Nondestructive Imaging of Individual Biomolecules

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Radiation damage is considered to be the major problem that still prevents imaging an individual biological molecule for structural analysis. So far, all known mapping techniques using sufficient short wavelength radiation, be it x rays or high energy electrons, circumvent this problem by averaging over many molecules. Averaging, however, leaves conformational details uncovered. Even the anticipated use of ultrashort but extremely bright x-ray bursts of a free electron laser shall afford averaging over 10^6 molecules to arrive at atomic resolution. Here, we present direct experimental evidence for nondestructive imaging of individual DNA molecules. In fact, we show that DNA withstands coherent low energy electron radiation with deBroglie wavelength in the Ångstrom regime despite a vast dose of 10^8 electrons/nm^2 accumulated over more than one hour.

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Exploring the three-dimensional structure of individual biomolecules, in particular, those of proteins, is the foundation for a basic understanding of biochemistry, molecular biology, and biophysics. Most of the protein structural information data available today have been obtained from crystallography experiments by averaging over many molecules assembled into a crystal. Despite this vast amount of available data, a strong desire for acquiring structural data from just one individual molecule is emerging for good reasons. Most of the relevant biological molecules exhibit different conformations; thus, averaging cannot reveal detailed structural information. Moreover, there is a large quantity of proteins, in particular, the important class of membrane proteins, featuring a pronounced reluctance to readily crystallize.

Because of the strong inelastic scattering of x-rays and high energy electrons, there is little hope for obtaining structural information from just one single molecule by conventional x-ray or high energy electron microscopy tools. Despite recent advances in cryo-electron microscopy, especially in image processing and reconstruction, averaging over typically 10 000 images is still necessary to build up a high signal-to-noise ratio image with structural features finally emerging [1]. This, in turn, smears out most of the details related to conformational flexibility. The necessity for averaging is given by radiation damage inherent to the interaction with high energy electrons and limits the obtainable resolution in conventional electron microscopy to $\sim$1 nm [2]. In order to obtain structures of individual biological molecules at atomic resolution, new concepts and technologies are envisioned. A major effort currently underway involves the development and implementation of the x-ray free electron laser (XFEL), as a source of ultrashort but extremely intense x-ray pulses. The overall idea is to take advantage of the principle of inertia: by keeping the interaction time of the intense x-ray burst with the molecule of interest extremely short, the site information of the atoms is carried to the detector before the molecule has been given time to finally decompose [3]. Unfortunately, in x-ray diffraction, inelastic scattering outweighs elastic scattering, but only the latter carries information about the structure of molecules. Hence, a very large number, of the order of 10^6, of diffraction patterns of identical molecules must be recorded in order to obtain structural detail at a resolution of 3 Å, even with a 10 fs x-ray pulse containing 2×10^{12} photons at 1.5 Å wavelength [4].

In the following, we show that a molecule as fragile as DNA withstands irradiation by coherent low energy electrons and remains unperturbed even after a total dose of at least 5 orders of magnitude larger than the permissible dose in x-ray or high energy electron imaging. The experimental setup for testing radiation damage is illustrated in Fig. 1. DNA molecules are stretched over holes by using freeze drying technology known from cryo-microscopy [5]. First, an array of 1 μm diameter holes in a thin carbon film is cleaned and rendered hydrophilic by UV-ozone treatment. Next, a droplet of λ-DNA solution of 2 μg/ml concentration is applied onto the carbon film. Following an incubation time of typically 10 minutes, blotting paper is used to remove excess water. The remaining thin water film is transformed into amorphous ice by rapid quenching in liquid ethane. Next, the sample is freeze dried at $-80\,^{\circ}$C under vacuum conditions within typically 20 minutes and monitored by a mass spectrometer above the sample. Finally, the sample is transferred into the low energy electron point source (LEEPS)-microscope chamber for obtaining DNA electron holograms [6] at various energies ranging from a few 10 eV up to about 300 eV.

