

3D electron diffraction for structure determination of small-molecule nanocrystals: A possible breakthrough for the pharmaceutical industry

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Funding information

Regione Toscana FELIX project, Grant/Award Number: Por CREO FESR 2014-2020

Edited by: Gareth Williams, Associate Editor and Nils G. Walter, Co-Editor-in-Chief

Abstract

Nanomedicine is among the most fascinating areas of research. Most of the newly discovered pharmaceutical polymorphs, as well as many new synthesized or isolated natural products, appear only in form of nanocrystals. The development of techniques that allow investigating the atomic structure of nanocrystalline materials is therefore one of the most important frontiers of crystallography. Some unique features of electrons, like their non-neutral charge and their strong interaction with matter, make this radiation suitable for imaging and detecting individual atoms, molecules, or nanoscale objects down to sub-angstrom resolution. In the recent years the development of three-dimensional (3D) electron diffraction (3D ED) has shown that electron diffraction can be successfully used to solve the crystal structure of nanocrystals and most of its limiting factors like dynamical scattering or limited completeness can be easily overcome. This article is a review of the state of the art of this method with a specific focus on how it can be applied to beam sensitive samples like small-molecule organic nanocrystals.

This article is categorized under:

Therapeutic Approaches and Drug Discovery > Emerging Technologies

KEYWORDS

3D electron diffraction, electron crystallography, pharmaceuticals, small-molecule organics, structure solution

1 | INTRODUCTION

The term nanomedicine is generally used for all medical applications that involve nanoscaled or nanostructured “questions,” from the development of nanomaterials to therapies based on molecular nanotechnology. The main issues concern the quantitative correspondence of macroscopic physical and chemical properties (e.g., toxicity, bioavailability, and delivery) with sub-micrometer features or apparatus. Recent advances, like the discovery and application of nanomaterials to medicine, significantly improved the efficiency of old and novel drugs and allowed the selective diagnosis through specific markers (Combes et al., 2021; Rakowska & Ryadnov, 2011).

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Computational studies help in predicting molecular interactions between drugs and biological macromolecules and significantly contribute in the development of the next-generation drug assets (Kim et al., 2021). However, practical applications revealed a high level of uncertainty, often related with a non-clear definition of the synthetic product. Therefore, the thorough physical and chemical characterization of medicaments is still a mandatory step for asserting actual safety, toxicity, and effectiveness. Moreover, it is important to ascertain the behavior of any product when in contact with the biological systems, since this may not reproduce the properties of free particles, and may eventually result in diminished curative outcomes or even in unexpected collateral effects (Wacker et al., 2016). In this regard, the most efficient method for understanding drug interactions with the biological environment involve the determination of their nanoscaled features, down to the determination of their three-dimensional (3D) atomic structure.

High resolution transmission electron microscopy (HRTEM) is one of the most used techniques for the direct imaging of nanostructures, providing information in the 1–2 Å resolution range. However, the electromagnetic lenses of a transmission electron microscope (TEM) are characterized by important aberrations that significantly restrict the resolution and compromise the immediate interpretation of HRTEM images. Only the latest generation of microscopes, equipped with energy filters and electromagnetic multi-pole correctors (Urban, 2008), can partially correct for such aberrations, reaching down to 0.39 Å resolution (Jiang et al., 2018). Unfortunately, all these fantastic performances are strongly sample dependent, in fact HRTEM imaging requires a rather strong illumination (high electron dose rate) on the sample. The need to orient the crystal for acquiring atomic-resolution information further increases the exposure time to the electron beam. Under such conditions, nearly all organic and most of inorganic materials suffer a fast deterioration due to beam damage, that may lead to a modification of the crystalline structure or to a complete amorphization (Reimer & Kohl, 2008; Spence, 2003) so very few materials can be studied at this high resolution.

Since beam damage is often the most limiting factor for TEM analysis, several methods for reducing beam damage have been developed. Cooling at liquid N₂ or He temperature is a well-known practice for stabilizing a sample inside a TEM, increasing its resistance to electron beam damage. Moreover, cryo-fixation or vitrification through cryo-plunging is crucial for the investigation of hydrated or biological samples, which cannot endure high vacuum inside the TEM column. This protocol is also important for fixing metastable materials that would otherwise deteriorate in a short time-frame (Danino, 2012).

In the recent years, single particle analysis using cryo-EM emerged as a very efficient technique for determining the 3D atomic structure of large macromolecules without the need of any crystallization. The typical resolution of single particle cryo-EM is about 4–6 Å (Mao et al., 2013; Yu et al., 2008; X. Zhang et al., 2008). Recent technical advances, like aberration-correctors and automatic algorithms for big data collection and processing, allowed in certain cases to achieve almost atomic resolution (down to 1.22 Å; Masiulis et al., 2019; Nakane et al., 2020; Uchański et al., 2021). However, cryo-EM works well only for macromolecular assemblies, while small molecules are too small for imaging techniques.

