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Rapid Structure Determination of Microcrystalline Molecular Compounds Using Electron Diffraction

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Abstract: *Chemists of all fields currently publish about 50 000 crystal structures per year, the vast majority of which are X-ray structures. We determined two molecular structures by employing electron rather than X-ray diffraction. For this purpose, an EIGER hybrid pixel detector was fitted to a transmission electron microscope, yielding an electron diffractometer. The structure of a new methylene blue derivative was determined at 0.9 Å resolution from a crystal smaller than $1 \times 2\ \mu m^2$. Several thousand active pharmaceutical ingredients (APIs) are only available as submicrocrystalline powders. To illustrate the potential of electron crystallography for the pharmaceutical industry, we also determined the structure of an API from its pill. We demonstrate that electron crystallography complements X-ray crystallography and is the technique of choice for all unsolved cases in which submicrometer-sized crystals were the limiting factor.*

Crystallography is an essential technology in most fields of chemistry.[1–4] The three-dimensional structure of a molecule is determined by the bonds between its atoms, which determine the molecule's function, reaction kinetics, and thermodynamics. In addition to the structure of a molecule, crystallisation as different polymorphs leads to different macroscopic properties: Different pigment polymorphs demonstrate different colour shades and fastness,[5,6] and the uptake of drugs improves or worsens depending on the polymorph. Therefore, different patents are granted for different polymorphs of the same compound.[7] Polymorphism explains the mechanics of "jumping crystals".[8] In catalytic chemistry, where the shape of the grains is very important, single-crystal diffraction data can reveal the grain morphology, even without solving the structure.[9] Pharmaceuticals frequently have one active isomer, while the other isomers are inactive or produce negative side effects.[10] Until now, single-crystal X-ray diffraction is the main technology for structure determination in chemistry. The availability of sufficiently large, good-quality crystals is one of the main bottlenecks.[11–14] Crystals must have a side length of at least 5–10 μm when using strong synchrotron radiation. With laboratory diffractometers, 50 μm is a more realistic lower bound, in particular to avoid a checkCIF A-alert when the resolution is worse than 0.84 Å.[2] In some cases, the structure may be determined by X-ray powder diffraction.[15] As opposed to X-ray diffraction, electron diffraction imposes virtually no lower size limit to the crystal. Electrons diffract strongly and with high resolution from crystals smaller than the individual grains of icing sugar. Electron diffraction enables structure determination even from non-crystalline carbon nanotubes that are only 3–5 nm wide.[16–20] Microcrystalline powders outnumber single-crystal compounds by a factor of 3 to 4 (we refer to the term "microcrystalline" as "composed of small crystals", for example, suitable for X-ray powder diffrac-

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tion).[21] Thus, when electron crystallography becomes broadly accessible, the amount of information for the development of chemicals with new properties will be expanded enormously.[22,23] Structure determination with electron crystallography comes at a price: An accurate description of the interaction process between electron radiation and a crystal is based on the dynamical theory of diffraction,[24,25] which is more complex than kinematic diffraction, the basis of X-ray crystallography.[26] Proper treatment of dynamic scattering effects can improve the level of detail of the structural model.[27] However, time and effort for dynamic refinement are generally impractical considering the throughput in most structural chemistry facilities both in academia and in industry. Fortunately, ignoring dynamic scattering does not lead to loss in model accuracy, but only to worse model and data statistics, so that additional validation criteria must be provided.[9,28–31] To date, electron crystallography only plays a minor role in structural chemistry. As of September 2018, the Inorganic Crystal Structure Database contained 425 single-crystal structures from electron crystallography compared with over 78500 structures from X-ray crystallography.[1] The Cambridge Structural Database of organic structures does not even provide a search mask for electron diffraction.[4] Many publications of the past decades concluded or described how close electron crystallography was to becoming a broadly applicable technology.[27,32–36] In this study, we created a prototype of an electron diffractometer based on an EIGER X 1M detector and a transmission electron microscope.[37,38] We present two results to demonstrate how this prototype meets the requirements of a modern X-ray facility: The structure of an active pharmaceutical ingredient of the drug Grippostad® is the first single-crystal structure determined out of a mixture of microcrystalline and non-crystalline compounds. The structure of a new methylene blue derivative highlights that electron crystallography solves large and complex structures as fast and reliably as X-ray crystallography.

