubes as a sample mount for individual biomolecules in a diffraction microscopy apparatus. You should consider this question in the context of developing a general-purpose method to replace the huge efforts currently employed in structural biology. For example, can you think of a way to make arbitrary proteins stick to a nanotube (at some low linear density)? Will there be problems getting the molecule-coated nanotube into the high vacuum environment of the electron microscope? Can the nanotube be rotated in order to obtain multiple views, or should one translate the tube along its length and thereby expose other molecules, with different orientations, to the beam? Please consider some of these practical issues and of course any others that occur to you. If it looks like this proposal has serious problems you are only expected to bring them to light, not solve them!

Determining protein structures is one of the most important challenges facing biologists today. Existing techniques such as x-ray crystallography require many months of work to image a single protein, and there are approximately $10^6$ human proteins that cannot be imaged at all using existing methods. Membrane proteins, for example, are very difficult to crystallize, but understanding their structure is critical for applications such as drug delivery (Spence and Doak, 2004).

One possible solution is to image individual proteins using electron diffraction. Because electrons have a larger scattering cross section than x-rays, it is possible to collect a diffraction pattern from a single molecule, as was recently demonstrated by Zuo et al. (2003) with a carbon nanotube. One of the major challenges in collecting diffraction data from a single protein is the method of holding the protein: the mount must be either removed from the electron beam or well-characterized at the atomic scale so that its diffraction pattern can be subtracted from the data. Here we consider the possibility of using a carbon nanotube as the mount for individual proteins in an electron diffraction experiment.

We will first introduce the principles of diffraction in Section 1 and will describe diffraction theory in more detail in Section 2. In Section 3, we will investigate how
electron diffraction can be used to image individual molecules. Finally, in Section 4 we describe an experimental procedure for using carbon nanotubes to support proteins for imaging with electron diffraction, along with the problems that may be encountered.

1 Introduction to Electron Diffraction

Diffraction occurs when any kind of coherent wave—a sound wave, an electromagnetic wave, or a particle like an electron or neutron—meets an obstruction. The basic principles of diffraction are the same for all of these waves; the main difference is the way the wave interacts with the obstruction. Waves of shorter wavelength, for example, generally produce higher resolution images. For electromagnetic waves, the wavelength is around 500 nm for visible light and 100 pm for x-rays, while the wavelength of electrons in a 200-keV transmission electron microscope (TEM) is 2.5 pm.

For diffraction imaging, another important distinction between different waves is the scattering cross section, which effectively measures how large an object appears to those waves. Since x-rays are scattered by individual electrons, the natural length scale for x-ray diffraction is the classical electron radius \( r_e = \frac{e^2}{4\pi\epsilon_0 m_e c^2} = 2.8 \times 10^{-15} \) m. Electrons, however, are scattered by the screened nuclear Coulomb potential, and so the relevant length scale is the Bohr radius \( a_0 = \frac{4\pi\epsilon_0 \hbar^2}{m_e e^2} = 0.53 \times 10^{-10} \) m (Lucas and Lambin, 2005). Electrons therefore have a larger scattering cross section than x-rays, which means it is possible to collect a signal from a much smaller sample.

The basic principle of diffraction imaging for an optical system is shown in Figure 1. A plane wave incident on an aperture creates a far-field diffraction pattern that is the Fourier transform of the aperture. A transform lens can be used to pull in this diffraction pattern to the lens’s back focal plane. The image of the aperture can then be seen at the image plane conjugate to the object plane, and a second lens can be used to put this image at a convenient distance. Alternatively, the far-field diffraction pattern can be directly recorded, either far from the aperture in a lensless system, or at the transform plane after a single lens. The image of the aperture can be recovered by taking the inverse Fourier transform of this diffraction pattern numerically, although in Section 2 we will see that there is an added complication of retrieving the phases that are lost in the measurement.