2 | DIFFRACTION TECHNIQUES: NEUTRONS, X-RAYS, AND ELECTRONS

Compared with direct space imaging, diffraction methods are generally applicable only to crystalline samples, but deliver structural information with a better resolution and require a much milder illumination. Diffraction studies can be carried out using neutrons, X-rays, or accelerated electrons. Even if the way these three radiations interact with matter is different, all deliver information about the fundamental unit cell parameters and the positions that the atoms occupy inside this cell. The most striking difference between these different radiations is the size of the typical crystal needed for the analysis. For neutrons it is common to use crystals that are about 1 mm³ (Piccoli et al., 2007). In-house X-ray diffractometers require single crystals that are some tens of microns (Glaeser et al., 2000), while synchrotron X-ray radiation may work with single crystals smaller than 5–10 μm (Yamamoto et al., 2017). Finally, electron diffraction performed by a TEM allows the study of much smaller crystals, with sizes generally below 1 μm (Gemmi et al., 2019).

Neutrons interact primarily with nuclei, while X-rays and electrons interact with nuclei and with electron clouds. For X-rays the contribution of nuclei is negligible and often ignored, that means in the presence of heavy atoms with many electrons, it may be difficult to detect light atoms (especially hydrogen atoms involved in highly polarized bonds). In contrast, neutron diffraction is able to detect light elements such as hydrogen, even in combination with heavy atoms. Neutron scattering from hydrogenated materials is anyway affected by a strong incoherent scattering that introduces a very high background and may hinder structural investigations unless the material is deuterated.

Both positively charged nuclei and negatively charged electron clouds contribute to the scattering of accelerated electrons which are deflected by the electric field created by these charges. The electromagnetic interaction of electrons

with matter is very strong (from 3 to 4 orders of magnitude) compared with X-rays (Vainshtein, 1964a) and provides diffraction patterns with good signal-to-noise ratio even for small and thin samples. Electron diffraction is also very sensitive to the presence of light atoms. Unfortunately, such strong interaction and the simultaneous diffraction of many reflections generate dynamical scattering effects (multiple scattering) that is not negligible and may compromise the overall reliability of reflection intensities, since it spoils the simple linear relation between the diffracted intensities and the square modulus of the structure factor (Peng et al., 2004; Spence & Zuo, 1992).

In the case of nanocrystalline materials, the crystal size is a crucial limit for the applicability of single crystal analysis by neutrons and X-rays. If crystals of sufficient size cannot be obtained, various methods (fiber diffraction, powder diffraction, and small angle scattering; Černý, 2017; Jacques & Trehwella, 2010; Marvin, 2017) can be used to obtain structural information, yet without a true 3D character. The most common method for nanocrystal structure characterization is powder X-ray diffraction (PXRD). PXRD allows for rapid and non-destructive analyses, without the need for complex sample preparation. Nowadays, PXRD is a widespread and routine technique able to provide relatively fast and well consolidated routes for structure solution of nanocrystalline materials (David et al., 2002; Kabova et al., 2018; Kaduk et al., 2021; Shankland et al., 1997; Shankland et al., 2013). Nevertheless, PXRD suffers from the essential reduction of the structural information to one-dimensional (1D). This significantly limits its applicability for crystals with small coherent domains, long cell parameters or pseudosymmetries, which result in reflection peak broadening and strong overlapping. Such flaws may hinder the correct measurement of the position and of the diffraction intensity of each reflection (McCusker & Baerlocher, 2013), which, in turn, casts uncertainties on reflection indexing and cell determination and ultimately hampers the structure determination. Furthermore, a powder diffractogram is strongly influenced by experimental factors (preferential orientation, non-homogeneous dimensions), which are not always predictable or can properly be modeled (David et al., 2002). Finally, this technique is hardly applicable to mixtures where the phase of interest is not dominant, because reflections of all phases overlap on the same dimension (Weirich, 2003).

The need for techniques which allow the acquisition of single crystal data even from sub-micrometric crystals, has encouraged scientists to search for an efficient electron diffraction method which should overcome the limitation imposed by dynamical scattering. Until a few years ago, electron diffraction data were only recorded after the sample was oriented along specific crystallographic axes, a protocol that requires a certain expertise and an unnecessary exposure of the sample to the electron beam. The crucial innovation that allowed the routine acquisition of more complete and less dynamical (close-to-kinematical) electron diffraction data, even from very beam sensitive samples, was the introduction of rotational methods for sequential data acquisition. This new way of collecting data is generally referred as three-dimensional electron diffraction (3D ED), but in the literature several other acronyms can be found: automated diffraction tomography (ADT; Kolb et al., 2007), rotation electron diffraction (RED; Zhang et al., 2010), electron diffraction tomography (EDT; Gemmi & Oleynikov, 2013), precession electron diffraction tomography (PEDT; Boullay et al., 2013), microcrystal electron diffraction (microED; Nannenga et al., 2014), integrated electron diffraction tomography (IEDT; Gemmi et al., 2015), continuous rotation electron diffraction (cRED; Wang et al., 2017), single crystal electron diffraction (SCED; Cichočka et al., 2018), low dose electron diffraction tomography (LD-EDT; Kodjikian & Klein, 2019), or fast and automated electron diffraction tomography (fast-ADT; Plana-Ruiz et al., 2020). In fact, all these acronyms refer to slightly different strategies and software for 3D ED acquisition, which anyway share the common idea to collect non-oriented diffraction patterns through a crystal tilt series. Several recent reviews cover the historical developments and the technical characteristics of 3D ED, highlighting its exponential spread for a wide range of applications (Clabbers & Xu, 2020; Gemmi et al., 2019; Gruene et al., 2021; Gruene & Mugnaioli, 2021; Huang et al., 2021; Nannenga, 2020). Here we will focus on the benefits of this method for the structure characterization of small-molecule organics that appear only in the form of sub-micrometric crystals and have potential pharmaceutical interest.