Figure 1 illustrates how our prototype diffractometer turns powder diffraction into single-crystal diffraction. It shows a crystal from the microcrystalline powder of the cold medicine Grippostad® (STADA). This small crystal would hardly diffract X-rays, even with the strongest X-ray sources (see the Supporting Information, Section S3). With electrons as the radiation source, the diffraction of this crystal reaches atomic resolution (Figure 1c). Grippostad® powder is a blend of several active and non-active ingredients, both crystalline and non-crystalline (see the Supporting Information). By means of the unit cell parameters and space group (Tables S1 and S2), this crystal was identified as a monoclinic polymorph of paracetamol. The data completeness from several parts of this crystal reaches only 40%. Yet, the structure was solved with direct methods, which confirmed it to be paracetamol. The data quality was sufficiently high for automated assignment of all non-H atom types except for two mismatches: The nitrogen atom was assigned as carbon, and one carbon atom was assigned as nitrogen. The first refinement cycle of the model reveals the positions of several hydrogen atoms (Figure 1d). As common in X-ray crystallography, the positions of all hydrogen atoms were calculated with the riding atom model, and not refined. As electrons interact with the electrostatic potential both from protons and electrons in the crystal, electron diffraction reveals the "real", internucleic distance as the hydrogen bond length. In X-ray crystallography, hydrogen bond lengths appear shortened because X-rays interact only with the electron cloud, which for a hydrogen bond is centred between the hydrogen atom and its bonding partner.[39] For the structure of paracetamol, this results in a significant improvement of the crystallographic R1 factor of more than one percentage point. The visualisation of hydrogen positions without specialised data collection or refinement techniques underlines the data quality produced with such a diffractometer.

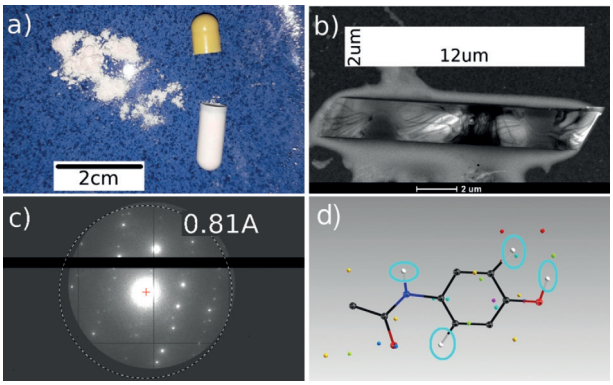

Figure 1. Single-crystal structure from a powder blend. a) Drugs in powder form (Grippostad®, STADA Arzneimittel AG) are usually a microcrystalline blend. b) The powder grains are too small for single-crystal X-ray analysis. The STEM micrograph shows a single crystal from the powder. c) The diffraction intensity is proportional to the crystal volume. Despite the minute volume, this crystal diffracts electrons to atomic resolution. d) High data quality localises some hydrogen atoms (encircled).

To show that electron diffraction meets the demands of modern structural chemistry, the system was challenged with the structure of a methylene blue derivative, **$MBBF_4$**. Methylene blue derivatives find wide applications in medicine, for example, for the treatment of drug-induced methemoglobinemia and as photo-activatable antimicrobials.[40,41] Crystals of **$MBBF_4$** grow only as extremely thin needles. A single needle was found to span two meshes on the sample grid. The width of the needle ranged from about 0.8 μm near its tip (bottom left inset of Figure 2) to approximately 1.5 μm (top right inset of Figure 2). The needle spans two meshes and is thus about 100 μm long.

Data were collected in segments along the needle (see Figure 3 and Section F.4 in the Supporting Information). As the prototype diffractometer was properly calibrated (see the Supporting Information), the data were processed in parallel to data collection. With data merged from the first nine segments, id09–id15, id17, and id18, the structure was solved on site within about 4 h starting from sample preparation (Figure 3, Table S4). This matches state-of-the-art X-ray structure determination.

