15 Physics of Biological Systems

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Our central activity, the structural investigation of individual biological objects with coherent low-energy electrons, involves in-line holography as well as coherent diffraction imaging and is assisted by micro-structuring techniques using a focussed gallium ion beam for miniaturized electron optics and sample preparation. In the past year, it became possible for the first time in the history of structural biology to image individual proteins as will be discussed in this report.

Our current activities are divided in the following interconnected individual projects:

- Electron Holography and Coherent Diffraction
  Major experimental challenges are ongoing to improve the interference resolution, establish methods for creating free standing thin films of graphene transparent for low-energy electrons as well as appropriate techniques to present a single protein to the coherent electron wave front. Next to these experimental issues, a second, equally important aspect for achieving high resolution structural information is the numerical reconstruction of the electron holograms respectively iterative phase retrieval in coherent diffraction. This is achieved by employing newly developed numerical algorithms to solve the integrals governing these coherent optics problems.

- Coherent Diffraction Imaging of Graphene-Supported Single Biomolecules at Atomic Resolution
  While this is an independent effort by Jean-Nicolas Longchamp, well worth a seperate and detailed report by itself, we include it here in view of the frame and scope of this report. Methods to deposit biomolecules onto freestanding graphene, in particular in-situ electrospray deposition have been explored and successfully implemented. The results, which led to the imaging of individual proteins in a collaboration with a group from the Max Planck Institute in Stuttgart are described further below.

- Detection of individual elementary charges and charge transfer processes
  A comparison of experimental observations with simulations reveals that it is not only possible to observe individual charges on free-standing graphene but that charge transfer processes involving a single charge and the mobility of moving charge carriers can be detected too. From the mean square displacement the diffusion coefficient has been derived.

- Direct mapping of the unoccupied states in freestanding graphene
  Electrons with kinetic energies below 30 eV originating from a coherent electron point source are transmitted through freestanding graphene. Under those angles where the *k*-vector component of the incoming electron parallel to graphene matches unoccupied states in graphene, high absorption is observed. This directly translates into a mapping of the unoccupied bands of graphene revealed by a hexagonal structure in the transmission image observed on a distant detector. These hexagons grow as expected when decreasing the beam energy from 30 down to 18 eV. The measurement principle is illustrated in Fig. 15.1.

- Instrumentation
  A new Low Energy Electron Point Source (LEEPS) microscope (Fig. 15.2) has been designed and build by our machine shop. It is currently operated to investigate the adsorption of individual alkali atoms onto free-standing graphene.

- Direct writing of sub-micron sized structures
  A so-called super ink jet printer was purchased by the R'Equip fund SPOT (Single Protein on Target), to be used to the benefit of research groups in physics and physical chemistry. So far, it has been possible to pattern surfaces with silver lines by direct printing in an application aiming at carrying out four point measurements to explore transport through graphene layers.

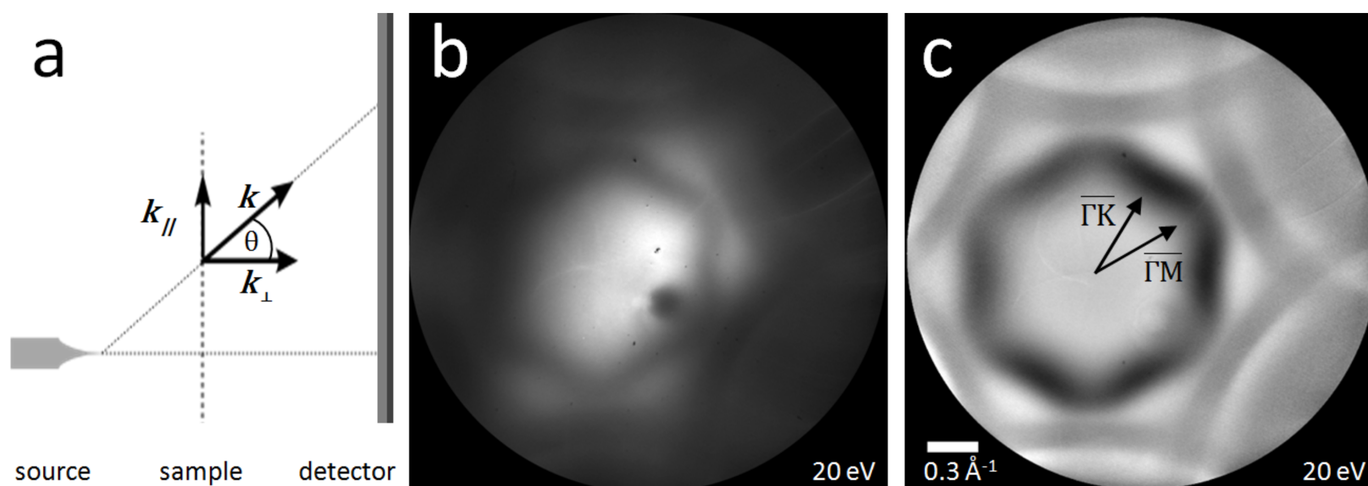
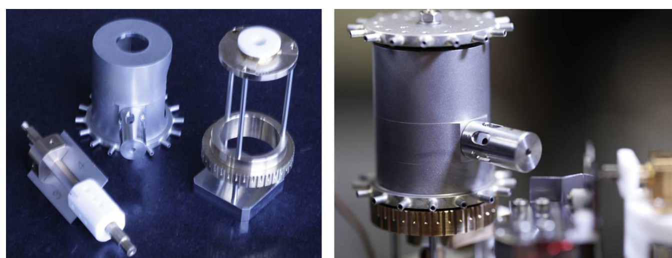


