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LVEM: CONTRAST-ENHANCING TECHNOLOGY FOR APPLICATIONS FROM 0D TO 3D MATERIALS

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Dr Jaromír Bačovský obtained his PhD in Physical Engineering at Brno University of Technology, specialising in low-voltage transmission electron microscopy (LVEM). His scientific interest was focussed on aberration correction of low voltage transmission electron microscope, and nowadays, he develops and innovates application areas of these unique instruments. With over a decade of experience, he is a senior application team member at Delong Instruments, where he works as an application scientist/physicist and continues innovating in LVEM technology.

1. *ABSTRACT*

Low-voltage transmission electron microscopy (LVEM) is a powerful tool in nanoscience and materials characterisation. This talk will focus on the key benefits offered by LVEM techniques, highlighting their significance in various application areas, especially material sciences, and emphasising their versatility for multimodal imaging.

LVEM operates at significantly lower accelerating voltages compared to conventional transmission electron microscope (TEM), typically below 25 kV. This lower voltage regime offers several distinct advantages, including increased contrast and gentle electron-sample interaction observed in several commonly used sample kinds.

Both spatial resolution and image contrast stand as the most crucial criteria for evaluating the micrograph quality. Low voltage provides improved contrast mechanisms, particularly invaluable for the samples composed of light elements. The reduced electron energy results in increased electron scattering, enhancing image contrast and revealing finer details within specimens. This capability is important in imaging polymers, graphene, biological samples, and generally low-contrast materials, where traditional TEM techniques may yield limited contrast.

Moreover, LVEMs mitigate radiation damage for a broad range of appropriately thin samples, preserving delicate nanostructures and enabling prolonged observation of beam-sensitive specimens.

The unique combination of TEM, scanning transmission electron microscope (STEM), scanning electron microscope (SEM) imaging modes in LVEM instruments provides researchers with a comprehensive toolkit for investigating diverse materials. Furthermore, LVEM's versatility extends beyond imaging, offering advanced analytical capabilities such as energy-dispersive X-ray spectrometry (EDS) and electron diffraction.

2. *INTRODUCTION*

While it may be tempting to assume that advancements in microscopy technology would inherently allow the examination of smaller and smaller objects, the reality is constrained by the finite diversity of natural specimens available for routine analysis. Modern electron microscopes have already revealed specimen details of all scales all the way down to the atomic structure. However, a resolution is only one parameter to evaluate micrograph quality. The second, no less critical parameter is image contrast, a significant feature of micrographs provided by low voltage transmission electron microscopes (LVEM).

The concept of enhancing contrast in biological specimens through the use of a (TEM) operating at low voltages has been discussed already more than six decades ago. As early as 1958, Nixon [1, 2] recognised the potential for increased contrast through heightened inelastic scattering at lower electron energies. Building upon this principle, pioneers such as Wilska and Mollenstedt embarked on practical implementations, decelerating electrons before their interaction with the specimen and subsequently accelerating them post-scattering. Their efforts yielded promising results, demonstrating notable improvements in contrast. Despite these advancements, the realisation of a low-voltage TEM remained elusive for decades [3].

The visionary origins of LVEM, are connected to the pioneering work of Prof Armin Delong. His dream of creating a compact, user-friendly electron microscope accessible to all scientists has been realised in the form of LVEM series microscopes produced by Delong Instruments [4].

LVEM has been developed as a technique utilising an electron beam with an energy range of 5 - 30 kV, corresponding to a wavelength interval of 17.3 - 7.1 picometres. Such a low primary beam energy gives this technology very specific imaging properties. The reduced electron energy increases electron scattering, significantly enhancing image contrast. This is particularly essential for investigating sensitive samples characterised by light atoms and weak bonds, where conventional transmission electron microscopes usually have difficulty achieving sufficient contrast.

While the initial benefits of low voltage technology were predominantly recognised in enhancing the contrast of biological specimens, it has become evident over time that its application area extends far beyond this and also includes material science and nanotechnology.

