Imaging proteins at the single-molecule level

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Imaging single proteins has been a long-standing ambition for advancing various fields in natural science, as for instance structural biology, biophysics, and molecular nanotechnology. In particular, revealing the distinct conformations of an individual protein is of utmost importance. Here, we show the imaging of individual proteins and protein complexes by low-energy electron holography. Samples of individual proteins and protein complexes on ultraclean freestanding graphene were prepared by soft-landing electrospray ion beam deposition, which enables acquiring subnanometer resolution images of individual proteins (cytochrome C and BSA) as well as of protein complexes (hemoglobin), which are not the result of an averaging process.

**Significance**

We report a method to image and reveal structural details of proteins on a truly single-molecule level. Low-energy electron holography is used to image individual proteins electrospray deposited on freestanding graphene. In contrast to the current state of the art in structural biology, we do away with the need for averaging over many molecules. This is crucial because proteins are flexible objects that can assume distinct conformations often associated with different functions. Proteins are also the targets of almost all the currently known and available drugs. The design of new and more effective drugs relies on the knowledge of the targeted proteins structure in all its biologically significant conformations at the best possible resolution.

Author contributions: J.-N.L. had the original idea to combine ES-IBD and low-energy electron holography and further elaborated the concept with K.A. and H.-W.F. J.-N.L. prepared the ultraclean freestanding graphene supports and recorded the holograms. J.-N.L. and S.R. performed the hologram reconstructions with her self-developed software package. J.-N.L. and S.R. interpreted the data. H.-W.F. invented the technology of lens-less holography with low-energy electrons based on atomic sized coherent electron point sources. J.-N.L., C.E., T.L., and H.-W.F. further developed the low-energy electron holographic microscope used in this study. S.R. and K.K. developed the ES-IBD technique. J.-N.L., C.E., and H.-W.F. wrote the manuscript main text and with S.R. the supplementary information, in discussions with all remaining authors.

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DNA, can withstand prolonged irradiation by electrons with a kinetic energy in the range of 50–250 eV. Even after hours of illumination and the exposure to a total dose of at least five orders of magnitude larger than the permissible dose in X-ray or high-energy electron imaging, biomolecules remain unperturbed as exemplified in a detailed study concerning DNA molecules (13). This, combined with the fact that the de Broglie wavelengths associated with this energy range are between 0.7 Å and 1.7 Å, makes low-energy electron microscopy techniques, especially holography, auspicious candidates for investigations at the truly single-molecule level. In this lens-less microscopy scheme inspired by Gabor’s original idea of holography (14), the samples are presented to a highly coherent beam of low-energy electrons generated by an atomically sharp field emitter (15–17) tip placed as close as 100 nm in front of the sample. The interference pattern formed by the scattered and unscattered electron waves, the so-called hologram, is recorded at an electron detector several centimeters away (for more details, see Low-Energy Electron Holography). Because in a hologram, the scattered and unscattered electrons are contributing to the image formation, acquisition times as short as 100 μs are sufficient for high signal-to-noise ratio records (18). Whereas highly coherent sources for low-energy electrons have been available for more than two decades, holography has long suffered from the lack of a substrate transparent to low-energy electrons but still robust enough that nanometer-sized objects can be deposited onto it. Recently, we have shown that ultraclean freestanding graphene fulfills these two requirements (19–21).

