15 Physics of Biological Systems

Conrad Escher, Hans-Werner Fink, Tatiana Latychevskaia, Jean-Nicolas Longchamp (until February 2017), Marianna Lorenzo, Flavio Wicki (until July 2016)

in collaboration with: Dr. Roger Morin, CNRS Marseille (France), Prof. Peter Hommelhoff, University of Erlangen (Germany), Prof. Klaus Kern and Dr. Stephan Rauschenbach, Max Planck Institute, Stuttgart (Germany), Prof. Christian Schönenberger, University of Basel (Switzerland).

15.1 Overview of Current Projects

The structural investigation of individual biological objects by employing coherent low-energy electrons is at the center of our research. It involves in-line holography with low-energy electrons as well as coherent diffraction imaging and is assisted by micro-structuring techniques using a focussed gallium ion beam device for miniaturized electron optics and sample preparation. Major challenging experimental developments 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. In this way, it has become possible to image individual proteins for the first time in the history of structural biology. During the past year, strategies for helping to bring this novel tool for single protein structural biology into routine operation to the benefit of medical and pharmaceutical research and development have been developed.

15.2 Novel applications of coherent point source beams

While holography and coherent diffraction applied to biological systems are the most prominent applications pursued by us, in the frame of the PhD thesis of Flavio Wicki, some quite different and novel applications of electron point sources have been developed. A miniaturized electron column exhibiting electrostatic lenses in the micrometer regime has been realized following the notion that the intrinsic lens aberrations of cylindrical symmetric electron lenses scale with their dimensions. This approach has led to a tiny electron microscope column with excellent beam shaping performance due to aberrations reduced by about a factor of thousand using this downscaling principle.

A further novel application is the in-situ combination of low-energy electron holography with Scanning Tunneling Microscopy (STM) technology in order to add a novel contrast mechanism to Low Energy Electron Point Source (LEEPS) microscopy. Holograms of clusters and their reconstruction were thus combined with image information arising from scanning the electron point source in close proximity to the free-standing graphene support to measure the absorption as well as the electron transmission through the very same individual clusters present on free-standing graphene.

This novel mode of operation leads to very low kinetic energies, below 30 eV, of the emitted electrons which has led to another previously unknown application of a coherent electron point source, namely a direct mapping of electronic structure details of free-standing 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 translates in a direct mapping of the unoccupied bands of graphene revealed by a hexagonal structure in the transmission image observed on a distant detector. Owing to the high numerical aperture of the detection system, the hexagons reflecting the unoccupied bands grow accordingly by decreasing the primary kinetic energy of the electrons from 30 down to 18 eV. The measurement principle and the observed pattern are illustrated in Fig. 15.1.

15.3 Imaging the potential distribution of individual charged impurities on graphene by low-energy electron holography

While imaging individual atoms can routinely be achieved in high resolution transmission electron microscopy, visualizing the potential distribution of individually charged adsorbates leading to a phase shift of the probing electron wave is still a challenging task. Low-energy electrons (30 - 250 eV) are sensitive to localized potential gradients. We employed low-energy electron holography to acquire in-line holograms of individual charged impurities on free-standing graphene. By applying an iterative phase retrieval reconstruction routine, we recover the potential distribution of the localized charged impurities present on free-standing graphene.

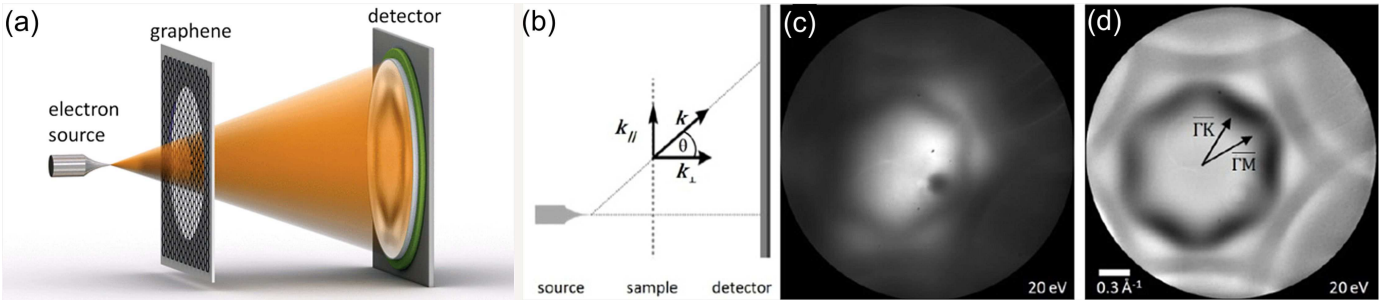

FIG. 15.1 – (a) Schematic of the experimental setup. The in-plane k-vector of the electrons impinging onto graphene under a certain angle is illustrated in (b). The in-plane momentum dependency of the transmission through free-standing graphene thus allows probing the unoccupied bands by observing the electron transmission pattern at a distant detector. In (c) raw data of such a transmission pattern for 20 eV electrons are shown while in (d) 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.