Moreover, certain technical aspects, which will be discussed upon later, also allow the LVEMs to be designed as a desktop concept, in contrast to the large dimensions typically associated with conventional TEM/STEM devices.

3. LOW VOLTAGE TRANSMISSION ELECTRON MICROSCOPY

The current scientific and laboratory instrument market offers a wide range of electron microscopes, each equipped with various instrumentation, capabilities, and accelerating energies.

The most widespread transmission electron microscopes typically operate at an accelerating voltage range of 50 kV to 300 kV. This device category is commonly referred to as conventional transmission electron microscopy. Such high beam energy provides specific properties to these devices that make them unremarkable, powerful investigative tools. However, this high voltage can also impose significant limitations on numerous potential applications.

Instruments featuring an accelerating voltage below 50 kV are called low-voltage electron microscopes (LVEM). Although the energy boundary is not strictly precise, it arises from an informal consensus based on the relative impacts of chromatic and spherical aberration. In the conventional transmission electron microscope, spherical aberration poses a significant limitation, while the impact of chromatic aberration is relatively less critical. On the other hand, at lower energies, the situation reverses. As the energy of the primary beam decreases, the significance of spherical aberration diminishes, and chromatic aberration becomes increasingly important. At approximately 50 keV, both aberrations exert equivalent influence, setting the threshold for low-voltage electron microscopy.

The insufficient contrast of conventional systems is usually solved by various methods indirectly highlighting the structures of the sample. It is usually necessary to use some level of defocus to introduce a phase contrast component. In addition, the sample must typically be selectively stained with heavy metal atoms (Mo, W, Os, U).

The cornerstone of common sample preparation protocols is a fixation by osmium tetroxide (OsO4), which exhibits rapid killing capabilities. It plays a crucial role in stabilising numerous proteins, transforming them into gels without compromising their structural integrity. Tissue proteins stabilised by OsO_4 remain uncoagulated by alcohol during the dehydration process, which is the essential step in the sample preparation protocol. Fixation by osmium tetroxide is considered to be the first staining level followed by post-staining with uranyl acetate. Aqueous solutions with concentrations ranging between 2 % and 5 % of this uranium salt are applied to the section. Uranyl ions bind extensively with phosphate groups found in nucleic acids, as well as phosphate and carboxyl groups present on cell surfaces, and provide selective high-level staining.

To reach sufficient contrast when imaging by LVEM is typically sufficient to perform the fixation and post-staining can be omitted [5].

Commonly used aforementioned staining agents pose significant health risks and are subject to stringent legal regulations regarding handling and storage. Consequently, efforts are underway to find less hazardous alternatives. One such solution is UA-Zero, a patented stain developed as a substitute for uranyl acetate. UA-Zero is non-radioactive and safe for use, transportation, and disposal. It can seamlessly replace uranyl acetate without requiring changes to standard protocols, offering high-contrast imaging capabilities.

Another alternative is UranyLess, a novel contrast stain solution tailored for transmission electron microscopy (TEM) applications, particularly effective for negative staining techniques.

In addition to these, more unconventional alternatives, such as extracts from green tea, are also utilised for staining purposes.

However, if it is possible to reduce or entirely eliminate staining, it is highly preferable. This approach allows for direct observation of the sample, eliminating the risk of introducing staining-related artefacts. With low-voltage electron microscopes, we usually achieve comparable results even without staining, as achieved by conventional microscopes with staining.

Another extensively discussed topic related to low energies is the interaction between the electron beam and the sample. Although it might seem that the lower energy of electrons results in reduced sample damage, the opposite holds true in theory; lower energies theoretically lead to more severe damage compared to the typical higher energies of conventional transmission electron microscopes.

This effect can be attributed to the smaller interaction cross-section at higher energies. Thus as the contrast increases, so does the potential damage to the sample. Despite this theory, in practice, observations often reveal that samples illuminated by the electron beam in low-voltage microscopes endure significantly longer. This can be explained by the fact that other mechanisms of sample damage predominate. For instance, in the case of low energy, the majority of sample damage is caused by ionisation (radiolysis) and by heating of the sample, with knock-on effects being minimal [6]. However, not all of these phenomena manifest themselves visibly in the sample and do not induce changes in the sample morphology. From this point of view, the most critical parameter is the total amount of energy absorbed by the sample, which can induce local thermal damage, leading to the breaking of supporting films.