In electron diffraction, which is often performed in a TEM, the curved glass lenses that bend light are replaced with electromagnetic lenses that bend beams of electrons. These lenses can be used to obtain a direct image of the object being probed, but inherent aberrations limit the resolution of the image to 1-2 Å, about 50 times larger than the de Broglie wavelength of a 100-200 keV electron (Wu et al., 2005). For high-resolution structure analysis, it is better to record the diffraction pattern and then use a computer instead of a lens to take the Fourier transform (Yonekura et al., 2002).

In the following section, we explain why the far-field diffraction pattern and the sample are related by a Fourier transform, and we introduce the phase problem that is inherent in this method. We will then see how electron diffraction can be used to image an individual molecule.
Figure 1: The light diffracted by a cutout of the letter E at the front focal plane of the transform lens converges to form the far-field diffraction pattern at the back focal plane, or the transform plane. This diffraction pattern is the Fourier transform of the letter E. A second imaging lens is used to project this pattern onto the image plane. Alternatively, the E could be imaged by taking the inverse Fourier transform mathematically, either from the focal plane of the transform lens, or from the far-field diffraction pattern in a lensless setup. (Upper image based on Figure 13.31 from Hecht, 2002.)

2 Electron Diffraction Theory

The Schrödinger equation for elastic electron scattering\(^1\) by a potential \(V(\vec{r})\) is

\[
-\frac{\hbar^2}{2m} \nabla^2 \psi - eV(\vec{r}) \psi = E \psi. \tag{1}
\]

We can express the electron wavefunction \(\psi\) as a superposition of the incident and scattered waves,

\[
\psi = \psi_0 + \psi_s \xrightarrow{r \to \infty} e^{i\vec{k}_0 \cdot \vec{r}} + A(\vec{k}, \vec{k}_0) e^{ikr}, \tag{2}
\]

\(^1\)In a diffraction experiment, electrons may undergo elastic scattering, in which they do not change the atomic states of the scatterers, or inelastic scattering, in which they transfer energy via plasmon, single electron, or phonon excitations. For high-energy electron diffraction, inelastic scattering processes are usually much less significant than elastic ones (Peng et al., 2004, p. 3-4), and they will not be discussed here. In experiments where the background noise from inelastically scattered electrons affects the quality of the signal, these electrons can be removed with an energy filter (Yonekura et al., 2002). In Section 4.3, we will see that the bigger problem with inelastic scattering is the resulting radiation damage to the specimen.
where we have assumed that the incident wave is a plane wave\(^2\) with wave vector \(\vec{k}_0\) and have taken the asymptotic form of the scattered wave (with wave vector \(\vec{k}\)) far from the scattering center (Shankar, 1994, p. 527). Note that by conservation of energy, \(|\vec{k}| = |\vec{k}_0|\). We call the function \(A\) the scattering amplitude, which has no dependence on \(\vec{r}\).

In scattering experiments, the quantity measured is not the wavefunction; it is the differential scattering cross section \(d\sigma/d\Omega\), which is defined by

\[
\frac{d\sigma}{d\Omega} \equiv \frac{\text{number of particles scattered into } d\Omega/\text{sec}}{\text{number of incident particles/sec/area}} = \frac{\vec{j}_s \cdot \hat{e}_r r^2 d\Omega}{|\vec{j}_0|}, \tag{3}
\]

where \(\vec{j}_s\) and \(\vec{j}_0\) are the probability current densities given by (Shankar, 1994, p. 166)

\[
\vec{j} = \frac{\hbar}{2m} (\psi^* \nabla \psi - \psi \nabla \psi^*). \tag{4}
\]

Using the expressions for the wave functions from Eq. 2, we find that

\[
\vec{j}_0 = \frac{\hbar \vec{k}_0}{m} \quad \text{and} \quad \vec{j}_s = \frac{\hbar k}{m r^2} |A|^2. \tag{5}
\]

When we substitute these expressions into Eq. 3, we find that the differential cross section is simply given by

\[
\frac{d\sigma}{d\Omega} = |A|^2. \tag{6}
\]