FIG. 15.1 – The in-plane k vector of the electrons impinging onto graphene under a certain angle is illustrated in (a). The in-plane momentum dependency of the transmission through free-standing graphene thus allows probing the unoccupied bands by observing the electron transmission pattern at a distant detector. In (b) raw data of such transmission pattern for 20 eV electrons are shown while at (c) the same data are displayed after background subtraction and transformed into coordinates of the k vector parallel to graphene. The arrows indicate the direction of the high symmetry points K and M in the Brillouin zone, determined from the first order diffraction disks observed at higher electron energies.

15.1 Overall Motivation and Research Goals

Most of the protein structural information available today has been obtained from crystallography experiments by means of averaging over many molecules assembled into a crystal. Since biological molecules exhibit different conformations, averaging smears out structural details. That is why a strong desire to gain structural data from just a single molecule is emerging. We are working towards the objective of deriving atomic structure information from experiments carried out on just one individual molecule subject to the interaction with a coherent low-energy electron wave. Meanwhile, it has been thoroughly established that electrons with kinetic energies below 200 eV are the only radiation known today where elastic scattering dominates. Radiation damage-free imaging of a single biological molecule is thus possible by recording holograms and coherent low-energy electron diffraction patterns. Recently, by adopting a method to deposit individual proteins onto free-standing graphene in a collaboration with colleagues from the Max Planck Institute in Stuttgart, it has become possible to actually derive structural information on a single molecule level.



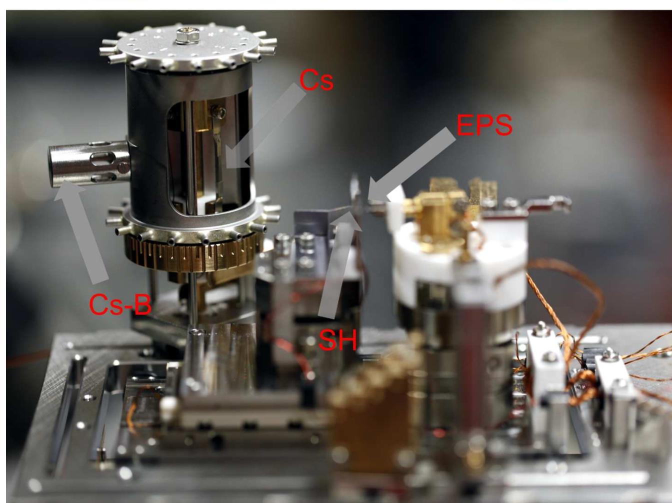


FIG. 15.2 – View into a new LEEPS microscope for exploring in situ adsorption of alkali atoms onto freestanding graphene. Top: dedicated rotatable alkali metal evaporator. Bottom: Cesium atom beam (Cs-B) tube to be directed towards free-standing graphene. Up to four cesium (Cs) sources are incorporated into the evaporator. The sample holder (SH) carries the free-standing graphene sample which can be transferred onto the system without breaking the ultra-high vacuum. The coherent electron point source (EPS) allows observing the adsorption processes by recording low energy electron holograms while Cs atoms settle onto the transparent graphene.

15.2 Imaging proteins at the truly single molecule level

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Imaging a single protein has been a long-standing aspiration for advancing structural biology along with various fields in natural science. In particular, revealing the distinct conformations of an individual protein is of utmost importance. Below, 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 allows chemical- and conformational-specific selection and gentle deposition. Low-energy electrons do not induce radiation damage, which enables acquiring sub-nanometer resolution images of individual proteins (cytochrome C and bovine serum albumin) as well as of protein complexes (hemoglobin), which are not the result of an averaging process.