Based on our experience, low voltage may damage the sample on the single slot grid with a thick formvar supporting layer because a lot of energy is absorbed due to the high total thickness of the entire sample. Consequently, these samples are not considered very suitable for LVEMs. On the other hand, in the case of optimal sample preparation using a standard grid with reasonable mesh size without supporting film or with ultra-thin carbon support if necessary, biological sections are highly stable and survive under the electron beam of low energy without visible damage. In contrast, such samples often break quickly with conventional microscopes under similar conditions.

3. LVEM'S FAMILY OF DELONG INSTRUMENTS

3.1. LVEM 5

LVEM 5 is the only commercially produced benchtop transmission electron microscope characterised by its remarkably compact design (Fig. 1). Operating at an accelerating voltage of approximately 5 kV, the LVEM 5 presents unparalleled versatility through its integration of four distinct imaging modes. Depending on the specific LVEM 5 configuration, the user can easily alternate between operating the microscope as a TEM, STEM and SEM.

Figure 1. Left: LVEM 5 and right: LVEM 25E.

Furthermore, the LVEM 5 is equipped to acquire electron diffraction, enhancing its utility in diverse crystallographic research applications. Despite having dimensions comparable to a light-optical microscope, it astonishes users with a resolution of 1.2 nm in the TEM boost mode.

3.2. LVEM 25E

According to the original intention of Prof Delong, LVEM 5's main application area was supposed to be the imaging of biological sections. However, the physical limitation of sample thickness (optimal sample thickness for 5 kV and biological sections is 20 - 30 nm) has proven to be quite challenging. Therefore, LVEM 25 and new generation LVEM 25E were designed for standard sample thickness of biological sections, typically within the range of 80 nm to 150 nm, while preserving the main advantage of low energy, i.e., exceptional image contrast (Fig. 1).

This instrument offers a comprehensive portfolio of imaging modes: Transmission electron microscopy (TEM) operating at an accelerating energy of 25 keV, scanning transmission electron microscopy (STEM) operating at 10 keV or 15 keV, and scanning electron microscopy (SEM) with detection of backscattered electrons (BSE) operating at 15 keV. Both transmission modes are capable of bright field and dark field imaging.

TEM is especially convenient due to its comfortable operation, with no waiting for scanning, offering the highest spatial resolution among available modes. STEM becomes advantageous when sections are not perfectly sectioned, and the TEM image seems slightly blurry due to chromatic aberration [2]. STEM deals better with thicker samples because it is not sensitive to the additional contribution of chromatic aberration given by the broadening of energy spread caused by the interaction of the primary beam with the sample itself.

The versatility of the instrument is supported by analytical modes, including energy-dispersive X-ray spectrometry (EDS) for elemental analysis, including mapping, and electron diffraction (ED) for crystallography. The combination of all of these modes makes LVEM 25 a perfect solution for multimodal imaging.

4. UNIQUENESS OF TECHNICAL DESIGN

The columns of the common transmission electron microscopes are usually quite massive, due to the fact that they contain ordinary electromagnetic lenses made from coils that cannot be simply scaled down because the high electric current and many turns in the coil are necessary to reach a sufficient magnetic field in the lens gap. Moreover, electromagnetic lenses generate considerable heat, and conventional microscopes must, therefore, be equipped with a cooling system, which is usually essential to thermally stabilise the electron optics and protect it against overheating.

All transmission electron microscopes developed by Delong Instruments are designed with different approach. They are equipped with objective lens based on permanent magnet technology. This construction allows for a remarkably high level of miniaturisation and enables operation without any cooling system of the column.