3 | ED FOR THE STRUCTURE ANALYSIS OF SMALL-MOLECULE ORGANICS

3.1 | Early attempts

As a consequence of their strong interaction with matter, electrons can be diffracted numerous times while crossing the crystal. Because of these “dynamical scattering,” for several decades electron diffraction collected by TEM was considered without application by most crystallographers (Heidenreich, 1964; Lipson & Cochran, 1966). Dynamical effects lead to a meddling of the intensities of reflections, which hampers structure solution and refinement. The mathematical

models able to describe this phenomenon are rather complex (Cowley, 1995; Fultz & Howe, 2002; Howie & Whelan, 1961; Peng et al., 2004), require a starting structural model and are anyway insufficient in the presence of defects or non-homogeneous thicknesses (Dorset, 1995). Thus, first attempts of structure solution were focused on weak-scattering materials, like organics, which were expected to produce nearly kinematical diffraction data (Rigamonti, 1936; Schoon, 1938; Thiessen & Schoon, 1937). Despite this, diffraction data could rarely be used alone for structural analyses, without the support of some a-priori information or of high-resolution imaging data.

Nevertheless, notable results were achieved by Pinsker and Vainshtein using an in-house developed electron diffraction camera (Pinsker, 1953) and assuming only a relatively simple dual beam dynamical scattering (Vainshtein, 1956; Vainshtein, 1964a; Vainshtein & Lobachev, 1956). Their data collection protocol, named oblique textured electron diffraction (OTED), was based on the illumination of a large amount of slightly disoriented flat crystals, emphasizing the mosaicity of the sample (Figure 1a). OTED experiments require a highly complex sample preparation procedure, because the obtained diffraction pattern derives from many crystals which need to be oriented approximately in the same way (Pinsker, 1968). Notably, with this method it is not possible to analyze a single nanocrystal, as it is normally the case using a TEM. Nonetheless, the OTED method significantly reduces dynamical effects and allowed the structure solution and refinement of a number of organic and inorganic phases, up to the precise localization of hydrogen atoms (Vainshtein, 1964b; Zhukhlistov et al., 1997; Zhukhlistov & Zvyagin, 1998; Zvyagin, 1967).

In the 1970s, the interest in electron diffraction crystallography grew significantly, when it was demonstrated that structure solution can be successfully achieved for individual nanocrystals. Electron diffraction data were collected by TEM after the sample was oriented along low index crystallographic axes (Dorset & Hauptman, 1976). Only very thin crystals (<50 Å) were used, in order to reduce the dynamical effects so much that a kinematical approximation ($I \approx F^2$), or at least a dual beam approximation ($I \approx F$), could be applied for structure solution. In the following years, many organic and inorganic structures have been extensively investigated using this strategy (Dorset, 1995; Dorset et al., 2005; Nicolopoulos et al., 1995; Voigt-Martin et al., 1995; Wagner et al., 1999; Weirich et al., 1996).

3.2 | CBED, SAED, NBED, and PED

While convergent beam electron diffraction (CBED) techniques deliver very accurate information about cell parameters, symmetry and fine structural details, electron diffraction data for structure determination are usually collected with a parallel illumination on the specimen (Tanaka & Tsuda, 2011). CBED produces diffraction discs (Figure 2a) rather than focused spot patterns. It has anyway almost no applications for beam sensitive materials, since a high beam convergence results in a high electron dose rate on the sample. Moreover, long crystallographic axes cause overlap of the diffraction disks and limit the useful information in CBED patterns.

In contrast, spot-like electron diffraction patterns can be obtained either by inserting a post-sample aperture positioned at the image plane (selected area electron diffraction [SAED]; Figure 2b), or by inserting a pre-specimen small condenser aperture (5–10 μm) to generate a quasi-parallel electron beam with a diameter down to 10 nm (nanobeam electron diffraction [NBED]; Figure 2c). SAED is the most practical and common way to collect electron diffraction data, because it is possible to easily switch from sample search in imaging mode to diffraction mode. However, in SAED a larger area of the sample is unavoidably illuminated, while the diffraction information is collected from the target area inside the selected area (Figure 1b). If the sample is beam sensitive, the electron beam damages much more sample than that visible inside the SAED aperture.