For a single molecule like a carbon nanotube, electron scattering is weak and can thus be well described by the kinematic (or first Born) approximation (Zuo et al., 2003).\(^3\) With this assumption of single-scattering events and by using a Green’s function, we find that the scattering amplitude is given by the Fourier transform of the scattering potential,

\[
A(\vec{q}) = -\frac{m}{2\pi \hbar^2} \int V(\vec{r}) e^{-i\vec{q} \cdot \vec{r}} d\vec{r}, \tag{7}
\]

where \(\vec{q} \equiv \vec{k} - \vec{k}_0\) (Shankar, 1994, p. 534-540).\(^4\) We can thus find the real-space potential, and the information it gives about atomic positions in the molecule being probed, by taking the Fourier transform of the scattering amplitude:

\[
V(\vec{r}) = -\frac{2\pi \hbar^2}{m} \int A(\vec{q}) e^{i\vec{q} \cdot \vec{r}} \frac{d\vec{q}}{(2\pi)^3}. \tag{8}
\]

\(^2\)For electron diffraction with a TEM, the incident wave actually has two components—a plane wave plus a peak wave packet—and its coherence is limited by lens aberrations (Zuo et al., 2003).

\(^3\)Even when scattering off a single atom, a high-energy electron actually frequently undergoes multiple scattering, but it turns out that this affects the phase far more than the scattering amplitude. Since only the amplitude is measured in conventional electron diffraction, the kinematic approximation is often sufficient. For techniques that are sensitive to the phase, such as electron holography, a more detailed treatment is necessary (Peng et al., 2004, p. 24-32).

\(^4\)You may often see \(A\) written as \(A = \sum_j f_j(\vec{q}) e^{i\vec{q} \cdot \vec{r}_j}\), where \(f_j(\vec{q})\) is called the atomic form factor and is the Fourier transform of the atomic potential for the \(j^{th}\) scatterer (see, e.g., Ashcroft and Mermin, 1976, p. 107).
The difficulty here is that, as we saw in Eq. 6, the measured quantity is $|A(q)|^2$, not the complex amplitude $A(q)$. Taking the square root gives the magnitude of $A(q)$, but not the phases. It is possible to recover these phases, however, by making certain assumptions about $V(r)$; this is known as the phase retrieval problem, and the development of efficient and accurate phase retrieval algorithms is an active area of research (see, e.g., Elser, 2003). Recent advances in phase retrieval have been as important as experimental developments for improvements in electron diffraction accuracy (Zuo et al., 2004).

3 Imaging Nanostructures with Diffraction

We have now seen that from a diffraction pattern, we can recover the structure of the scattering atoms, and we know that the higher scattering cross section for electrons means that it is possible to collect scattering data from a much smaller sample than for x-rays. In fact, Zuo et al. (2003) have recently shown that the structure of an individual double-walled carbon nanotube (DWNT) can be determined by electron diffraction with a 200-keV TEM, and their experimental setup and data are shown in Figure 2. The diffraction pattern of a 50-nm diameter electron beam incident on a suspended DWNT is collected with digital imaging plates, and the nanotube structure is then determined using a phase retrieval algorithm. Zuo et al. refer to this measurement technique as coherent nano-area electron diffraction (NED).

![Figure 2: Electron diffraction from a DWNT. (A) Schematic ray diagram. (B) Recorded diffraction pattern. (C) Reconstructed DWNT image and a structural model based on the diffraction pattern. (Zuo et al., 2003)](image)

Another technique for obtaining electron diffraction from nanoscale objects is electron nanodiffraction (END), which is performed with a dedicated STEM. In this technique, a convergent (rather than parallel) electron beam is focused on a small disk in the sample, and the diffraction pattern of this disk is collected (Crowley, 2004). END has been used to study various nanoparticles, including individual CdSe quantum rods.

---

5In their original Science paper, they use the acronym NAED, but in a longer paper the following year this is shortened to NED (Zuo et al., 2004).
in the Silcox lab at Cornell (Yu et al., 2005), but it is difficult to obtain structural information about an entire sample through this method (Zuo et al., 2004).