Inline optics of LVEMs begin with a Schottky-type electron source followed by a permanent magnet doublet. The first gap of the doublet is considered to be the condenser lens 1. Due to the inlens character of the immersion objective lens, the second gap, situated farther from the electron source, forms both condenser lens 2 by the pre-field and also the objective lens by the part of the field following after the specimen [7, 8]. The presence of a magnetostatic lens necessitates a different approach to solving many traditionally encountered solutions, for instance, focussing and adjusting illumination.

All electron optics are enclosed in a significantly shortened column compared to the conventional instruments. The possibility of designing the system in such a compact form is a direct consequence of low beam energy.

The reason for using electrons for microscopy is to overcome the limitation given by the wavelength of the light. However, since Scherzer published his theorem, it was clear that electron optics do not qualitatively match light optics and do not reach its physical limits. Consequently, some electron microscopes use the additional magnification of the image created with electrons by light optics.

Low voltage microscopes are designed in the same way, but there are distinct advantages. The first stage of magnification is done by electron optics, which forms the initial image on a fluorescent YAG screen. This nearly structureless scintillation crystal without any granular

structure, processed to a flat surface plate with high optical homogeneity, converts the electron-optical image into the initial light image, which is further magnified by light optics with a selection of proper light objectives [3, 7].

Electron with low energy excites significantly smaller volume in the YAG screen. Thus, it is possible to magnify the initial electron image much more by the following light optics before the empty magnification is reached. The higher energy of incident electrons typical for conventional transmission electron microscopes leads to a bigger excited volume in the fluorescent screen material, and consequently, it is not reasonable to magnify the initial light image by the light optics too much.

Conventional transmission electron microscopes have to reach higher magnification by the electron optical part, leading to longer columns, because it is not possible to design a short optical system with such a high magnification without severe aberration deterioration.

Compact columns offer numerous advantages beyond enabling the device to be built in a compact or benchtop form. They also contribute to mechanical stability. A shorter column with a dimension ratio closer to a cube is less sensitive to vibrations compared to longer, rod-like columns, which are less solid. This entire configuration is further optimised by the upside-down design, which situates the cathode at the bottom to ensure a lower centre of gravity.

5. LVEM FOR CHARACTERISATION OF LIGHT ELEMENT SAMPLES

Low voltage transmission electron microscopy (LVEM) offers a wide range of applications across diverse scientific domains, spanning from material science to life science. Its unique advantages become particularly pronounced when imaging samples are predominantly composed of light elements, which often pose contrast challenges for conventional transmission electron microscopes. However, it is important to note that LVEM is not limited solely to such samples. In fact, with proper sample preparation, it can also effectively image other types of specimens, including material science FIB lamellas or metallographic samples, although these may not represent its primary application domain.

Due to the sufficient resolution in transmission modes and versatility of the instrument equipped with backscattered electron (BSE) detector, it is possible to investigate samples from 0D quantum to 3D bulk samples with volume up to approximately 1 mm^3. Moreover, it is possible, if it makes sense for a given sample, to combine signals from different modes and extract the maximum amount of information from the interaction of the beam electrons with the sample.

Considering the reasonable cost, the LVEMs stand out as popular screening devices that serve as a valuable complement to high-resolution TEMs or provide basic nanomaterial characterisation directly within the production facility. This makes them efficient tools for quick assessments and initial screenings, allowing for timely decisions without the need for outsourcing or waiting for results from overloaded big TEMs.

In order to demonstrate the ability of the low voltage instruments on typical samples drawing on the advantages of the device, several typical applications were selected through different scientific fields and application directions.

As evidence that low voltage transmission electron microscopy is not limited in application by its resolution, it can be considered the imaging of various kinds of quantum dots (Figs. 2 and 3).