On the contrary, in NBED mode, the information is collected only from the area of interest that is directly illuminated by the electron beam (Ohnishi & Hiraga, 1996), avoiding any damage on the area of the crystal that is not illuminated (Figure 1c). Additionally, with NBED it is possible to have a significantly smaller probe, which is very helpful in the case of nanocrystalline samples with low crystallinity, high mosaicity, nanotwinning, or tendency to form agglomerates. Therefore, SAED is less flexible for beam sensitive materials than NBED, as beam damage can be still a major issue in electron diffraction for organics or water-containing materials.

The invention of the beam precession method in 1994 (Vincent & Midgley, 1994) was a significant step forward for electron crystallography. In precession electron diffraction (PED) data, dynamical effects are significantly reduced. The crystal is still oriented along low index zone axes, but the electron beam is slightly tilted and undergoes a fast rotation movement around the optical axis, comprising a conical path. This way, the sample is never fully oriented along the crystallographic zone. Being off-axis, means that only a few reflections are excited simultaneously (i.e., only those that

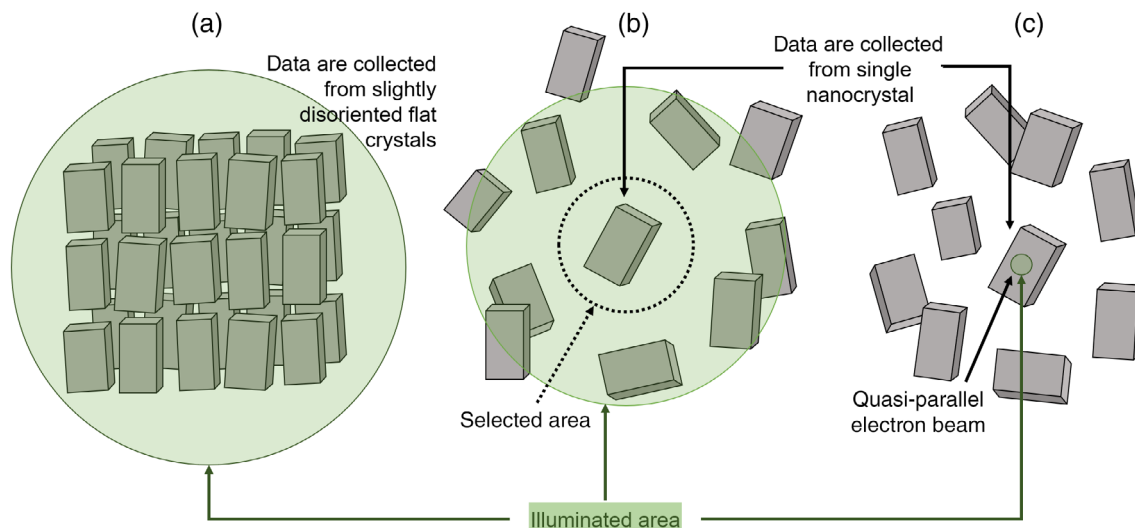


FIGURE 1 Schematic illustration of a typical sample for OTED (a), SAED (b), and NBED (c) techniques. Illuminated area for each experimental set up is highlighted in light green.

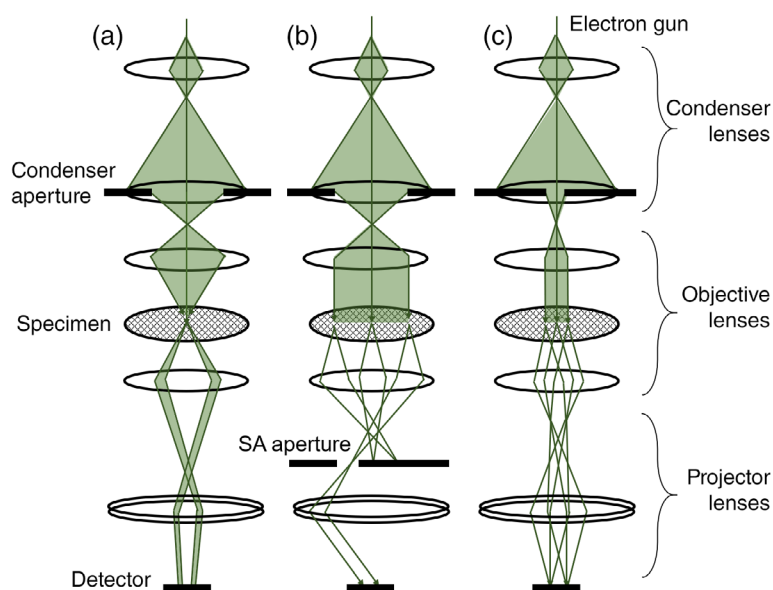


FIGURE 2 Electron beam geometry: (a) convergent beam electron diffraction (CBED), (b) selected area electron diffraction (SAED), and (c) nanobeam electron diffraction (NBED).