The experimental setup is depicted in Fig. 15.2. Electrons are field emitted from a sharp tungsten tip and the electron wave passes through the sample while part of the wave is scattered by the object. Interference between the scattered and non-scattered wave leads to a hologram recorded at a distant detector. The sample is free-standing graphene with some residual adsorbates after sample preparation. Since transparent free-standing graphene provides an overall equipotential plane, a neutral adsorbate present on graphene results in an ordinary hologram, as illustrated in Fig. 15.2(b). However, a charged impurity locally creates a high electric field deflecting the passing electrons. The presence of a positively charged impurity thus leads to a distinctive signature in the hologram, a bright spot, as illustrated in Fig. 15.2(c).

In the case of imaging a charged object with an electron wave, the distribution of the electron wave is altered by the object. A charged impurity deflects even those electrons which are passing the localized charge at some distance. Therefore, a clean separation between the reference wave (not affected by the object) and the object wave (affected by the object) is not possible any more. Consequently, the lack of a well-defined reference wave precludes an ordinary hologram reconstruction. A sensible hologram reconstruction becomes thus more involved. The problem of object reconstruction from the recorded interference pattern is similar to the phase retrieval problem in coherent diffraction imaging (CDI), where only the intensity of the scattered wave is detected but no reference wave is present. In CDI, typically, an iterative procedure is applied to retrieve the missing phase distribution in the detector plane and consequently to reconstruct the object distribution. We therefore apply such an iterative procedure to reconstruct holograms of charged impurities. It starts with an initial random phase distribution in the detector plane. In this way, it could finally be

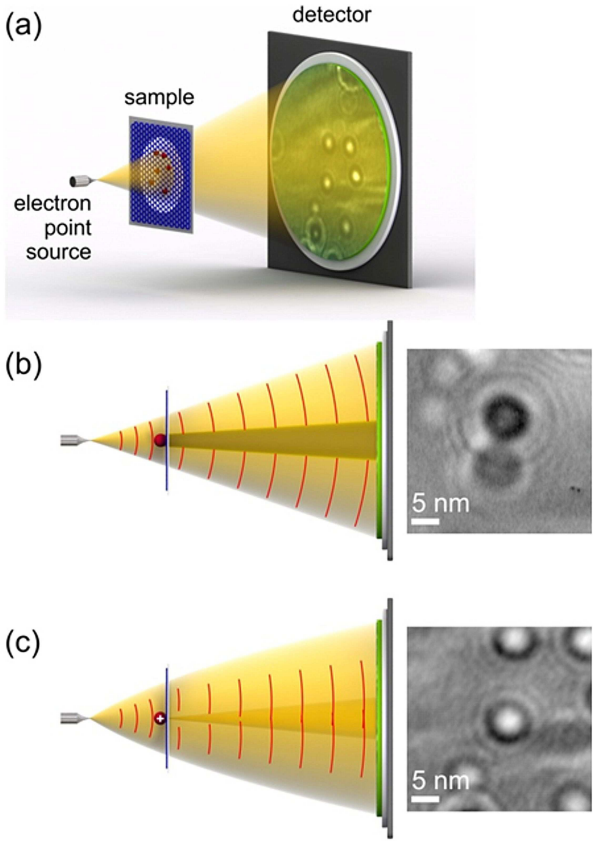

FIG. 15.2 – Experimental arrangement. (a) Low-energy electron holographic microscope. The distance from the source to the sample is in the range of tens of nanometers and the distance from the source to the detector is 47 mm. (b) Imaging of a neutral adsorbate whereby part of the beam is absorbed resulting in an overall dark interference pattern. (c) Imaging of a positively charged adsorbate. The trajectories of electrons are deflected inward by the presence of a positive charge resulting in a bright spot on the detector.