In the following, we show how subnanometer resolution images of individual proteins are obtained by means of low-energy electron holography. Although the damage-free radiation of coherent low-energy electrons and the conceptual simplicity of the experimental scheme for holography are appealing, this tool for single-protein imaging critically relies on the sample preparation method. The proteins in their native state must be brought into a ultrahigh vacuum (UHV) environment and fixed in space for an appropriate period to accumulate sufficient structural information on the one hand, while avoiding the emergence of contaminants on the other hand. Here, native protein ions are transferred from aqueous solutions to the gas phase (22–27) and deposited onto ultraclean freestanding graphene in an UHV environment by means of soft-landing electrospray ion beam deposition (ES-IBD) (28–30). The workflow for imaging a single protein involves several steps, as illustrated in Fig. 1. An ultraclean freestanding graphene sample containing 500 × 500-nm² apertures milled in a 100-nm-thick SiN membrane is prepared following the recently developed platinum metal catalysis method (31) and is characterized in the low-energy electron holographic microscope (Fig. 1, Left). The sample is subsequently transferred to an ES-IBD system (Fig. 1, Center and Fig. S1) under permanent UHV conditions by means of a UHV suitcase operating in the 10⁻¹¹–mbar regime (see UHV Transfer for more details). Native cytochrome C (CytC), BSA, and hemoglobin (HG) ion beams are generated by electrospray ionization and mass filtering. The charge states z = 5–7 are selected for CytC (22, 26) and the charge states z = 15–18 are selected for BSA (32). In the case of HG, the charge states z = 16 or z = 17 of the intact complex are known to be of native conformation (33) and hence the corresponding m/z region is selected (the corresponding mass spectra are displayed in ES-IBD and Fig. S2). In all three cases, the ions are decelerated to a very low kinetic energy of 2–5 eV per charge, which ensures retention of the native state upon deposition onto ultraclean freestanding graphene (26). Preparative mass spectrometry of proteins (28, 34, 35) followed by electron microscopy has already been reported in the literature, but there the deposition was made on thick carbon films and not performed under UHV conditions (36, 37). Furthermore, the imaging by high-energy electrons in a TEM required exposure to atmospheric conditions and negative staining of the proteins.

Results
Cytochrome C. After deposition, the samples are transferred again under preserved UHV conditions from the ES-IBD system back to the low-energy electron holographic microscope (Fig. S3), where holograms of individual proteins are recorded. In Fig. 2, holograms of three distinct entities found on freestanding graphene after deposition of native CytC ions are presented (Fig. 2 A–C). In these images, the characteristic fringes of a holographic record, resulting from the interference between the unscattered and elastically scattered electrons, are observed. From the hologram itself it is impossible to recognize the shape of the object. Numerical hologram reconstruction involving back propagation of the wave front from the hologram to the sample plane (38–40) is required to finally reveal the object’s structure (Fig. 2 D–F). For instance, in Fig. 2D, an individual CytC is displayed after hologram reconstruction. The diffuse rings around the object are due to the presence of the out-of-focus twin image inherent to in-line holography (38). As apparent from the high-contrast images shown in Fig. 2 D–F, not only are the globular structures with the correct overall dimensions of the protein revealed, but also details of the shape of CytC in different orientations even while forming agglomerates of two (Fig. 2E) and, respectively, three proteins (Fig. 2F). The spatial resolution attained in a hologram can be estimated using the Abbe criterion (41, 42) and by measuring the largest angle under which interference fringes are observable (40, 43, 44). In Fig. 2, a resolution of 7–8 Å is calculated and the same value is found in the reconstructed images by measuring the edge response (45) over the protein structures.
A similar resolution is estimated for all other micrographs presented below. In a hologram, the spacing between consecutive interference fringes gradually decreases toward higher orders. Hence, high-order interference fringes and consequently high-resolution structural details are most susceptible to mechanical vibrations. The latter currently limit the resolution, and intense efforts are ongoing to increase the mechanical stability of the low-energy electron holographic microscope to overcome this limitation and approach atomic resolution. Whereas the current resolution already reveals the outer shape of single proteins and protein subunits, an enhanced resolution of 2 Å will permit imaging internal structural details as well. The inner contrast variations apparent in the images presented here demonstrate that proteins are sufficiently transparent to low-energy electrons (Fig. 2 D–F).