The structure (Figure 4) is in full accordance with the chemical constitution as expected from the synthetic route of preparation and the known structures of the starting materi-

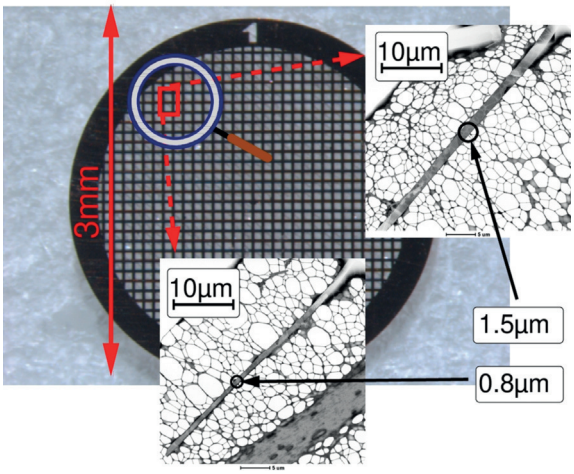

Figure 2. STEM micrographs of the **MBBF$_4$** needle (contrast inverted for better visibility), lying on the TEM grid (background image). Data were collected in segments along the needle (Figure 3).

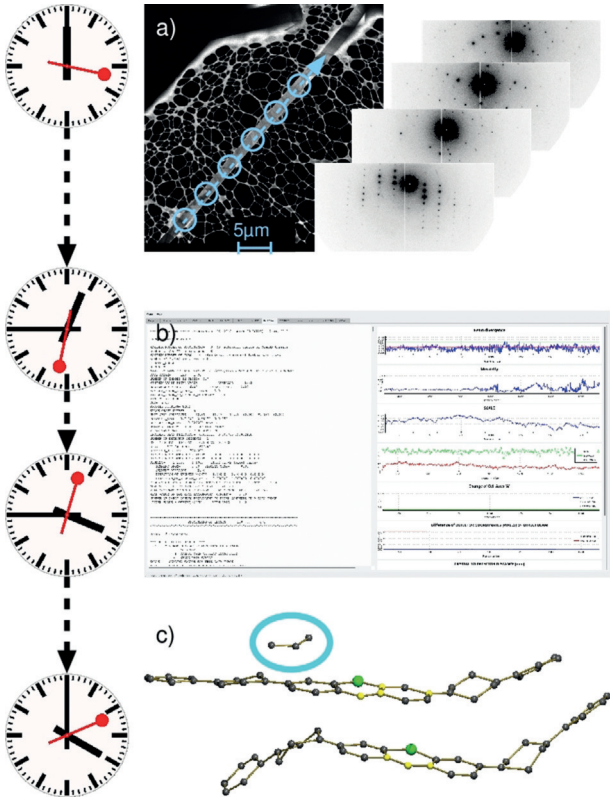

Figure 3. Structure determination of **MBBF$_4$** with electrons in 4 h, comparable with modern X-ray instruments: a) Circles along the blue arrow illustrate the exposure along the crystal needle of compound **MBBF$_4$**. Data from 15 segments collected in 45 min. b) Data of the first nine segments were processed in 3 h and were sufficient for c) structure solution in 15 min. The fragment encircled in cyan is an incomplete fragment. It was identified as BF_4^- counterion during refinement.

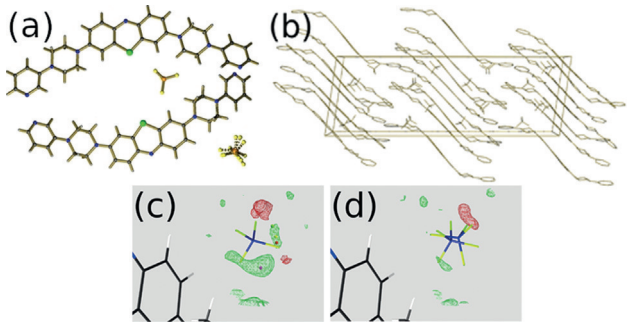

Figure 4. Crystal structure of **MBBF$_4$** solved with electron crystallography. a) Structure of **MBBF$_4$**. b) Stacking of **MBBF$_4$** in the crystal. c) The data quality shows the disorder of a counterion. d) The difference map reduces upon modelling of two conformations.

