Single Molecule Diffraction

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For solving the atomic structure of organic molecules such as small proteins which are difficult to crystallize, the use of a jet of doped liquid helium droplets traversing a *continuous* high energy electron beam is proposed as a means of obtaining electron diffraction patterns (serial crystallography). Organic molecules (such as small proteins) within the droplet (and within a vitreous ice jacket) may be aligned by use of a polarized laser beam. Iterative methods for solving the phase problem are indicated. Comparisons with a related plan for pulsed x-ray diffraction from single proteins in a molecular beam are provided.

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The structure determination of the approximately 10^6 human proteins which cannot (or are very difficult to) crystallize is perhaps the most important challenge currently facing structural biology. Examples include the class of membrane proteins (which comprise about 30% of all proteins), important for drug delivery. It has been estimated that 70% of drug molecules interact with a membrane protein. Our understanding of drug delivery and action (for example, by cocrystallization of the drug molecule bound to a protein) is thus critically limited at present by the great difficulty in finding appropriate detergents which will facilitate crystallization of the hydrophobic membrane proteins, only about 45 of which have so far been solved.

Research in cryoelectron microscopy [1] has established that diffraction from at least 10^4 proteins is needed to produce a statistically significant three-dimensional reconstructed charge-density map at 0.3 nm resolution. This redundancy is necessary if the radiation dose to each molecule is to be kept below the "critical dose" D_c which would destroy features of a given resolution. The redundancy provided by two- or three-dimensional crystals is therefore responsible for the great success of both x-ray and electron crystallography for these highly radiation-sensitive molecules. In this Letter a method of building up such a transmission electron diffraction pattern is described, by accumulating patterns from successive oriented single molecules in a molecular beam. (Because of the "coherent superposition" advantage of Bragg scattering, a larger number of molecules must be used in this "serial crystallography" scheme). The recent experimental observation of transmission electron diffraction patterns from a jet of molecules, partially oriented by a polarized laser beam, suggests that this may be a useful approach [2]. In place of a gas-phase molecular jet, a jet of submicron liquid helium droplets is proposed here, each containing one hydrated organic molecule. This jet passes across a focused, high energy electron beam, where it is also immersed in a polarized focused laser beam for molecular alignment. The nozzle expansion of gas-phase helium is known to produce ultracold (<1 K) helium droplets, whose low temperature will lead to better laser-induced molecular alignment (and less radiation damage) than in previous gas-phase experiments. The molecules may be aligned about one or two axes by the action of an elliptically polarized laser beam. Each droplet is subject to a fluence of less than the critical dose (about $500e^-\ \mathrm{nm}^{-2}$ for proteins at 0.3 nm resolution). Methods for obtaining a jet of such molecules aligned along one or two axes are discussed. The resulting electron diffraction patterns may be acquired using existing electron area detector systems. For molecules aligned along one axis they will resemble fiber-diffraction patterns. Patterns from fully aligned molecules may be inverted to molecular charge-density maps using the new iterative phasing algorithms, which solve the phase problem for the continuous distribution of scattering from an isolated object. The ultimate goal is a comparison of the three-dimensional structure, at near-atomic resolution, of a protein with, and without, a small drug molecule attached.

A related proposal had been made based on pulsed x-ray beams and the free electron laser (FEL) [3]. In that proposal, the dose supplied in each 50 fs pulse destroys the molecule, after first providing a diffraction pattern. Considerable difficulty can be anticipated in finding the orientation relationship between successive noisy diffraction patterns from successive randomly oriented molecules. The continuous-beam scheme proposed here offers several advantages. (i) As pointed out by Zewail and others in the field of time-resolved diffraction [4], the electron elastic scattering strength is at least 10^4 times greater than x rays, producing much greater signal. Damaging inelastic scattering is also relevant, but the balance strongly favors electron diffraction [5]. (ii) The molecular alignment we plan eliminates the need to determine the orientation relationship between patterns from randomly orientated molecules, which may not be possible. Our scheme allows a large number of two-dimensional diffraction patterns to be added together

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into the detector from successive molecules in the same orientation before each time-consuming detector readout event. (iii) The extremely challenging problem of creating pulses faster than 100 fs (required by the Neutze scheme) is avoided, since we propose the use of a continuous electron source. The field-emission electron source is brighter than current undulator-based continuous synchrotron x-ray sources [6], but not brighter than the peak of the FEL.