15.2.1 Introduction

Most of the currently available information on protein structures has been obtained from either X-ray crystallography experiments or cryo-electron microscopy investigations by means of averaging over many molecules assembled into a crystal or over a large ensemble selected from low signal-to-noise ratio electron micrographs, respectively. Despite the impressive coverage of the proteome by the available data, a strong desire for acquiring structural information from just one individual molecule is emerging. The biological relevance of a protein lies in its structural dynamics, which is accompanied by distinct conformations. The associated structural details however, remain undiscovered when averaging is involved. Moreover, a large subset of the entirety of proteins, in particular from the important category of membrane proteins, are extremely difficult, if not impossible, to obtain in a crystalline form. If just one individual protein or protein complex can be analyzed in sufficient detail, those objects will finally become accessible.

For a meaningful contribution to structural biology, a tool for single molecule imaging must allow for observing an individual protein long enough to acquire a sufficient amount of data to reveal its structure without altering it. The strong inelastic scattering cross-section of high-energy electrons as employed in the state-of-the-art aberration-corrected Transmission Electron Microscopes (TEMs) inhibits accumulation of sufficient elastic scattering events to allow high-resolution reconstruction of just one molecule before it is irremediably destroyed. Staining proteins with heavy metal atoms is unfortunately not a viable alternative; it is well known that the chemical processes involved alter the protein structure. Moreover, heavy metal atoms are highly mobile under high-energy electron beams leading to ambiguous images. A new approach to the problem of structural biology is associated with the X-ray Free Electron Laser (XFEL) projects. With this impressive technological development and novel experimental tool, it is now possible to elucidate the structure of proteins brought in the form of crystals of just nanometer size. This method even originally appeared as a way of gaining information at the atomic scale from just a single biomolecule. Meanwhile it has become clear that averaging over a large number of molecules will unfortunately not be avoidable. In contrast to the radiation problem experienced when using high-energy electrons or X-rays, biomolecules, for instance DNA, can withstand prolonged irradiation by electrons with a kinetic energy in the range of 50 – 250 eV. Even after 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. This, combined with the fact that the de Broglie wavelengths associated with this energy range are between 0.7 and 1.7 Å, makes microscopy techniques taking advantage of this energy range, especially low-energy electron holography, auspicious candidates for structural biology at the single molecule level. In this lens-less microscopy scheme inspired by Gabor's original idea of holography, the samples are presented to a highly coherent beam of low-energy electrons generated by an atomically sharp field emitter tip placed as close as 100 nm in front of the sample. The interference pattern formed by the scattered and un-scattered electron waves, the so-called hologram, is recorded at an electron detector several centimeters away. While 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 fulfils these two requirements. While the damage-free radiation of 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 protein must be brought into an ultra-high vacuum (UHV) environment and fixed in space for an appropriate period of time for to accumulate sufficient structural information on the one hand, while avoiding the emergence of contaminants on the other hand. In the following, we show how sub-nanometer resolution images of individual proteins are obtained by means of low-energy electron holography. Native proteins are transferred from aqueous solution to the gas phase and deposited onto ultraclean freestanding graphene in an UHV