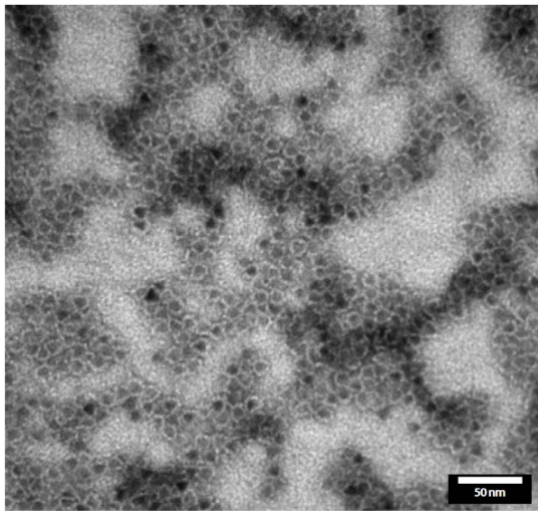

Figure 2. 10 nm quatum dot (QD), 605 ITK carboxyl, inorganic core and carboxyl shell, stained by 1.2 % UAc (LVEM 25).

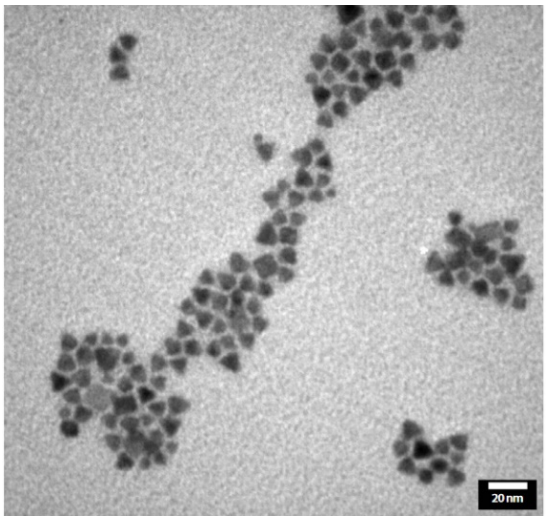

Figure 3. CdSe core with CdS shell covered with oleic acid (LVEM 25).

Particularly problematic from the contrast point of view for conventional TEMs are carbon quantum nanodots (CQDs). Carbon quantum nanodots are a novel class of 0D fluorescent carbon nanomaterials that have gained significant attention in recent years. They are composed of sp^2/sp^3 carbons and measuring less than 10 nm in size. Notably, they exhibit high stability, excellent conductivity, biocompatibility, low toxicity, environmental friendliness, dispersibility in water, and minimal photobleaching. One of the most remarkable features of CQDs is their strong and adjustable fluorescence emission properties, which can be finely tuned by manipulating the excitation wavelength. This adaptability renders CQDs highly versatile and suitable for a wide array of applications, including biomedicine, optoelectronics, catalysis, sensing, gene delivery systems, and theranostics. An example of CQDs application is the covalent modification of carbon quantum dots with polyamidoamine dendrimers (Fig. 4). These nanohybrids may have potential applications in biomedicine as promising gene carrier system of triple negative breast cancer gene therapy. Complementary imaging of dendrimers with an AFM operating in tapping mode has shown the morphology of the dendrimers, revealing a height ranging from 1 to 2 nm above the amorphous carbon substrate. Because of this subtle variation in the sample morphology excellent contrast was obtained [9].

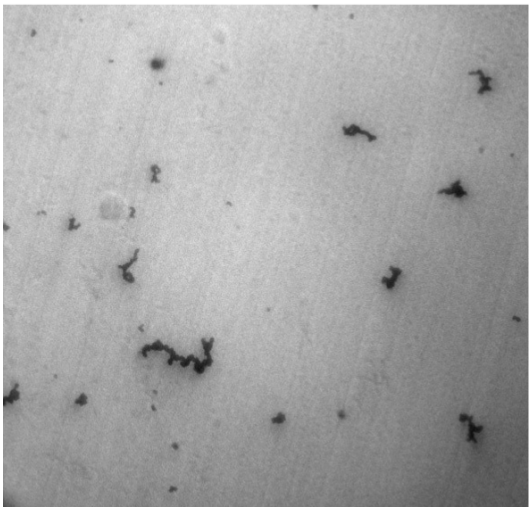

Figure 4. Dendrimers imaged by LVEM 5.