Figure 1 shows the orthogonal arrangement of beams proposed. Helium droplets are generated by expansion through a submicron aperture from gas above liquid helium into a pickup chamber, where hydrated organic molecules are collected. Details of a typical pulsed droplet source are given elsewhere [7], where a valve is demonstrated which provides control of the droplet generation rate. Continuous sources are also available. An electron gun with beam deflectors and magnetic focusing lenses is used — we consider the type to be used in the following section. The laser system shown uses a quarter-wave plate to generate elliptically polarized light, which fixes the orientation of passing molecules. Following continuous accumulation of data from many molecules in a single orientation, the area detector is read out, and both the laser and quarter-wave plate are rotated about the optical axis to provide a new molecular orientation. We now consider the relationship between the diffraction pattern thus obtained from moving molecules and the pattern which the inversion algorithm requires from a stationary molecule. It is readily shown that recording times for schemes involving a coherent nanoprobe which is pulsed [5] at the arrival of a single droplet are prohibitively long. (For such a probe, the diffracted intensity distribution which accumulates as the molecule traverses the probe is independent of molecular coordinate if the phase variation across the molecule is small. The contribution from zero crossings, where abrupt phase changes occur, is small. The situation is equivalent to scanning transmission electron microscope imaging).

A more practical scheme, however, may be based on a larger, high-current, partially coherent probe. The lowest velocities obtainable with helium droplets are about $v = 50\ \mathrm{ms}^{-1}$, so that, for a probe diameter d_2, each molecule is exposed for $T = d_2/v = 20$ ns per micron of travel. If we consider a simple single-lens optical system imaging a source of brightness B, diameter d_1, and beam divergence θ_1 onto a source image of size d_2 with divergence θ_2 and magnification $M = d_2/d_1 = \theta_1/\theta_2$, the fluence ("dose") received by each molecule is

$$D = \frac{B\pi\theta_2^2}{q}\left(\frac{d_2}{v}\right) \tag{1}$$

electrons per unit area, with q the charge on the electron. The coherence requirements of the reconstruction algorithm demand spatial coherence across the width of one molecule only, which fixes the beam divergence $\theta_2 = \lambda/L = 1$ mrad at 100 keV. (There is therefore no coherent scattering between different molecules.) Consistent values for an LaB6 source [8] are brightness $10^7\ \mathrm{A\,cm^{-2}\,str^{-1}}$ at 100 keV, $d_1 = 0.1\ \mu$m, and $\theta_1 = 5.6$ mrad (for current $I = 10^{-7}$ A). With $d_1\theta_1 = d_2\theta_2$, this fixes the probe size d_2 at 0.56 μm. Then Eq. (1) gives $D = 0.022e^-/\mathrm{nm}^2$, which is well below the critical dose $D_c = 500e^-/\mathrm{nm}^2$ which causes damage at the 0.3 nm level. It is now possible to include, say, 1000 or more helium droplets in a probe of this size simultaneously.

In these experiments, the energy otherwise concentrated into Bragg peaks in crystalline diffraction is spread over all of diffraction space, so that detection above noise requires longer exposure. The coherent superposition of Bragg scattering from M molecules in a two- or three-dimensional crystal produces Bragg reflections whose intensity is proportional to M^2 (for diffraction data