are close to the Ewald sphere in a given instant). Thus, dynamical effects are strongly reduced and PED patterns have a more kinematical imprint compared with steady SAED and NBED patterns, and normally deliver clear information about systematic absences and symmetry. Another significant and primary advantage of PED is the improvement of reflection intensity integration, which drastically reduces the uncertainty caused by excitation error (i.e., the intensity reduction determined by distance between the Ewald sphere and the exact Bragg position; Williams & Carter, 1996). An increasing number of structure solutions were achieved by using PED data, even for relatively thick samples (Dorset, 2007; Gemmi et al., 2003, 2010; Gjønnnes et al., 1998; Weirich et al., 2006). Nevertheless, PED can hardly be considered a routine path for structure analysis, as any working case needs a specific, different approach, and a highly expert TEM user. Moreover, this approach does not improve the overall coverage of sampled reflections, which remains rather poor as long as data are recorded only after the sample is oriented along specific crystallographic directions.

3.3 | The rise of 3D ED

The incompleteness of intensity data sets has been a classical limitation for electron diffraction. In fact, this depended only on the fact that the standard way of electron diffraction data collection was based on the acquisition of oriented zones. Conventional SAED, NBED, or PED patterns were acquired by tilting of a pre-oriented nanocrystal around a main crystallographic axis keeping a row of reflections in Bragg condition (Figure 3a). Only prominent zones were recorded, losing most high index reflections. Additionally, dynamic effects are maximized in oriented zones, where many reflections are simultaneously excited and all are related by easy geometrical relationships.

In 2007, Kolb et al. proposed a new protocol for collecting diffraction data, tilting the sample along an arbitrary axis (principally avoiding oriented zones; Kolb et al., 2007). They did not aim for oriented zone patterns, and data were instead taken sequentially after fixed and small tilt steps, for example, 1° (Figure 3b). In this way, it is possible to sample the whole reciprocal space attainable inside the mechanical limits of the TEM goniometer (normally about 120°). This allows a large reciprocal space coverage, shortens the total exposure time and the total electron dose (there is no dead time for sample orientation), and most importantly, provides reflections from off-axis cuts of reciprocal space, significantly reducing dynamical effects. In this regard, it is important to avoid the tilt around a specific crystallographic axis, otherwise oriented zones are collected accidentally and dynamical effects are enhanced.

This method spread very fast and found applications for different classes of nanocrystalline materials. Because of the non-existence of dedicated instrumentation, researchers developed and are still developing, different but conceptually similar ways for data acquisition. Therefore, different terms can be found in the literature indicating often very similar, if not identical, data collection protocols (Boullay et al., 2013; Cichocka et al., 2018; Gemmi et al., 2015; Gemmi & Oleynikov, 2013; Kodjikian & Klein, 2019; Kolb et al., 2007; Nannenga et al., 2014; Plana-Ruiz et al., 2020; Wang et al., 2017; Zhang et al., 2010). All of them are based on the same idea and are designed to increase the number of observed reflections, reducing dynamical effects, and decreasing the data collection time when compared with classical oriented-patterns. In the following we will therefore use for all of them the term 3D ED.

3.3.1 | Stepwise versus continuous data acquisition

The stepwise acquisition is the first and simplest protocol for 3D ED data collection. It consists of a homogenous sampling of reciprocal space in fixed tilt steps. The recorded diffraction intensities suffer from an imprecise integration (excitation error) due to the gap between sequential goniometer positions. In order to perform a proper integration of reflection intensities, PED can be applied during stepwise acquisition (Figure 3c). This data collection procedure is often referred to as precession or precession-assisted electron diffraction tomography (PEDT; Boullay et al., 2013). The ED data collected in this way have a large reciprocal space coverage and have been successfully used for ab initio structure solution by direct methods with a fully kinematical approximation (Andrusenko et al., 2019; Jiang et al., 2011; Klein et al., 2020).

Beam precession requires dedicated TEM routines or an external device. An alternative solution to account for the excitation error is filling each (relatively large) mechanical tilt step with fine beam tilt steps (about 0.1°), performed with the TEM deflection coils. This approach is generally referred as rotation electron diffraction (RED; Zhang et al., 2010) and also provides ED data of suitable quality for kinematical structure solution and refinement (Martínez-Franco et al., 2013; Willhammar et al., 2012).

The last and more recent approach for 3D ED is based on continuous data collection (Nederlof et al., 2013; van Genderen et al., 2016). ED patterns are acquired while the goniometer is rotating at a controlled speed (Figure 3c). Due to its relatively fast data collection time and to the avoidance of tracking the crystal position with imaging, this protocol is characterized by a lower electron dose. ED data collection in continuous mode is generally referred in the literature as microcrystal electron diffraction (microED; Nannenga et al., 2014), integrated electron diffraction tomography (IEDT; Gemmi et al., 2015), or continuous rotation electron diffraction (cRED; Wang et al., 2017).