Moving to 2D samples, LVEM provides very good differentiation between the individual polymer layers of very similar physical properties and almost identical chemical composition. The morphology of polymer blends can be observed in thin films without additional pre-treatment, such as staining, when utilising low-voltage electron microscopy techniques – TEM or STEM imaging modes. The contrast in the image is considered to be created by differences in density between individual components within the polymer blends. The sufficient density variations for identifying phase structure are as small as 0.04 g/cm^3 [10].

Considering the contrast creation mechanism when investigating ultrathin sections by low-voltage transmission electron microscopy, relief of the section plays a crucial role [11]. To understand this phenomenon, experiments were conducted to monitor the processes involved in ultrathin sectioning using a polymeric material consisting of a hard matrix and soft particles, specifically focusing on high-impact polystyrene. The results revealed volume changes leading to surface relief on both cut surfaces: ultrathin section and maternal sample. The cutting response of sample components was investigated during room-temperature and cryo-sectioning. The volume relaxation effect occurs especially when cryo-sectioning is utilised. These differences in thickness at the sites of individual phases contribute to the enhancement of image contrast in transmission electron micrographs, a particularly advantageous effect in LVEM imaging [11].

Another large application area of low voltage transmission electron microscopy is a graphene materials group. Genuine diamane is a novel member of this nanocarbon material group, which includes, among others, such promising materials as fullerenes, nanodiamonds, carbon nanotubes or graphene. The structure of this unique material consists of two crystalline sp^3-bonded carbon layers, with half of the carbon atoms hydrogenated and the other half bonding the layers together. It exhibits a wide band-gap semiconducting property, promising for various applications in nanoelectronics, nanooptics, quantum information processing, protective coatings, resonators, composite materials, and biomedicine. Stable diamane has been synthesized by the group of Piazza [12] for the first time. The synthesis is based on a hot-filament-promoted hydrogenation process at low pressure and temperature, which guarantees that bilayer graphene is efficiently hydrogenated, becoming stable diamane. If we change the production process, it is possible to increase number of layers and prepare another 2D carbon material called diamanoid with different properties [13].
The number of graphene layers strongly determines the properties of the material, so it is very important to have a method able to evaluate the properties of the material.

Thanks to the electron diffraction mode operating as low as 5 kV, it is possible to identify a number of layers and stacking sequences of pristine bilayer graphene films. The work of Prof Piazza revealed that the distribution of spot intensities might vary significantly with electron energy. They realized that using standardly high electron energy, the surface of the Ewald sphere is approximated as a plane; on the contrary, when using electrons with low energy, it starts to behave more like a sphere, providing a diffraction pattern of the same structure but with variable spot intensities or even with some spots disappeared. It allows distinguishing between single-layer domains with AA or AB stacking, as well as twisted bilayer graphene (2LG), consisting of two randomly stacked single-layer graphene. Notably, 2LG-AB is distinguished from 2LG-AA by its three-fold symmetric spot intensity distribution on the inner diffraction ring, and 2LG-AA differs from single-layer graphene (1LG) by lower outer ring spot intensity (Fig. 5).