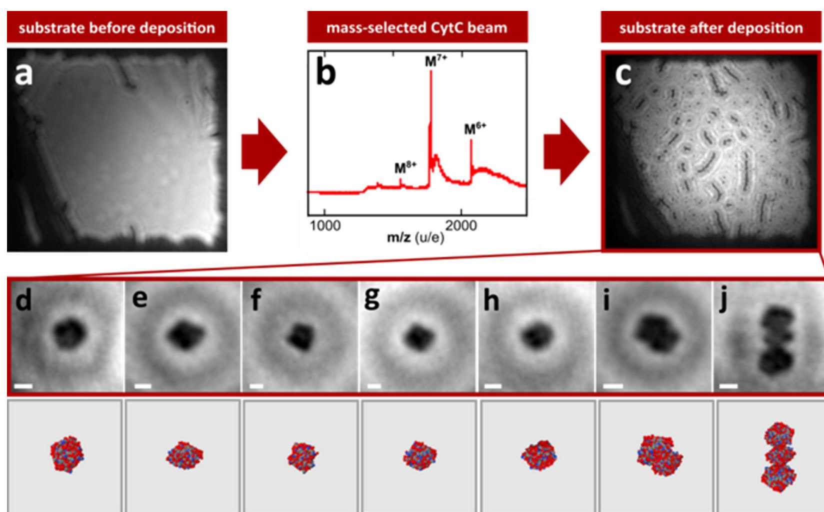

Fig. 15.3 – Complete data set for the imaging of CytC. (a) Low-energy electron image of ultraclean graphene before protein deposition. (b) Mass-spectrum of the mass-selected CytC beam. (c) A survey image of the very same freestanding graphene region after the deposition of CytC. (d) – (j) Low-energy electron micrographs with suggestions for possible protein orientations based on the averaged protein structure derived from X-ray crystallography data and documented in the protein data bank (pdb id: 1HRC). The scale bars correspond to 2 nm.

environment by means of soft-landing electrospray ion beam deposition (ES-IBD).

The workflow for imaging a single protein involves several steps. An ultraclean freestanding graphene sample covering 500×500 nm^2 apertures milled in a 100 nm thick SiN membrane is prepared following the recently developed platinum metal catalysis method and is characterized in the low-energy electron holographic microscope. The sample is subsequently transferred to an ES-IBD system under permanent UHV conditions by means of a UHV suitcase operating in the 10 – 11 mbar regime. Native cytochrome C (CytC), bovine serum albumin (BSA), and hemoglobin (HG) ion beams are generated by electrospray ionization and mass filtering. The charge states $z = 5 - 7$ are selected for CytC and the charge states $z = 15 - 18$ are selected for BSA13. In the case of HG, the charge states $z = 16$ or $z = 17$ of the intact complex are known to be of native conformation and hence the corresponding m/z region is selected. In all three cases, the ions are decelerated to a very low kinetic energy of 2 - 5 eV per charge, which ensures the intact deposition and retention of the native state upon deposition onto the ultraclean freestanding graphene substrate

15.2.2 How to image a single protein

After the deposition of a fraction of a monolayer of proteins, well-separated globular objects of similar size as well as agglomerations thereof, probably due to surface diffusion, are found on the graphene substrate. Control experiments involving the complete transfer process between the two vacuum chambers but without deposition have been performed, demonstrating that this process does not introduce any contamination onto freestanding graphene. At high magnification, the shape of the individual CytC proteins is revealed in several distinct orientations on graphene (Fig. 15.3(d) – 3(j)). It is not surprising to find the protein in different orientations since the deposition process is random in this respect.

The high-magnification low-energy electron micrographs of CytC presented in Fig.15.3 are of sufficiently high resolution to allow comparison with the structural data information obtained from X-ray crystallography investigations and available from the protein data bank. The overall size of the imaged CytC corresponds to the expected dimensions, and the low-energy electron images can clearly be associated with proteins in several distinct orientations. Imaging single objects over an extended period of time never led to any changes in the images. In particular, no sign of decomposition of the protein during electron exposure was observed, which is similar to what was demonstrated previously with DNA.

The same experimental workflow was used in the case of imaging BSA, a much larger protein than CytC (66 kDa versus 12 kD). A collection of low-energy electron micrographs of BSA is presented in Fig.15.4 (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 three-dimensional 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 but very characteristic orientations. The agreement between the micrographs and the atomic model for a protein like BSA, not purely globular but exhibiting very pronounced structural features, clearly demonstrates that proteins are found in UHV in structures closely related to their native structure.

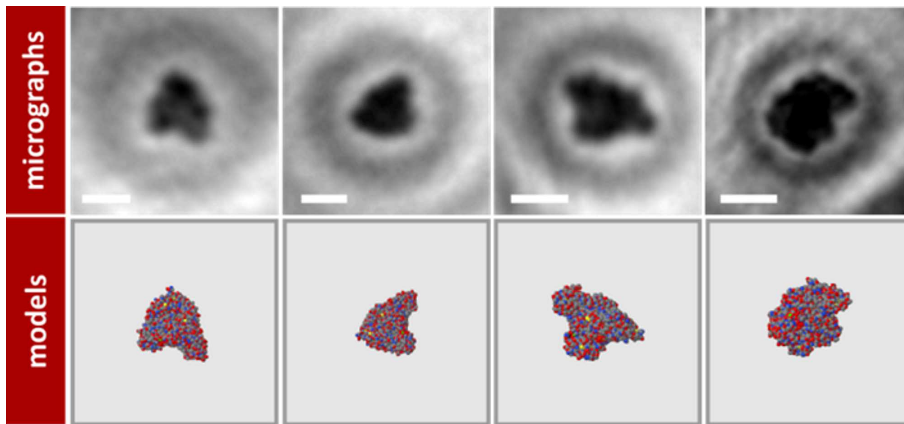

Fig. 15.4 – Low-energy electron micrographs of BSA in different orientations on graphene. Top: low-energy electron micrographs of BSA. Bottom: the atomic model of BSA (pdb id: 3V03) in the corresponding orientations. The scale bars correspond to 5 nm.