Selecting the best way for ED data collection strongly depends on the specific characteristic of the investigated sample. Beam sensitivity is the biggest limitation for stepwise data collection, while crystal size, shape, and agglomeration are critical issues for continuous acquisition, because the difficulty or impossibility of efficient crystal tracking during rotation and the, until now, unavoidable mechanical instabilities of the TEM goniometer.

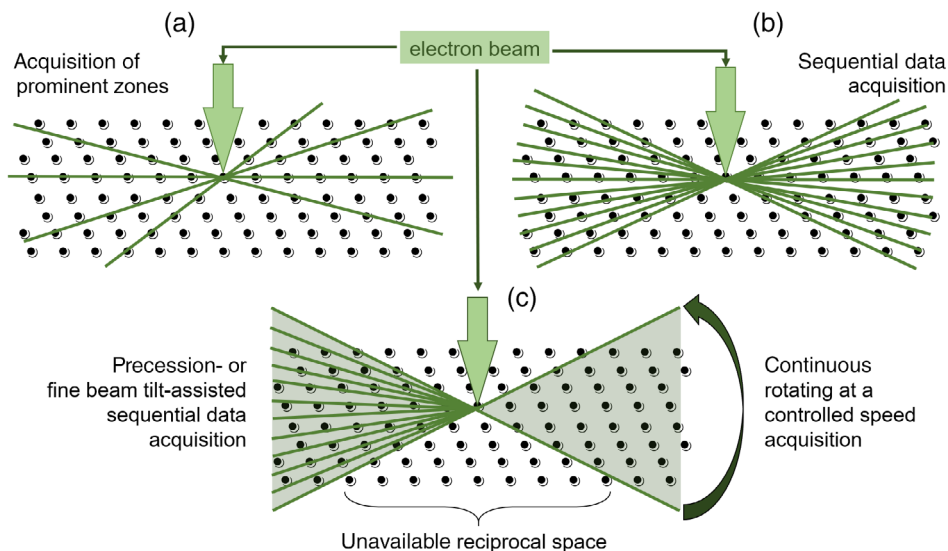


FIGURE 3 Electron diffraction data collection: (a) only prominent zones from a pre-oriented nanocrystal, (b) homogenous sampling of the reciprocal space in fixed tilt steps, and (c) proper integration of reflection intensities using precession, fine beam tilt, or continuous rotating.

3.3.2 | Strategies for crystal tracking

Adjusting the crystal height helps to minimize sample movements during rotation (Dierksen et al., 1992). Nevertheless, working at the nanoscale, any minimal mechanical instability of the goniometer can move the target crystal out of the illuminated area. Even crystals perfectly adjusted at the correct eucentric height slightly move during crystal tilting. This may be particularly critical for crystals smaller than 1 μm or characterized by significant variations in thickness. For a proper exploitation of the whole goniometer tilt range, the crystal position generally needs to be tracked by correlation with a reference image taken in TEM or scanning transmission electron microscopy (STEM) mode. Eventual crystal shift can be checked and, if needed, corrected manually or automatically. This is obviously possible only for step-wise acquisitions.

Crystal tracking introduces unwelcome additional electron dose on the sample. In this respect, STEM imaging needs a lower illumination and induces a lower electron dose when compared with TEM imaging. STEM is also preferable because, if the imaging is performed with a parallel nanobeam, there is no need to switch the lens system for acquiring diffraction data, which further reduces the total acquisition time and eliminates possible misalignments due to lens hysteresis. Additionally, in order to minimize the electron dose, Kodjikian and Klein (2019) proposed to blank the beam during tilt and to irradiate the sample exclusively during the data acquisition.

The first semiautomatic crystal tracking by STEM imaging that appeared in the literature, generally referred as automated diffraction tomography (ADT), allowed compensating for the mechanical drift through an equivalent shift of the electron beam (Kolb et al., 2007). This routine reduces dramatically the electron dose on the sample, bringing valuable improvements for hybrid and organic materials (Bellussi et al., 2012; Denysenko et al., 2011; Feyand et al., 2012; Gorelik et al., 2012). Recently, a new acquisition method has been implemented, fast and automated diffraction tomography (fast-ADT). This last development is based on two subsequent tilt scans (Plana-Ruiz et al., 2020). The first one is used to check the crystal drift during the tilt and sample it through imaging, while the second one is the actual acquisition of the diffraction data with the drift correction automatically implemented.

During continuous acquisition, crystal drift is the main limiting factor. The position of the crystal can be roughly corrected by monitoring the intensity of the diffraction patterns during data collection. An alternative crystal tracking strategy comprises in the defocusing the diffraction pattern via the intermediate lens at regular intervals (Wan et al., 2013). This routine has been implemented in the software *Instamatic* (Cichocka et al., 2018), simplifying the process of the crystal re-centering.

On the other hand, using a SAED aperture that is large enough to allow the sample to be entirely inside the selected area during the experiment, but not too large to avoid blooming due to the transmitted beam, it is possible to acquire diffraction data without crystal tracking (Gruene et al., 2018; Jones et al., 2018).