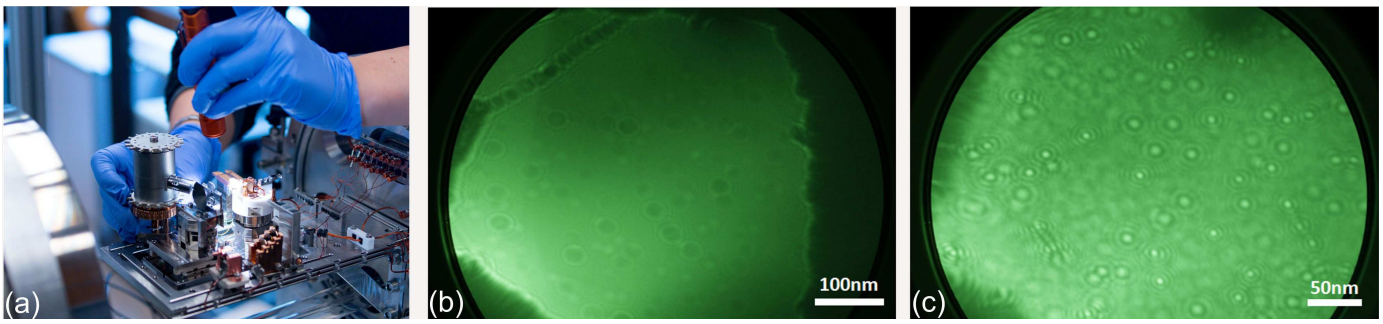

FIG. 15.3 – (a) View into the new LEEPS microscope featuring a rotatable metal evaporator with up to four different metal sources. (b) Clean graphene imaged with 145 eV electrons. (c) Image taken with 100 eV electrons after Cesium deposition onto graphene.

demonstrated that projected potentials of individual adsorbates can be obtained from their low-energy electron holograms by iterative phase retrieval reconstruction.

15.4 Metal nucleation on free-standing graphene

A modified LEEPS microscope has been employed to study in situ metal nucleation on free-standing graphene in the frame of the PhD thesis of Marianna Lorenzo. Up to four different metal sources allow the in-situ deposition of alkali metals under UHV conditions. Alkali atoms were chosen because of an expected significant charge transfer from the atoms to the free-standing graphene support. The transfer of the s-electron to graphene should lead to a strong dipole which should be detectable with low-energy electrons. The experimental setup is shown in Fig. 15.3(a), together with an image of the clean, 15.3(b), as well as a Cesium covered graphene window, 15.3(c). It seems that the transfer of the s-electron causes a remaining positive charge attracting the low-energy electrons leading to bright spots as signatures for charged objects. Detailed studies with different alkali atoms, namely Potassium and Lithium, have also been carried out as well as control experiments with Palladium atoms leading to compact cluster formation.

Figure 15.4 shows a region of a graphene sample where a transition between a single layer and a double layer is displayed which allows studying intercalation effects with alkali atoms. It turns out that Cesium is highly mobile on graphene at room temperature as apparent from observing the situation in-situ during the evaporation process. Upon stopping the evaporation, an equilibrium with constant overall coverage is eventually established. Apparently, as evident from the bottom part of Fig. 15.4, the density of Cesium atoms intercalated in between the double layer appears to be significantly higher than that on the single graphene layer. This translates into an enhanced free energy of binding of intercalated Cesium which is the subject of ongoing studies.