A very important question for the imaging of soft-landed proteins by means of low-energy electron holography is the charge state of the adsorbed particles after landing. Whereas several studies have shown retention of the charge states for protein depositions because the deposition process is random in this respect. To understand the origin of the darker region in the center of the images presented here it is possible to address how the protein ions, produced during the electrospray process, are neutralized after landing on the graphene substrate. A fast quenching of the ions, produced during the electrospray process, is posited onto self-assembled monolayer (46–48), other studies have shown that the protein ions, produced during the electrospray process, are neutralized after landing on metallic surfaces (49, 50).

BSA. The same experimental workflow was used in the case of imaging BSA, a much larger protein than CytC (66 kDa vs. 12 kDa). An image of a low-energy electron micrograph of BSA is presented in Fig. 4 (Top). Similar to CytC, high-contrast images reveal features that suggest a globular structure with the correct dimensions of the protein. In contrast to CytC, which is nearly spherical in shape, the 3D shape of BSA is traditionally described as heart shaped. The micrographs of individual BSA molecules reflect this structure as well as the protein in other features, clearly demonstrating that proteins can be found in UHV in structures closely related to their native structures.

A further possibility to qualitatively analyze our micrographs is to compare them with simulated electron density maps at the corresponding resolution. In Fig. 5, two of the BSA micrographs presented in Fig. 4 are therefore compared with electron density maps simulated at a resolution of 8 Å with the software Chimera (52), originally developed for the analysis of cryo-EM images. We find a considerable correspondence between the simulated density maps and the micrographs and the resolution estimate made from the holographic record is in good agreement with the simulation.
proteins are actually adsorbed on the graphene substrate. Of particular interest is the side view presented in Fig. 5, Right as it shows that in this specific orientation, BSA is of strongly varying thickness with a maximum toward the center. The darker region observed in the micrograph evidently corresponds to a higher absorption due to an increased protein thickness in this area. This observation and analysis demonstrate that already with a resolution of 8 Å, information on the 3D structure of the protein can be gained. In the prospect of an improved resolution of the order of 1–2 Å, it also illustrates the future ability to gain a complete 3D structure from a single low-energy electron hologram of proteins at least as large as 60 kDa.

In biology, a noncovalently bonded complex of several proteins rather than a single protein is performing a function. Next to the atomic structure of a protein, the composition and structure of protein complexes are of utmost importance. The data of Fig. 3 show that protein agglomerations formed of two and three CytCs can be resolved. It has been extensively shown that by means of electrospray ionization, it is possible to ionize entire protein complexes while keeping their native conformations (24, 53–55).

Hemoglobin. In Fig. 6, two micrographs of individual hemoglobin, a complex of four protein subunits, are presented, demonstrating that with our method, entire protein complexes in their native configuration can be deposited and individually imaged. Whereas for the cases of CytC and BSA the agreement between the low-energy electron images and the atomic models is almost perfect, differences can be observed for the case of HG. Because HG is a protein complex composed of four subunits, it exhibits a large conformational flexibility, actually required for its function in a living organism. When an averaging process over millions of molecules is involved in the imaging of a highly flexible protein, discrete conformations cannot be distinguished and only an average structure evolves. However, with a technology capable of imaging individual proteins, like low-energy electron holography, the entire conformational landscape is revealed. It is therefore not surprising that structural differences between the low-energy electron micrographs and the atomic model are apparent. Furthermore, a much larger set of images will be needed to address the full conformational landscape of flexible proteins, for instance hemoglobin, to be able to determine a protein structure, and to judge the abundance of the variants. Here, we present two images of hemoglobin that could be associated to its atomic model in a specific orientation.

Fig. 3. Complete dataset for the imaging of CytC. (A) Low-energy electron image of ultraclean graphene covering a 500 × 500-nm² aperture before protein deposition. (B) Mass spectrum of the mass-selected CytC beam. (C) A survey image of the very same freestanding graphene region after deposition of CytC. (D–J) Low-energy electron micrographs with suggestions for possible protein orientations based on the averaged protein structure derived from X-ray crystallography data and documented in the PDB (PDB ID: 1HRC). (Scale bars, 2 nm.)