3.3.3 | Data analysis

The sequence of collected patterns can be analyzed by several software to obtain unit cell parameters, crystal symmetry and a three-dimensional set of diffraction intensities, exactly as in single crystal X-ray diffraction. Some of these software packages are specifically designed for electrons, like *ADT3D* (Kolb et al., 2011), *EDT-PROCESS* (Gemmi & Oleynikov, 2013), *RED* (Wan et al., 2013), and *PETS* (Brázda et al., 2019); others, originally written for X-rays, have been adapted to treat also 3D ED data, like *MOSFLM* (Nannenga et al., 2014), *XDS* (Clabbers et al., 2017), or *DIALS* (Clabbers et al., 2018).

The derived 3D ED intensities can be processed by the same structure solution software used for X-rays, that generally embeds scattering factors for electrons. The structure solution can be achieved fully ab initio by direct methods with programs like *SIR* (Burla et al., 2015) or *SHELXT* (Sheldrick, 2015) or by charge flipping using *SUPERFLIP* (Palatinus & Chapuis, 2007). If the data quality is not optimal, either because the sample is beam sensitive or poorly crystalline, the structure solution can be performed using simulated annealing, an algorithm which requires the starting coordinates of the molecules. Each molecule can be modeled as a unique fragment, where the atomic distances and coordination are known. This global optimization method works especially well for organic compounds, where the symmetry is commonly low, no atom is in a special position and there are strict geometrical constraints imposed by intramolecular chemical bonding (Andrusenko et al., 2020, 2021; Das et al., 2018; Lightowler et al., 2022; Zhang et al., 2013).

The fact that the data analysis is not really different from X-ray diffraction and that the same software can be applied to both radiations will likely facilitate the spreading of 3D ED methods among any X-ray crystallography laboratory.

3.4 | Specifics experimental requirements for beam sensitive materials

3.4.1 | Dedicated detective devices

For the first 5 years after its first appearance, 3D ED was mostly applied to inorganic materials, generally less sensitive to beam damage (Andrusenko et al., 2011; Birkel et al., 2010; Mugnaioli et al., 2009; Rozhdestvenskaya et al., 2010; Sedlmaier et al., 2011). Only with the introduction of protocols for continuous rotation data collection and of faster and more sensitive detectors, structure analysis of small-molecule organics and macromolecules also started to be performed on a routine basis (Bruhn et al., 2021; Gemmi et al., 2015; Gruene et al., 2018; Jones et al., 2018; Nannenga et al., 2014; Nederlof et al., 2013; van Genderen et al., 2016).

ED patterns are traditionally acquired by a post-column charge-coupled device (CCD) based on *p*-doped or intrinsic metal-oxide-semiconductor (MOS) capacitors. There are several examples of beam sensitive compounds determined on the basis of 3D ED data acquired with CCD cameras (Brázda et al., 2019; Gorelik et al., 2012; Kolb et al., 2010). However, even most recent CCD models have some disadvantages. First, due to their high read-out time, they do not allow a full acquisition frame rate at fast rotation speeds, often necessary to fulfill data acquisition on beam sensitive materials. Second, the high read-out noise requires a relatively long exposure time for measuring good signal-to-noise ED patterns. Third, an overexposure to the transmitted (central) beam may damage the sensitive CCD scintillator, so a beam stop is normally required, complicating the whole data collection process and possibly masking low resolution reflections.

Complementary metal-oxide-semiconductor (CMOS) sensors are less quantitative compared with CCD, but have also several significant advantages when used for the 3D ED experiment: better sensitivity, lower background, faster read-out time. The different CMOS circuit styles are nowadays available and can be fully integrated inside TEMs and used for the recording of 3D ED data (Simon Prabu et al., 2020).

The recent introduction of direct hybrid-pixel detectors (DHPDs) that can operate in counting mode detecting the single electron has further improved the efficiency of the 3D ED method (Georgieva et al., 2011; Tinti et al., 2018). In particular, DHPDs allows ED data collection with high dynamic range, zero read-out time and practically low background. This drastically improves the signal-to-noise ratio compared with a conventional CCD for the same electron dose, a crucial aspect for beam-sensitive materials. In addition, DHPDs are radiation hard for electrons and do not require a beam stop even for long time exposures. An example of how effective DHPDs are in collecting an ED pattern in low dose mode compared with a conventional CCD is displayed in Figure 4. With DHPD, an extremely low dose

illumination, corresponding to about $0.01 \text{ e}^- \text{ \AA}^{-2} \text{ s}^{-1}$ electron dose rate, can be sufficient, avoiding beam damage even for extremely beam sensitive materials. The total electron dose for data collection depends on several experimental parameters, such as exposure time, image tracking mode and number of frames. A rough estimation of the electron dose during a continuous data collection is about $1 \text{ e}^- \text{ \AA}^{-2}$, with a total exposure time that can be less than 1 min. With a standard CCD, the dose should be at least one order of magnitude higher.