15.5 Imaging proteins at the truly single molecule level

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

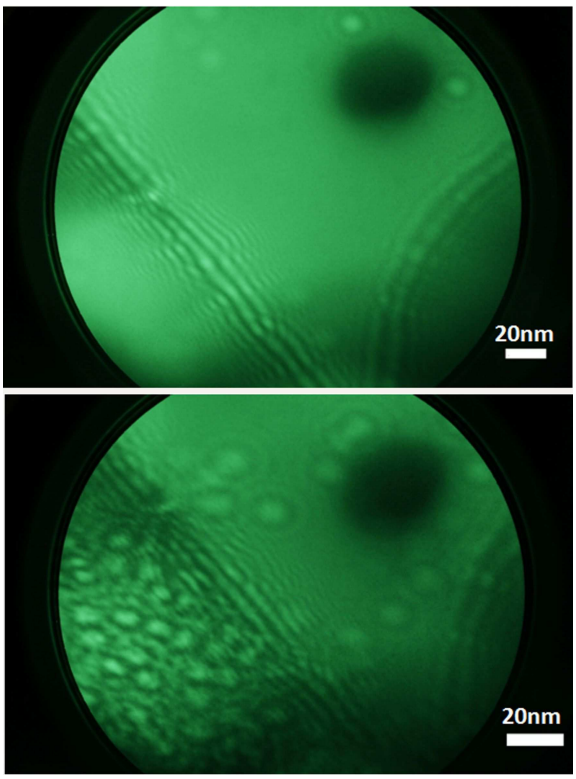

FIG. 15.4 – Top: Graphene sample exhibiting a transition from single to double layer graphene imaged with 66 eV electrons. Bottom: Same region, again imaged with 66 eV electrons, after evaporation of Cesium. After an appropriate waiting time, the system assumes an equilibrium state which is apparently characterized by an enhanced density of intercalated Cesium atoms compared to the density observed for the single layer.

importance. We have shown that imaging individual proteins and protein complexes has become possible 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, carried out by our colleagues from the Max Planck Institute in Stuttgart. Their technology allows for 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. It was thus the first time that an individual protein has ever been observed at a meaningful spatial resolution.

15.5.1 Current state of the art and perspectives

In Fig. 15.5, two micrographs of individual Bovine Serum Albumin (BSA) are presented and confronted to electron density maps simulated at a resolution of 8 Å with the software package Chimera, originally developed for the analysis of Cryo-EM images. We find a considerable conformance between the simulated density maps and the micrographs and the resolution estimate made from the holographic record is in good agreement with the simulation. To demonstrate that even proteins as large as BSA are sufficiently transparent for low-energy electrons to deliver three-dimensional structural information from a single hologram, the contrast in these micrographs has been enhanced.

Especially in the low-energy electron image presented in the right column, variations of the inner contrast can easily be recognized. To understand the origin of the darker region in the center of the protein image and to associate it with structural features, one may take a look at the protein from a direction parallel to the plane of graphene. The red arrows in the middle row of Fig. 15.5 indicate the direction of observation corresponding to the side views presented in the third row of this image. With the help of these side view representations of the density maps, in which graphene is indicated by a solid blue line, it is possible to address how the proteins are actually adsorbed on the graphene substrate. Of particular interest is the side view presented in the right column as it shows that in this specific orientation, BSA is of strongly varying thickness with a maximum towards the center. The darker region observed

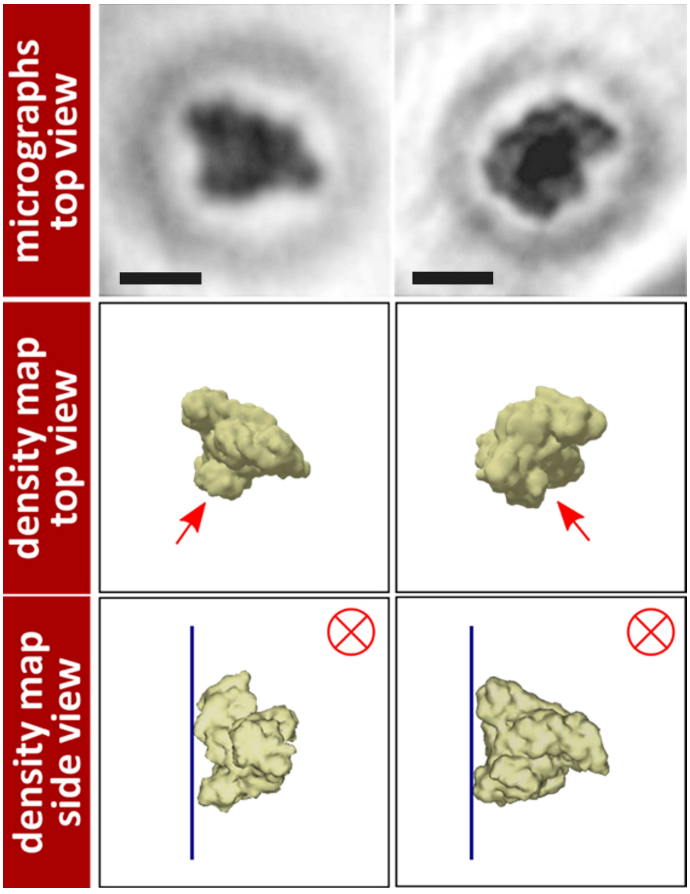

FIG. 15.5 –
Comparison of low-energy electron micrographs of BSA with simulated electron density maps. Top: Low-energy electron micrographs of BSA. The scale bars correspond to 5 nm. Middle: Electron density maps simulated at a resolution of 8 Å and rotated to match the orientation of the proteins presented in the top row. Bottom: Side view of the density map along the directions of observation indicated by the arrows shown in the middle row.