4 Carbon Nanotube Diffraction Mounts

To study an individual molecule using electron diffraction, it must be isolated from any large structures that would interfere with the diffraction pattern. Zuo et al. (2003) were able to look at an isolated section of nanotube by suspending the nanotube across a TEM grid. A similar trick would not be possible for proteins, since they lack the high aspect ratio and periodic structure of carbon nanotubes. In the following sections we investigate the possibility of using an individual nanotube as a mount for a protein. Since the structure of the nanotube could be characterized (e.g., by looking at the diffraction pattern from a section without any attached molecules), it should be possible to isolate the protein diffraction pattern from that of its nanotube mount. We will now examine the specific steps that would be needed to perform this experiment: growing carbon nanotubes, attaching proteins to them, measuring the diffraction pattern, and analyzing the data with phase retrieval algorithms to obtain the protein structure.

4.1 Nanotube Growth

The nanotubes that Zuo et al. (2003) used for their diffraction experiment were grown directly on a gold TEM grid using chemical vapor deposition (CVD). This is a logical choice, since the nanotubes must be suspended (so that the substrate does not interfere with the diffraction pattern) and since the diffraction experiment will be performed in a TEM. It is not even necessary that the nanotube reach across an entire gap: there only needs to be enough of the nanotube over vacuum so that the electron beam can diffract off the nanotube without hitting the TEM grid.

Zuo et al. (2003) grew their nanotubes by spinning an Al₂O₃-supported Fe₂O₃ catalyst on TEM grids and then putting the grids in a CVD furnace and flowing methane and hydrogen at 700-900 °C. They chose to image a DWNT because the atomic structure of single-walled carbon nanotubes (SWNTs) has already been characterized. If the nanotube is only being used as the sample holder, however, the simpler SWNT structure would be preferable to aid in separating the nanotube and protein diffraction patterns. The nanotube growth process used by Zuo et al. is very similar to that used in the McEuen lab at Cornell, where nanotubes have also been grown on TEM grids. Obtaining SWNTs for this diffraction experiment should therefore be straightforward.

4.2 Attachment of Proteins to the Nanotubes

There are a number of techniques for attaching biomolecules to the surfaces of carbon nanotubes. Tsang et al. (1995) first immobilized proteins on and inside open-ended multi-walled nanotubes by simply putting the NTs and proteins in the same solution and finding that they adsorbed to each other. They repeated these experiments with several other proteins and with DNA molecules, and used the nanotube supports to
Figure 3: Two approaches for attaching a protein to a nanotube. (A) Nonspecific binding. Many proteins spontaneously adsorb to NTs due to hydrophobic interactions (Chen et al., 2003). (B) Functionalizing a SWNT with an arbitrary protein using 1-pyrenebutanoic acid, succinimidyl ester. The pyrenyl group interacts noncovalently with the nanotube through π-stacking, and the succinimidyl ester reacts with amines on the protein surface to form an amide bond (Chen et al., 2001).

image the biomolecules with a transmission electron microscope (see, for example, Guo et al., 1998). Chen et al. (2003) later found that many proteins (streptavidin, avidin, bovine serum albumin, α-glucosidase, and staphylococcal protein A) spontaneously adsorb on SWNTs, and Bradley et al. (2004) found the same result when electrically sensing streptavidin with a SWNT-FET. An illustration of globular protein adsorption to a nanotube is shown in Figure 3A.

A second approach to functionalizing SWNTs with proteins was developed in Hongjie Dai’s laboratory by Chen et al. (2001), and is depicted in Figure 3B. They incubated the nanotubes in a solution of 1-pyrenebutanoic acid, succinimidyl ester, which is the molecule in the upper left of Figure 3B. Just as one graphene sheet is attracted to another, the pyrenyl group in this molecule is attracted to the nanotube sidewall; this interaction between the π electrons in the aromatic rings is known as π-stacking. The nanotubes were then soaked in a protein solution, and the amines that exist on the surface of most proteins reacted with the succinimidyl ester to form an amide bond. Chen et al. demonstrated this method by functionalizing SWNTs with ferritin, streptavidin, and biotinyl-3,6-dioxaocanediadmine (biotin-PEO-amine), and Besteman et al. (2003) later copied their protocol to attach glucose oxidase to a SWNT-FET for electrical measurements.