Fig. 4. Low-energy electron micrographs of BSA in different orientations on graphene. (Top) Low-energy electron micrographs of BSA. (Bottom) The atomic model of BSA (PDB ID: 3V03) in the corresponding orientations. (Scale bars, 5 nm.)
Conclusion and Outlook

The ultimate goal of directly uncovering the structure of unknown proteins or protein complexes and describing their conformations at the atomic level still requires experimental efforts toward a better chemical and conformational selectivity of the deposition process. This could be attained by adding ion-mobility capability to the ES-IBD device. With this more elaborated deposition device, it would be possible to select the objects on the basis of not only their charge state but also their gas phase conformation. By this, an assessment of the relation between the conformation in the gas phase and on the surface, influenced by the charge state (22–25), deposition conditions (49, 56), and surface properties, would be possible.

The mapping of the proteins with unknown structure will also require an improved imaging resolution, fundamentally limited solely by the electron wavelength. Furthermore, as 3D information is encoded in a single in-line hologram, improved spatial resolution will already permit to determine the (x,y,z) spatial coordinates of every atom of a protein from this very holographic record. A complementary strategy to reveal the complete 3D structure of a single protein is to add tomographic capability to the experimental setup. At this stage, the comparison of the low-energy electron micrographs with atomic models available at the PDB has the character of a supplementary strategy to reveal the complete 3D structure of a single protein from this very holographic record. (Scale bars, 5 nm.)

Fig. 5. Comparison of low-energy electron micrographs of BSA with simulated electron density maps. (Top) Low-energy electron micrographs of BSA. (Scale bars, 5 nm.) (Middle) Electron density maps simulated at a resolution of 8 Å and rotated to match the orientation of the proteins presented in Top. (Bottom) Side view of the density map along the directions of observation indicated by the arrows shown in Middle.

Materials and Methods

Ultraclean freestanding graphene is prepared by the Pt-metal catalysis method described in detail elsewhere (31). Before the transfer of the ultraclean substrate from the UHV chamber of the low-energy electron holographic microscope to the UHV chamber of the ES-IBD device, the cleanliness of the substrate is characterized and reference images are recorded for comparing the very same region of freestanding graphene before and after protein deposition.

During the whole experimental workflow, the samples are kept under strict UHV conditions with the help of a UHV suitcase for transfer between the two experimental chambers. Details of the ES-IBD procedure and of the low-energy electron holography experimental scheme are described in ES-IBD and Low-Energy Electron Holography.

Fig. 6. Low-energy electron micrographs of two individual HG molecules and the atomic model in the corresponding orientations. (Top) Two micrographs of HG soft landed onto freestanding graphene. (Bottom) Suggestions for possible orientations based on the averaged protein structure derived from X-ray crystallography data and documented in the PDB (PDB ID: 2QSS). (Scale bars, 5 nm.)

From ion-mobility/mass-spectrometry investigations, demonstrating that proteins and protein complexes can be transferred from the liquid phase to a vacuum environment while maintaining their tertiary, respectively quaternary, structures unperturbed (25, 57–60). The low-energy electron micrographs presented here are further strong evidence that proteins in a folded state are stable in UHV. As recently demonstrated it is possible to add water molecules to small peptides (61) directly in an UHV environment. Low-energy electron holography with its ability to image proteins individually will also allow us to study the effects of adding hydration shells to the protein. Furthermore, questions related to transport, such as diffusion of proteins and subsequent association into protein complexes, will be addressed. First observations of the diffusion of folded proteins on freestanding graphene by means of low-energy electron holography are presented in Fig. S5, illustrating that the method described here is also capable of accessing dynamic processes.

To conclude, we have shown here how to image a single protein by combining ES-IBD technology with low-energy electron holography. This method has led to a tool for revealing structural details of single native proteins and protein complexes without destroying them. With the recent advances in electrospray ionization and mass spectrometry of large protein complexes (62), and in particular membrane proteins (53, 55), even the structure of these biologically important but reluctant to readily crystallize entities may become accessible in the future.
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