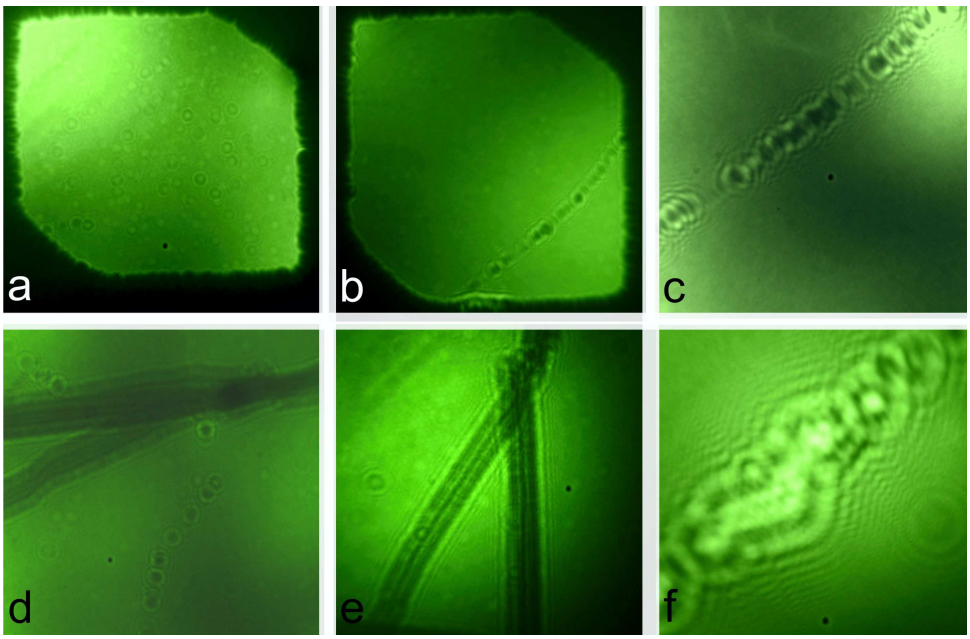

FIG. 15.6 – Low-energy, typically around 100 eV here, transmission images of free-standing graphene. (a) Perfect 500 nm by 500 nm graphene window representative for about 60% of all 400 windows inspected. An apparent grain boundary with some adsorbates is shown in (b) and with higher magnification in (c). Ribbons are shown in (d) and (e) and occasionally holes in otherwise perfect graphene are also apparent (f).

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 three-dimensional structure of the protein can be gained. In-prospect of an improved resolution of the order of 1-2 Å, it also illustrates the future ability to gain a complete three-dimensional structure from a single low-energy electron hologram of proteins at least as large as 60 kDa.

In this context, it should be noted that the protein data taken so far have been obtained with a LEEPS microscope equipped with the feature of linking up a UHV suitcase for transporting samples to and from Stuttgart where the protein deposition took place. Since its original design was optimized for coherent diffraction imaging for which residual vibrations are not relevant, it was not optimized for best possible vibration damping. It appears that our newest generation LEEPS microscope is already capable to deliver interference resolution in the 3 Å regime at least. Further improvement is to be expected from modifications of the electron point source leading to an increased emission angle.

15.5.2 Properties of free-standing graphene

It seems that graphene, at least until no other two-dimensional structure with high electron transmission properties becomes known, constitutes an optimal support for deposing proteins onto it for subsequent imaging with low-energy electrons. It can be considered as equivalent to the cover slip in light optical microscopy. And, just like for a cover slip in light optical microscopy, one wishes to render it as featureless as possible in order to image just the object that it is supposed to hold fixed in space. So far, we have been using commercially available graphene placed over micro-structured square windows with a typical side length of 500 nm in a SiN membrane. Next to single layer graphene, these commercial samples also feature double and triple layers as well as defects and grain boundaries. While it is not too difficult identifying appropriate windows covered with just a single layer, it seems desirable to maximize the number of perfect graphene windows for imaging proteins deposited onto them. It turns out that our colleagues in the mesoscopic physics group, led by Christian Schönenberger in Basel, are capable of growing superior high quality graphene on copper substrates. Samples from Basel were provided to us and some transmission images are shown in Fig. 15.6.