Dai and co-workers have also developed a number of protocols to allow only specific proteins to bind, such as coating the nanotube with a polymer which is attached to the receptor of the protein of interest, so that only that protein will bind to the nanotube (Shim et al., 2002; Chen et al., 2003, 2004). While this is important for sensing a particular protein in a solution of many biomolecules, it would be easier to simply start with a pure protein solution for an electron diffraction experiment. Other methods for
covalently attaching proteins to nanotubes (e.g., Huang et al., 2002; Bhattacharyya et al., 2005) are also more complicated than is necessary.

Depending on the protein of interest, we conclude that one of the two approaches shown in Figure 3 should be used to attach it to nanotubes on a TEM grid. For most proteins, non-specific adsorption is probably easiest. For proteins like ferritin that do not adsorb non-specifically to nanotubes (Chen et al., 2001), a linker molecule should be used.

We must also consider the linear density of proteins along the nanotube, which must be low enough that only one protein is in the electron beam at a time. The beam diameter used by Zuo et al. (2003) to image a DWNT was about 50 nm, so we will use this as a guideline. The linear density of adsorbed proteins has not been quantitatively considered in nanotube functionalization experiments so far, and since the goal of these experiments has often been to use the functionalized nanotube as a sensor, a high linear density was usually preferred. We can get a rough estimate, however, from some published images of functionalized nanotubes, shown in Figure 4. In both AFM images of nanotubes grown on SiO$_2$ substrates (one showing non-specific adsorption and one showing attachment with a linker molecule), there are protein

Figure 4: Images of proteins adsorbed to SWNTs. (A) AFM image of staphylococcal protein A nonspecifically adsorbed on a nanotube. NTs were grown on SiO$_2$ and then immersed in a 10-50 nM protein solution for 1 hour (Chen et al., 2003). There are roughly 30 nm/protein. (B) AFM image of ferritin molecules on a 4.5-nm bundle of SWNTs that have been functionalized with the linker molecule 1-pyrenebutanoic acid, succinimidyl ester. NTs were grown on SiO$_2$, immersed in a 6 mM linker molecule solution for 1 hour, and then immersed in a 10 µg/mL (20 nM) ferritin solution for 18 hours (Chen et al., 2001). There are roughly 65 nm/protein. (C) TEM image of ferritin on an individual SWNT that was first functionalized with the linker molecule. The dark round spots are the ferritin iron cores. Suspended NTs were grown on TEM grids, immersed in a 6 mM linker molecule solution of the linker molecule, and then immersed in a 5 mg/mL (10 µM) ferritin solution for 18 hours (Chen et al., 2001). There are roughly 8 nm/protein.
molecules separated from their neighbors in a 50 nm gap, wide enough for the electron beam. The spacing between proteins is lower (roughly 8 nm per protein) in the image of a suspended nanotube on a TEM grid, but Chen et al. (2001) used a 500-times more concentrated protein solution for their TEM-imaged NTs than for the AFM-imaged ones. It should therefore be possible to achieve our desired linear density by adjusting the protein concentration and the amount of time the NTs are immersed in the protein.

4.3 Measurement of the Diffraction Pattern with a TEM

Zuo et al. (2003) obtained the diffraction pattern from a carbon nanotube with a 200-keV JEOL2010 TEM in the nanodiffraction mode. The Cornell Center for Materials Research (CCMR) Microscopy Facility maintains two 200-keV TEMs—a LEO 922 EFTEM and a FEI Tecnai G20 TEM/STEM—which could be set up for imaging our nanotube. The most serious difficulties with this whole experiment, however, will be in actually acquiring the protein diffraction pattern without changing the structural configuration of the protein or completely destroying it with the radiation from the electron beam.