als. As also large crystals were available, X-ray data of **MBBF$_4$** to subatomic resolution were collected for comparison using highly brilliant synchrotron radiation. The crystal used for X-ray diffraction had an about 6000 times larger volume than the one used for electron diffraction. Both structures are identical within coordinate error (Figure S7). **MBBF$_4$** crystals are surprisingly stable against electron radiation. Data were collected at room temperature, and showed diffraction even after about 40 s exposure. As hydrogen radicals are the first to be formed during electron irradiation, it is the stability of the dehydrogenated radical that finally determines the structure resistance to electron irradiation. By forming a large, conjugated, and rather stable radical, the stability of **MBBF$_4$** against electron radiation most likely originates from the strongly delocalised, redox-active aromatic system, based on the known dye methylene blue with a push–pull chromophore.[42] Figure 4c illustrates the modelling of a twofold disordered counterion. Similar to the visibility of hydrogen atoms in paracetamol, this demonstrates the reliability of the model.

In conclusion, we have presented results acquired with a dedicated electron diffractometer. The instrument is based on the combination of an EIGER hybrid pixel detector with a transmission electron microscope and the reliable calibration of the experimental parameters. The power of the instrument was demonstrated by the single-crystal structure of paracetamol out of a mixture of crystalline and non-crystalline compounds as typically found in pharmaceutical drugs. This result shows one great advantage of electron crystallography over structure determination with single-crystal X-ray diffraction, as the latter would require recrystallisation to grow sufficiently large crystals. The general applicability of the method was further demonstrated with the structure determination of a new methylene blue derivative, a large organic structure. The results make electron crystallography available to all fields in chemistry that rely on X-ray structure determination. It complements the use of X-rays when small or intergrown crystals are the limiting factor for structure determination. Modern structure validation is currently essential as classical statistics are little meaningful. Future work towards the full integration of hardware and a scaling model adapted to the physics of interaction between electron and crystal, as well as the combination of data from such an instrument with dynamical refinement, may lead to even more detailed possibilities for structural analysis.

Experimental Section

An EIGER X 1M detector (DECTRIS Ltd.) was housed in a vacuum-tight steel chamber and mounted on-axis to a Philips CM200 (C-CINA, University Basel) and to a Tecnai F30 (ScopeM, ETH Zurich) transmission electron microscope. Both microscopes were equipped with a Schottky-type field emission gun. The EIGER was controlled separately from the main instrument through a python interface provided by DECTRIS Ltd. All data were collected at 200 keV, corresponding to a wavelength of $\lambda = 0.02508$ Å. The system was validated with several data sets from the zeolite ZSM-5 of MFI framework.[9]

The synthesis of **$MBBF_4$** is described in the Supporting Information. X-ray diffraction data for **$MBBF_4$** were collected at PETRA III at DESY, a member of the Helmholtz Association (HGF).[43] Grippostad® hard capsules were purchased at a pharmacy.

Electron diffraction data are available from Zenodo (DOI https://doi.org/10.5281/zenodo.1297083). CCDC 1856579, 1856580, and 1856585 (paracetamol from Grippostad®, electron structure of **$MBBF_4$**, X-ray structure of **$MBBF_4$**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre. Further details on the crystal structure investigation may be obtained from the Fachinformationszentrum Karlsruhe, 76344 Eggenstein-Leopoldshafen, Germany (fax: (+49) 7247-808-666; e-mail: crysdata@fiz-karlsruhe.de), on quoting the depository numbers CSD-1856581 (ZSM-5 x2_27), -1856582 (ZSM-5 x3_31), -1856583 (ZSM-5 x7_9), and -1856584 (ZSM-5 x8_11).

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Conflict of interest

This work was supported by the loan of an EIGER X 1M detector (Dectris Ltd.) for 15 days. Two authors, S.D.C. and R.P., are employed by Dectris. The setup of the prototype was used by Crystallise! for an industrial structure determination, which unfortunately cannot be published, due to pending patents. Crystallise! also contributed a couple of test samples. Crystallise! is owned by two co-authors, G.S.-Q. and G.S.

Keywords: chemical crystallography · electron crystallography · structure determination · methylene blue derivatives

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