For the holographic imaging, solely the elastic scattered electrons contribute to the hologram, while the inelastic scattered electrons lead to an incoherent diffuse back-

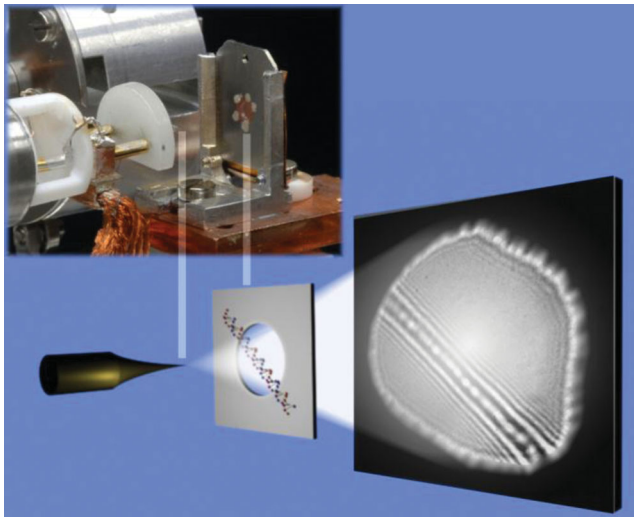

FIG. 1 (color). Schematic of the setup. For exploring radiation damage effects on DNA, the molecules are exposed to coherent low energy electrons. A spherical wave emitted from a coherent electron source is scattered at DNA molecules stretched over holes in a thin film and positioned at about 1 μm beyond the source. At a 10 cm distant micro-channel-plate screen detector, the interference between the elastic scattered wave (object wave) and the unscattered wave (reference wave) produces the hologram captured by a 14 bit dynamic range CCD camera. Magnification is provided by the geometry of the setup alone and can be adjusted by the source-sample distance. The smallest interference fringe spacing in the hologram amounts to 0.7 nm and is a measure for the achievable resolution.

ground at the detector level. The fraction of inelastically scattered electrons by an object can be estimated by negatively biasing the front of the detector. While there is a significant amount of inelastic scattering in imaging metals with low energy electrons, measurable inelastic scattering in imaging DNA has not been observed. This could be a first, albeit just qualitative, hint for little or no radiation damage caused by low energy electrons. To actually measure the electron dose leaving DNA molecules unperturbed, we have carried out quantitative experiments. Evidence for damage-free imaging is provided in Fig. 2 showing a hologram of DNA molecules subject to 60 eV electron radiation. In this experiment, DNA has continuously been exposed to a 200 nA electron current for 70 minutes. A set of DNA holograms has been recorded every 10 minutes (see supplementary materials [7]). Next, three regions in the DNA holograms have been selected, marked by red squares in Fig. 2. Thereafter, the cross-correlation function between the first holographic record and subsequent holograms taken at 10 min intervals have been computed for these very regions.

As evident from Fig. 2, the cross-correlation coefficient varies between 0.93 and 1.0 indicating a high degree of similarity between the first and all subsequent holograms. While the fluctuation of the cross-correlation coefficient is