As Figure 4C shows, it is possible to image proteins attached to a suspended carbon nanotube in a high-vacuum TEM environment; this image, however, is at low-resolution, and the proteins are identified only by their dark iron cores. The natural environment of a protein is an electrolyte solution, so high vacuum can cause some structural deterioration. Furthermore, a protein can be quickly damaged by the radiation resulting from inelastic scattering of the electron beam. Most biomolecules cannot withstand an electron dose greater than 10 e/Å², whereas Zuo et al. (2003) needed a dose of 10³ e/Å² to image their DWNT at 1-Å resolution. They note that a higher molecular weight molecule would require a lower dose, but their DWNT had a similar weight to many proteins.

One technique that may help is cryogenic cooling, in which the sample is rapidly frozen by plunging it into liquid ethane and then transferred to the electron microscope while cooled to liquid nitrogen temperatures. This technique was developed in the 1980s to limit radiation and dehydration damage to a biological specimen in a TEM, and it has been used to image crystallized proteins with resolutions of a few angstroms. Cooling a biomolecule can increase its threshold electron dose by factors of 10-30 (van Heel et al., 2000). The LEO 922 EFTEM run by the CCMR has a cryo-transfer stage that we could use to image our sample at low temperatures.

By combining cryogenic cooling with the choice of a very high molecular weight protein, it may be possible to collect a diffraction pattern at a slightly lower resolution (say, 3 Å instead of 1 Å) before there is significant structural damage to the protein. It might be necessary, however, to incorporate more tricks for decreasing radiation

---

6The electron nanodiffraction experiments used to study individual CdSe quantum rods at Cornell were performed in an STEM with assistance from CCMR research specialist Mick Thomas (Yu et al., 2005).

7From the dimensions given by Zuo et al. (2003), the 50-nm section of the DWNT that they imaged weighed about 0.8 attograms, corresponding to a molecular weight of 500 kDa, which is a typical value for proteins.
damage, like the techniques in electron nanodiffraction for recording diffraction from only the first electrons to strike a sample (Crowley, 2004), before electron diffraction of individual proteins will become feasible.\(^8\)

Finally, if it is possible to obtain a diffraction pattern from an individual protein attached to a carbon nanotube, this will provide only a two-dimensional image of the structure. To obtain the full three-dimensional structure, one could rotate the nanotube in order to expose different views, but this would exacerbate the problem of frying the protein with radiation. A better solution would be to image different sections of the nanotube that are attached to proteins in different orientations, and then to combine these images to form the three-dimensional structure.

4.4 Phase Retrieval

If we are able to overcome the problem of radiation damage and obtain a diffraction pattern, then it will be necessary to use a phase retrieval algorithm to reconstruct the phases, as discussed in Section 2. Although there will be an added difficulty of separating the diffraction from the nanotube and the protein, an algorithm used for phase retrieval on x-ray diffraction data (Shapiro et al., 2005) should be able to be adapted for this purpose (P. Thibault, personal communication).

5 Conclusion

We have seen that using a carbon nanotube as a sample mount for individual biomolecules should be a straightforward application of existing procedures: the nanotubes could be grown on a TEM grid and then functionalized with an arbitrary protein at a specific linear density. It would be a challenge, however, to then obtain a diffraction pattern from an individual protein before it is destroyed by radiation damage. Although cryogenic cooling and the use of a large protein would mitigate this damage, it will probably not be feasible to produce a very high resolution image with current techniques. If electron diffraction of a single biomolecule becomes possible, however, a carbon nanotube should be an ideal candidate for holding the sample.

\(^8\)A different idea that has been proposed to overcome the radiation damage problem is to combine the data from a large number of identical proteins, each of which sees only a small number of electrons. Each protein could be enclosed in a liquid helium droplet and aligned using a polarized laser, and the diffraction pattern could be measured as the jet of helium droplets traverses the electron beam (Spence and Doak, 2004). Although each component of this proposal has been experimentally demonstrated, there are many technical challenges involved and they have not yet been combined.
References