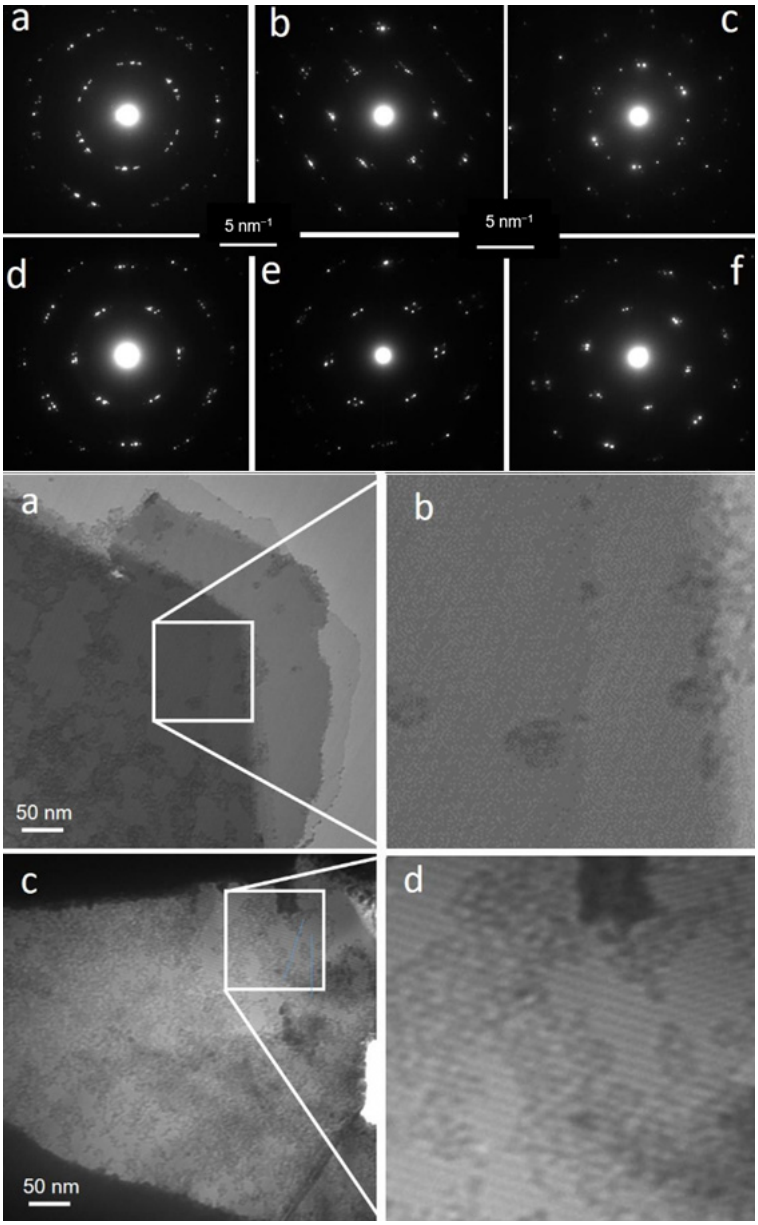

Figure 5. Electron diffraction patterns of film areas post hot-filament-promoted hydrogenation reveal characteristic satellite peaks, indicating superimposed coherent domains with similar periodicities twisted at slight angles. a) and c) TEM images with highlighted moiré periodicities corresponding to the diffraction patterns at the top (a to f) [13].

Graphene-based materials are generally a great example of the sample benefiting from the high contrast of the LVEM method, resulting in a well-visible number of overlapping layers (Figs. 6 and 7).

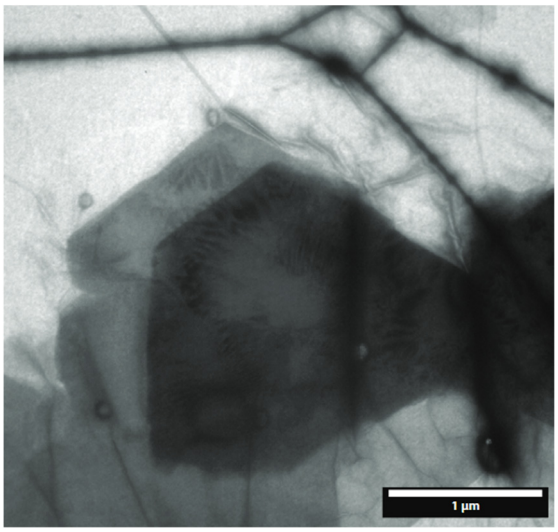

Figure 6. Graphene flakes LVEM5.