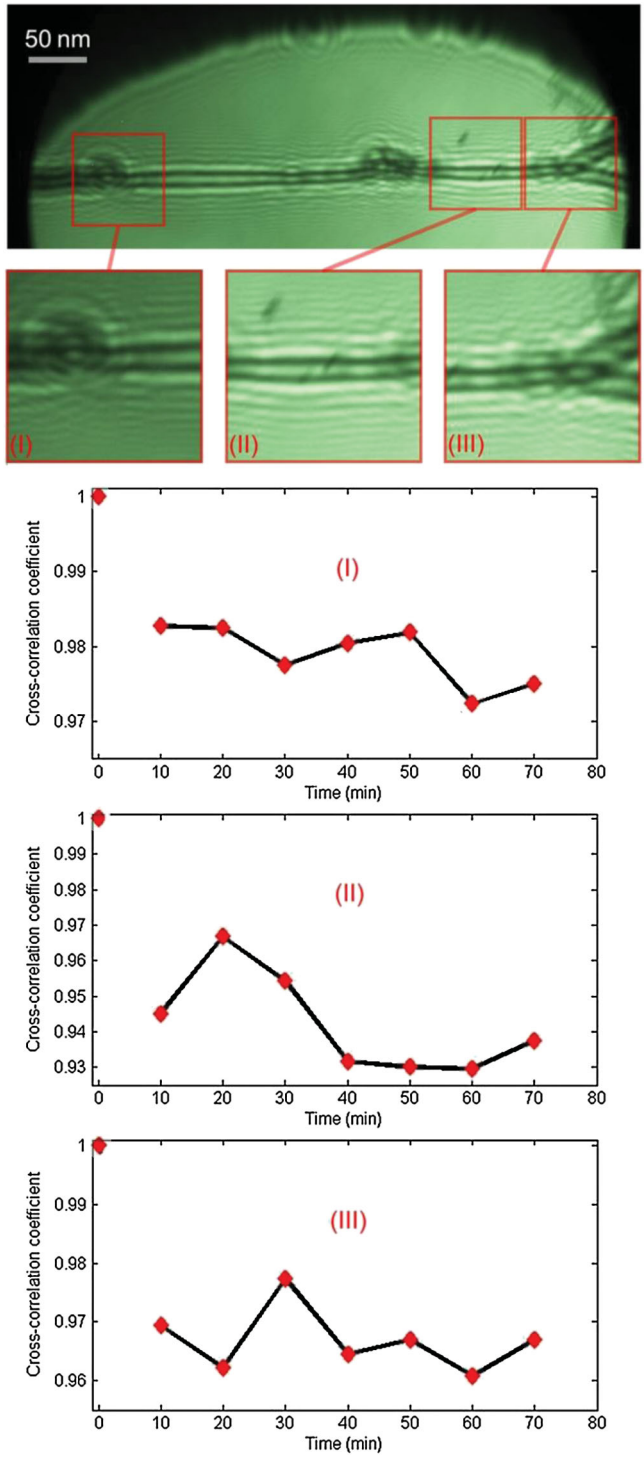

FIG. 2 (color). Nondestructive imaging of DNA. The low energy electron hologram of DNA molecules stretched over a hole in a thin film imaged continuously for 70 minutes using electrons of 60 eV kinetic energy and a total current of 200 nA is shown on top. Part of the rim of the 1 μm diameter hole, spanning the freestanding molecules, is visible at the very top of the image. Three regions of the hologram, marked in red, have been chosen to evaluate the cross-correlation function of subsequent holograms. The evolution of the cross-correlation coefficient is shown in the diagrams corresponding to the three regions.

apparent, its time dependence differs in all three DNA hologram regions. These fluctuations are due to statistical noise which varies from hologram to hologram and is attributed to the intrinsic stochastic process of field emission and to detector noise. As a consequence, the cross-correlation coefficient reaches values just below unity at best. However, there is no tendency of the cross-correlation coefficient to decay in time. In fact, the coefficient persists above 0.93 even after 70 minutes of continuous exposure. Thus, the molecule's structure remained intact during 70 minutes of exposure to 60 eV electrons. The total accumulated dose during that time amounts to 10^8 electrons/nm^2. This remarkably high electron dose certainly provides enough scattering events in a single molecule to be able to extract structural information at Ångstrom resolution. Moreover, there is nothing to be said against increasing the imaging current from 200 nA into the μA range or prolonging the exposure time. The kinetic energy of 60 eV has been chosen here because the corresponding deBroglie wavelength is close to the 1.5 Å x-ray wavelength used in XFEL simulations [4] setting the boundaries for single molecule imaging. However, similar experiments as described above have also been carried out at 110 eV electron energies and quantitatively analyzed revealing the same result of no observable damage to DNA. To demonstrate that our findings depend only on electron energy but not on any particularities of our setup, we would now like to present a control experiment, done in the same manner but at higher electron energies of 260 eV where DNA actually decomposes rapidly within a few seconds.

In fact, a detailed analysis as in the nondamaging situation described above has not been possible here because of a too rapid decomposition of the molecules within the first 10 seconds of the observation process. The situation is illustrated in Fig. 3. Again, DNA is stretched over a 1 μm diameter hole in a carbon film but now imaged with 260 eV energy electrons. Even before completing the data acquisition for the first image by the slow-scan CCD camera, the molecule had already been partly damaged as apparent in Fig. 3(a). Evidently, the 260 eV electrons cause bond braking in DNA. Since the DNA does not rest on a support but is freestanding, small molecular fragments created while DNA is decomposing sublimate into the vacuum. As a consequence, the remaining DNA gets shorter and shorter within a few seconds of observation.