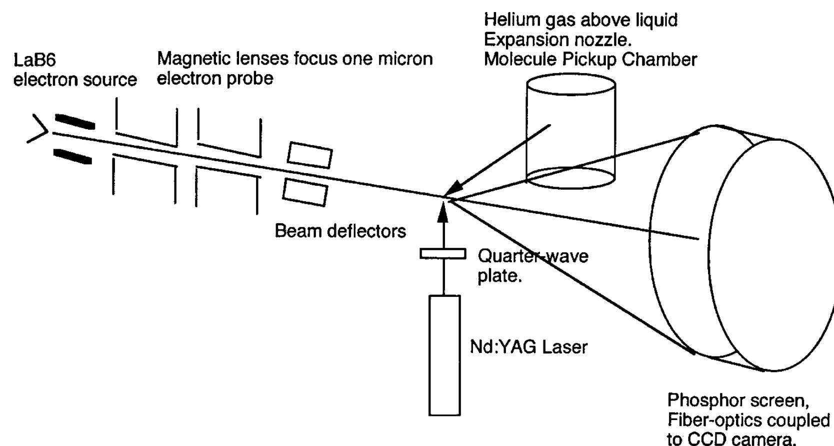

FIG. 1. Diffraction camera for single-molecule electron diffraction. A continuous electron beam is used. The laser polarization is rotated after each data readout for a new molecular beam orientation. Hydrated organic molecules are picked up within liquid helium droplets to form a molecular beam traversing the electron beam.

which is not angle integrated). The incoherent superposition of patterns from M successive molecules in our molecular beam produces diffuse scattering whose intensity is proportional to M. It follows that in order to produce the same signal-to-noise ratio as a cryomicroscopy crystal experiment using M molecules, the molecular-beam exposure time must be multiplied by M. Thus, for example, about 10^8 molecules would be needed to achieve the same signal-to-noise ratio as the 10 000 molecule crystal suggested earlier as the minimum for two-dimensional cryomicroscopy at 0.3 nm resolution. If the droplet beam has density n and thickness d_2, the exposure time becomes $T = 10^8 D_c q/(n d_2 I) = 80$ s for $I = 10^{-5}$ A, $d_2 = 10$ μm, $n = 10^{18}$ m^{-3} (for closely spaced 0.5 μm droplets). This is the time needed for each molecular orientation, prior to detector readout.

For two-dimensional image reconstruction, the dose needed to recognize a feature of size d increases steeply as the inverse square of the resolution d (according to the Rose criterion [9]), and in three-dimensional tomographic imaging it increases as d^{-4} [9] with increasing resolution (decreasing d).

The use of polarized nonresonant light to align (on one axis) and trap molecules (as distinct from particles or atoms) was proposed by Friedrich and Herschbach [10]. Recently, molecular alignment of a polyatomic molecule has been achieved about all three Euler angles using intense fields from an elliptically polarized laser (see Stapelfeldt [11] for a review). For our experiments using a molecular beam, the nozzle expansion into vacuum produces rotation temperatures of about 10 K. The degree of alignment has been measured by both optical spectroscopy or use of a second laser pulse to cause photoionization. The photofragments are ionized and detected, and their directions measured. Here it is found that the expectation value $\langle \cos^2\theta \rangle = 0.88$, where θ is the angle between the laser polarization axis and the molecular axis. Thus about 60% of the molecules have their axis located within a cone of 30° full width half maximum (compared with 3.4% for random orientations). Experimental transmission electron diffraction patterns have also been obtained recently from such a molecular beam, resulting in an anisotropic Debye-Scherrer ring pattern [2].

A much greater degree of alignment can be expected using these large molecules seeded into ultracold liquid helium droplets, because of their lower temperature and because of the molecular volume dependence of the anisotropic polarizability. In addition, this alignment will be achieved at much lower laser power, so that the use of a pulsed laser may not be needed. Other effects, such as nonspherical droplets, may act to reduce alignment. These doped droplets are known to be superfluid at temperatures of 0.37 K or less. The recent discovery of the formation of ultracold helium or ice droplets by expansion through a micron-size aperture into vacuum provides a remarkably simple experimental arrangement for generating droplets, and has produced a rapidly growing new field of isolation spectroscopy. It is important that the laser operates in the spectral window around 1 μm wavelength between overtone (anharmonic) vibration spectra — these windows broaden at low temperature [12]. Amino acids [13], phthalocyanine, and pentacene have all been studied in this way, which relies on the gentle nonbonded interactions of the inert helium to confine the organic molecule. Detailed calculations for the value of $\langle \cos^2\theta \rangle$ for organic molecules within helium droplets have yet to be undertaken; however, the reported 1.6 K rotation temperatures of molecules in helium droplets are much lower than those in free molecular beams. As with the alignment problem for single-particle molecular imaging [14], imperfect alignment can be expected to limit resolution $d = L\theta$ for a molecule of length L. In recent work, helium droplets have been doped with water [15], which suggests the future possibility of superfluid helium droplets containing hydrated organic molecules. We anticipate that the form of ice will be amorphous (as used in cryomicroscopy and x-ray crystallography) if cryoprotectant is used. The variable conformations of the proteins can be expected to limit resolution to the same extent as that which occurs in cryomicroscopy.