15.5.3 Perspectives for the first compact single protein structural biology tool

Up to now, protein imaging has been done ex-situ. Holographic imaging and hologram reconstructions were carried out in Zurich while protein deposition has been performed at the May-Planck Institute in Stuttgart. Given the interest of the live science community to eventually arriving at a routine single protein imaging tool, also exemplified by the significant efforts associated with the worldwide XFEL projects, it seems appropriate to work towards a single structural biology tool featuring protein deposition and imaging in-situ. During the past year, extensive interaction with colleagues in the field of protein mass spectrometry as well as with companies active in the field have been undertaken. The goal is to help bringing this novel tool for single protein structural biology into routine operation to the benefit of medical and pharmaceutical research and development. One possible

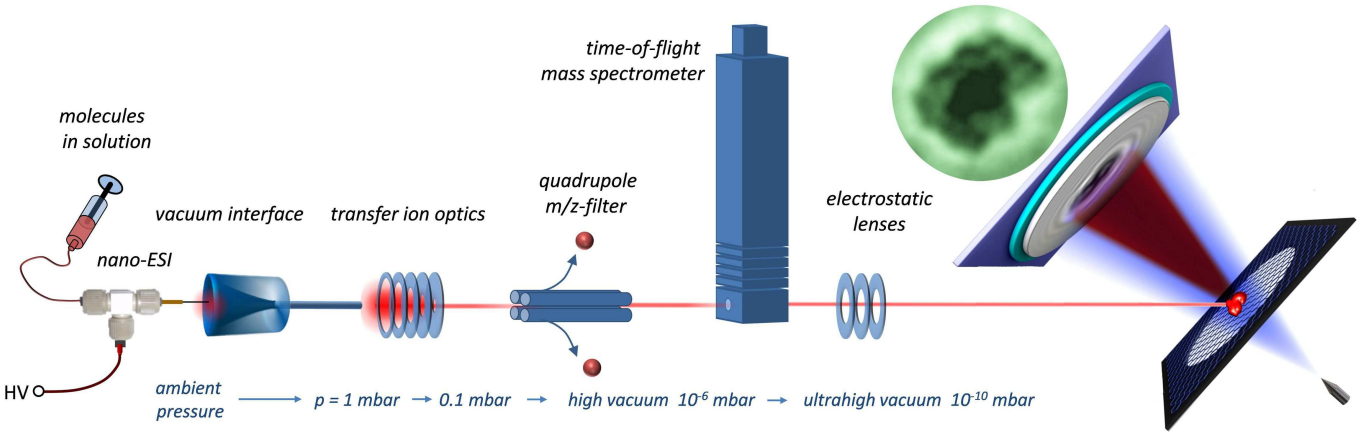

FIG. 15.7 – Schematic, showing the elements of an envisioned future compact single protein structural biology tool. From left to right: Protein ion source and beam shaping part for selecting a specific mass to charge ratio of a selected protein. Time of flight mass-spectrometer to verify the desired protein state. Coupling of the protein beam into the imaging chamber featuring soft landing and holographic imaging of single proteins.

route is to engage companies active in the field of protein mass spectrometry, also to the benefit of the University of Zurich that holds a patent for the combination of low-energy electron holography with protein mass spectrometry. A schematic showing how such a future system might look like, is depicted in Fig. 15.7. If the proteins, as hinted in the schematic here, might even be observable right during the process of landing on the graphene support, still remains to be explored in some more detail.

15.5.4 References

More details about properties and applications of coherent low-energy electron sources can be found here:

- Holography and coherent diffraction with low-energy electrons: A route towards structural biology at the single molecule level, by Tatiana Latychevskaia, Jean-Nicolas Longchamp, Conrad Escher, Hans-Werner Fink, Ultramicroscopy, Volume 159, Part 2, December 2015, Pages 395-402.

The imaging of single proteins using low energy electron holography is described in:

- Imaging proteins at the single molecule level, by Jean-Nicolas Longchamp, Stephan Rauschenbach, Sabine Abb, Conrad Escher, Tatiana Latychevskaia, Klaus Kern, Hans-Werner Fink, PNAS, February 14, 2017, vol. 114 no. 7.