For comparison to the nondamaging case, the evolution of the cross-correlation coefficient has also been computed and is shown in Fig. 3(e). As expected, the process of DNA decomposition is accompanied by a rapid decrease of the cross-correlation coefficient between subsequent DNA images. The red bar displayed at the top of Fig. 3(e) illustrates the range of fluctuations of the cross-correlation coefficient in the nondamaging experiment. While 60 eV electrons apparently cause no detectable damage to DNA at all, a rapid decomposition on a 4000 times shorter time scale is

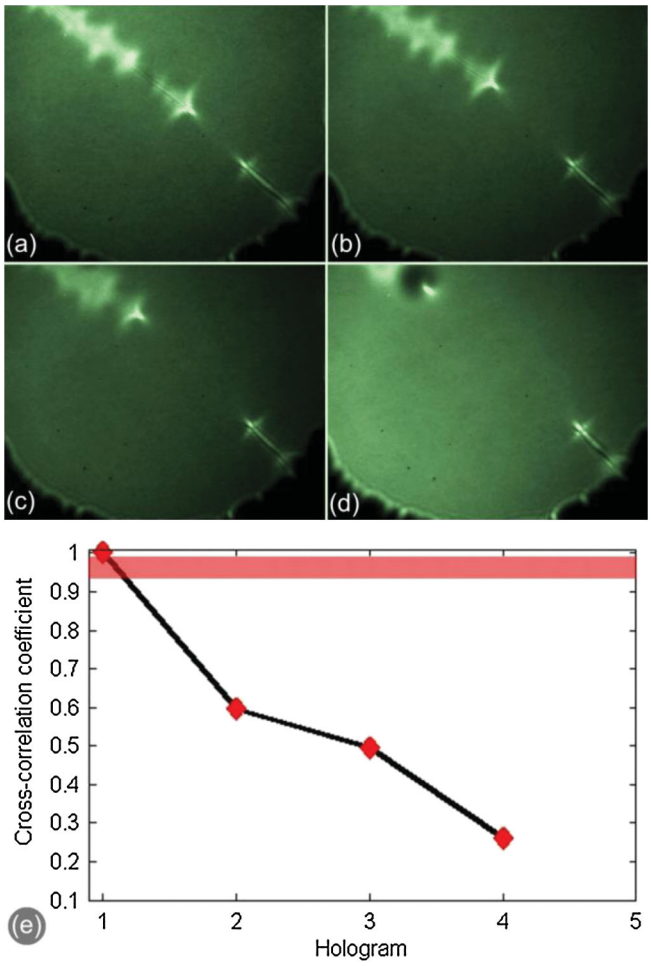

FIG. 3 (color). Decomposition of DNA. (a)–(d) Control experiment showing the rapid decomposition of DNA stretched over a 1 μm diameter hole in a carbon film during imaging with 260 eV electrons. At (e), the associated change of the cross-correlation coefficient is plotted. The red bar at the top indicates the range in which this coefficient fluctuated in the nondamaging experiment with 60 eV electrons.

observed when DNA is subject to the interaction with 260 eV electrons. We would like to point out that 60 eV kinetic energy of the imaging electron wave is not a unique value or condition for gently imaging DNA molecules. Equally nondestructive imaging conditions have empirically been found also at 115, 140, 215, and 230 eV kinetic electron energy, for example.

While high energy electrons in all keV kinetic energy ranges [1] cause unavoidable damage to biological molecules, it has also been shown that very low kinetic energy electrons between 3 and 20 eV initiate strand breaks in DNA molecules [8]. However, it is important to note that these studies [8] suggest that there is not a simple threshold for damage, but a rather pronounced energy dependence pointing at distinct energies between 3 and 20 eV where resonance effects lead to damage. Apparently, there are very low electron energy ranges around 10 eV, where DNA

experiences rapid damage as well as higher energy resonances leading to damage at 260 eV as we have observed it and described above. But, fortunately, there are also regimes in between where DNA can readily be imaged using an extremely high dose without any damage at all. The permissible dose leaving a molecule unperturbed is at least 5 orders of magnitude greater than in conventional x-ray or high energy electron imaging, demonstrating that coherent low energy electrons are the only nondamaging Ångstrom wavelengths radiation. Still, it is important to note that indirect radiation damage effects arising from the environment, such as very low secondary electrons for molecules adsorbed on a surface or radical formation for molecules in solution, must be ruled out. In this sense, freestanding biomolecules in vacuum are ideal. With coherent low energy electrons, it shall thus be possible to look at truly just one entity if it comes to high resolution diffraction microscopy of individual biomolecules.

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