An important issue concerns the dissipation of energy resulting from alignment. In work on laser levitation of particles in vacuum [16] it is found that the decay time of particle oscillations is several months, and the dissipative action of a surrounding medium is essential for practical laser tweezers. For doped ultracold droplets, the dissipative mechanism is evaporation, and the droplet provides an ideal constant temperature thermal reservoir. It is also known that small molecules rotate freely in the superfluid, which has zero viscosity at low velocity. The mechanisms by which a larger molecule such as a protein couples to the superfluid through the excitations of the superfluid remain to be investigated in detail.

New iterative numerical techniques have recently been developed which are ideally suited to solving the phase problem for this type of data. These are based on the Fienup-Gerchberg-Saxton hybrid input-output (HiO) algorithm [17]. This has now been applied to several experimental x-ray and electron diffraction patterns from nonperiodic objects, from which faithful images have been reconstructed [18]. The algorithm, which requires a Fraunhoffer diffraction pattern, an approximate estimate of the object's size, and knowledge of the sign of the scattering potential, iterates between real and reciprocal space, applying known constraints in each space until convergence is reached and a solution to the phase problem found. In one recent x-ray application an estimate of the object's boundary was obtained from its available autocorrelation function [19](so that little or no *a priori* information on the object is needed), while in another the

method was used to provide the first atomic resolution image of a carbon nanotube [20], using an electron nanodiffraction pattern.

While the HiO method works best in three dimensions for tomographic reconstruction, difficulties can arise for "complex objects." In this context, a complex object is one in which multiple scattering is important. The conditions under which complex objects can be reconstructed are summarized elsewhere [21]; see also [18], in which a new "shrinkwrap" HiO algorithm treats complex objects of unknown shape. We anticipate results which are consistent with the success of protein crystallography by TEM cryomicroscopy, where multiple scattering is normally a small perturbation [22].

In summary, the use of hydrated molecules within helium droplets is proposed for the structure analysis of small proteins. The use of a continuous electron beam and doped ultracold droplet source is suggested. Molecules are aligned using an elliptically polarized laser. (For molecules aligned with simple plane-polarized light along a single axis, a fiber-diffraction pattern will be obtained, similar to that used to solve the structure of DNA and tobacco-mosaic virus). Limited alignment may limit resolution to the secondary structure of proteins, which have recently been "solved" from powder diffraction data [23]. The low temperature obtainable within droplets is proposed in order to improve the degree of alignment, and to obtain this alignment at lower laser power, obviating the need for pulsed lasers. Although each separate component of this proposal has been demonstrated experimentally (laser alignment of molecular beams, droplet formation and doping with amino acids and water, electron diffraction from an aligned molecule jet), the experimental demonstration of their combination would be a formidable technical feat. The need to provide hydrated proteins in the gas phase (as presently undertaken for spectroscopy) may require a pickup chamber to be designed which provides collection of small hydrated proteins or micelles; however, in specific liquid jet implementations this may not be needed. The scientific urgency of addressing the problem of protein structures which cannot be crystallized is very great, justifying this effort. Among other benefits, we note the well-documented reduction in radiation damage which occurs at very low temperatures, from which ultracold droplet electron diffraction will benefit.

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