In biology, a non-covalently bonded complex of several proteins rather than a single protein is often 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.15.4 shows that protein agglomerations formed of two and three CytC 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. In Fig.15.5, two micrographs of individual hemoglobin (HG), 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. While for the cases of CytC and BSA the agreements between the low-energy electron images and the atomic models are almost perfect, differences can be observed for the case of HG. As mentioned above, HG is a protein complex composed of four sub-units. Therefore, HG has a large conformational flexibility related to its function in a living organism. When an averaging process over millions of molecules is involved in the imaging of a highly flexible protein, distinct conformations cannot be revealed and an average structure is obtained. In contrast, the micrographs of HG presented here are obtained from individual entities and the structural differences between our images and the model confidently demonstrate the benefit of a method for single protein imaging. In Fig.15.5 (top right) structural features of 7 – 8 Å can clearly be identified and may serve as a resolution estimate for this low-energy electron image. A similar resolution is found for all other micrographs. In a hologram, the spacing between consecutive interference fringes gradually decreases towards 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 in order to overcome this limitation and approach atomic resolution. While the current resolution already reveals the outer shape of single proteins and protein sub-units, an enhanced resolution of 2 Å will permit the inner structural details to be imaged as well, as hinted by the inner contrast variations apparent in the images above. As three-dimensional information is encoded in a single inline hologram, improved spatial resolution will permit these structural data to also be uncovered. Furthermore, tomographic capability could be added to the experimental setup to reveal the complete three-dimensional structure of a single protein.

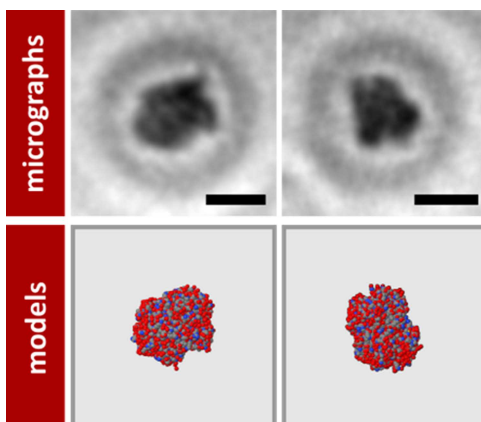

Fig. 15.5 – Low-energy electron micrographs of two individual hemoglobin molecules and the atomic model in the corresponding orientations. Top: two micrographs of hemoglobin soft-landed onto freestanding graphene. Bottom: suggestions for possible orientations based on the averaged protein structure derived from X-ray crystallography data and documented in the protein data bank. The scale bars correspond to 5 nm.

The ultimate goal of directly uncovering the structure of unknown proteins or protein complexes and describing their conformations at the atomic level still requires efforts towards improving the experimental setup and the imaging resolution. It is, however, not limited by fundamental physical constraints. At this stage, the comparison of the low-energy electron micrographs with atomic models available at the protein data bank has the character of a control experiment, proving the feasibility of this novel methodology. Nevertheless, fundamental questions remain. Most crucial is the influence of the environment on the protein's structure as graphene in vacuum represents for proteins a different environment than the aqueous medium of the cell. There is an important body of evidence, especially from ion-mobility/mass-spectrometry investigations, demonstrating that proteins and protein complexes can be transferred from a liquid phase to a vacuum environment while maintaining unperturbed their tertiary and quaternary structures, respectively. The low-energy electron micrographs presented here are further strong evidence that proteins in a folded sate are stable in UHV. Nevertheless, the possibility of adding a protein hydration shell under UHV conditions, as recently demonstrated for a small peptide and the possible induced conformational changes imaged by low-energy electron holography will be pursued in the future. 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 illustrate 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 has led to the first ever tool for revealing structural details of native single proteins and protein complexes without destroying them. With the recent advances in electrospray ionization and mass spectrometry of large protein complexes, and in particular membrane proteins, even the structure of these biologically important but reluctant to readily crystalize entities may become accessible in the near future.

For a complete list of references concerning our recent achievements in imaging single proteins using low energy electron holography, see:
`http://arxiv.org/abs/1512.08958`.