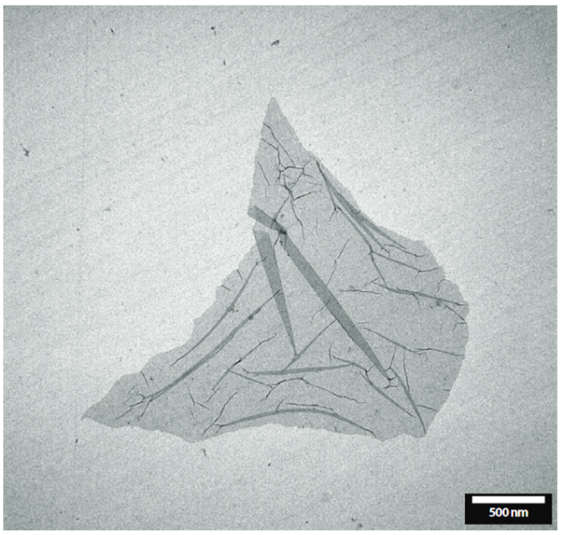

Figure 7. Graphene oxide crystal. LVEM5.

6. *CONCLUSION*

Low voltage transmission electron microscopy (LVEM) is a revolutionary technology with a broad range of applications across material and life sciences. Operating at voltages below 25 kV, LVEM provides enhanced contrast, making it a perfect choice for imaging light element samples where conventional methods struggle.

LVEM 5 and LVEM 25E offer compact designs with versatile imaging modes and advanced analytical capabilities. These instruments integrate multiple imaging modes, including TEM, STEM, and SEM, along with advanced analytical capabilities like energy-dispersive X-ray spectrometry (EDS) and electron diffraction, providing researchers with a comprehensive toolkit for multimodal imaging and analysis.

LVEM's unique technical features, such as the concept of electron optics based on permanent magnets and two-stage optics, enable miniaturisation without the need for cooling systems, ensuring stability and user-friendly operation.

Its ability to provide high-contrast imaging with reduced staining or completely without the need for staining makes it particularly suitable for studying delicate biological specimens and other low-contrast materials, including polymer and carbon-based materials.

7. *REFERENCES*

[1] Nixon W C 1960 Low voltage electron microscopy. in: *Verhandlungen*. (Bargmann W, Möllenstedt G, Niehrs H, Peters D, Ruska E and Wolpers C (Eds.). [Berlin- Heidelberg, Germany: Springer] 302-306. doi:10.1007/978-3-662-01991-7_95

[2] Delong A, Coufalová E and Štěpán P 2002 *Low voltage STEM*. in: Proc. 15th Int. Congress Electron Microscopy (ICEM-15). (Durban, South Africa).
[3] Delong A 1992 Low voltage TEM. *Electron Microscopy* **1** 79-82
[4] Delong A and Kolařík V 1998 *Low voltage transmission electron microscope LVEM-5*. in: Proc. 14th Int. Congress Electron Microscopy (ICEM-14). (Cancun, Mexico) 493-494
[5] Coufalová E and Delong A 2000 *Low voltage electron Microscope II. - Applications*. in: 12th Eur. Congress on Electron Microscopy (EUREM 2000). (Brno, Czech Republic)
[6] Egerton R F, Li P and Malac M 2004 *Micron* **35** 399-409
[7] Delong A, Hladil K, Kolařík V and Pavelka P 2000 *Low voltage electron microscope I. - Design*. in: 12th Eur. Congress on Electron Microscopy (EUREM 2000). (Brno, Czech Republic)
[8] Štěpán P and Delong A 200 *Low voltage electron Microscope III. - Present and future possibilities*. In: 12th Eur. Congress on Electron Microscopy (EUREM 2000). (Brno, Czech Republic)
[9] Drummy L F, Yang J and Martin D C 2004 *Ultramicroscopy* **99** 247-256
[10] Lednický F, Coufalová E, Hromádková J, Delong A and Kolařík V 2000 *Polymer* **41** 4909-4914
[11] Lednický F, Hromádková J and Pientka Z 2001 *Polymer* **42** 4329-4338
[12] Piazza F, Monthioux M, Puech P and Gerber I C 2020 *Carbon* **156** 234-241
[13] Piazza F, Monthioux M, Puech P, Gerber I C and Gough K 2021 *C* **7** 9