3.4.2 | Working at low temperature

Thanks to the low electron dose required for a 3D ED experiment with an DHPD, it is generally possible to perform efficient data collection already at room temperature, before critical sample deterioration appears. Several pharmaceutical compounds have been recently determined this way (Andrusenko et al., 2019, 2020, 2021). Obviously, cooling the sample slows the radiation damage process for highly beam sensitive materials. The easiest experimental protocol consists of the simple sample cooling after its insertion in the TEM column. In this case, cooling can be obtained by using a simple cryo-transfer holder with a small dewar filled with liquid N_2 .

A more efficient, but also more experimentally challenging, sample preparation protocol consists of embedding the sample in amorphous ice before its insertion in the TEM (Shi et al., 2016), the same procedure adopted in single particle cryo-EM. This can be achieved by the fast plunging of a drop of a crystal suspension inside liquid ethane. In this way the fast cooling of the cryogenic liquid in which the crystals are dispersed, vitrifies it and protects the crystals from the damaging effects of the electron beam. Before its insertion into the TEM column, the sample must be kept at liquid nitrogen temperature to avoid ice recrystallization and transferred first on a cryo-transfer sample holder. Cryo-plunging

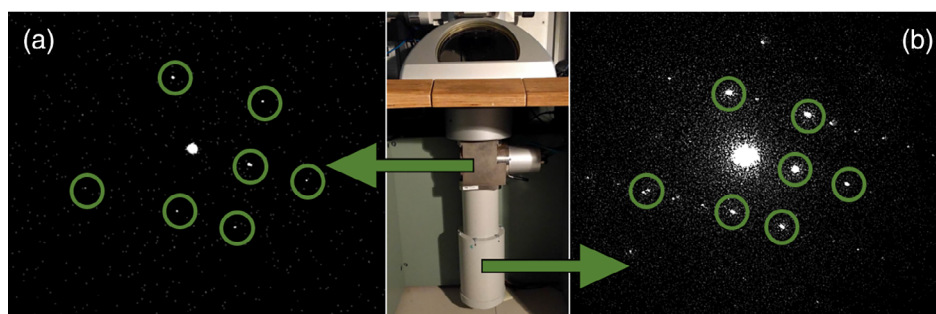


FIGURE 4 Examples of ED patterns from a beam sensitive organic material acquired with a conventional CCD (a) or a DHPD working in low dose mode (b). For a proper comparison both patterns are taken in the same illumination condition and have the same exposure.

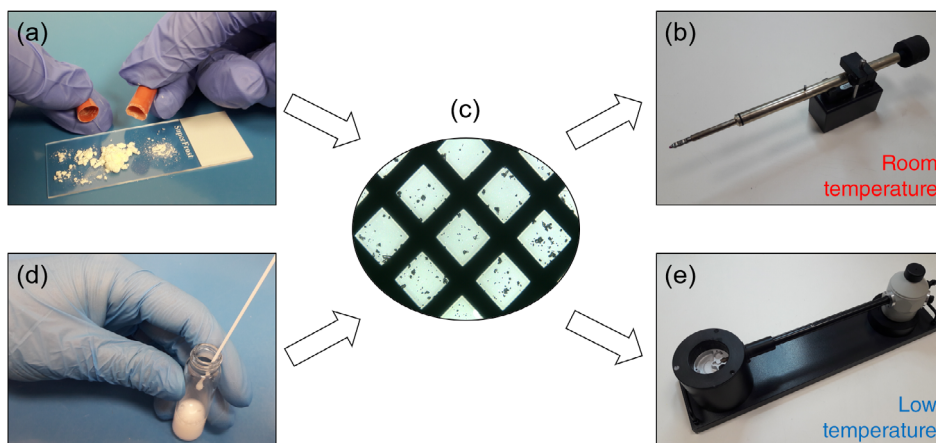


FIGURE 5 An example of preparing TEM grid experiment (c) from “dry powder” (a) or “liquid suspension” (d) sample for a 3D ED. A single tilt holder (b) for working at room temperature and cryo-transfer holder (e) for working at low temperature.

is also used for protecting vacuum-sensitive samples, like macromolecules or other hydrated materials, which otherwise get amorphous or undergo phase transition as soon as they enter the TEM column.

An ideal cryogen should have a high thermal conductivity to allow for rapid vitrification during plunging and a high heat capacity for minimizing the Leidenfrost effect. When possible, the first choice is just demineralized water, which does not significantly evaporate at N₂ temperatures (Taylor & Glaeser, 1976). Still, certain nanocrystalline powdered samples dissolve or modify in contact with water, therefore alternative and more specific cryogens may be required according to the sample one aim to investigate.

Cryo-plunging and cryo-transfer are quite complex procedures that may generate a number of artifacts during sample preparation or electron beam interaction. Additionally, any cryo-experiment requires the use of specific cryo-equipment. In particular, the cryo-transfer holder is prone to drift during rotation due to the weight of the filled dewar on its rear (Figure 5e). Moreover, presence of the protective amorphous ice layer limits the signal-to-noise ratio and may reduce the imaging capabilities for sample search and tracking.

4 | 3D ED APPLICATIONS FOR COMPLEX SMALL-MOLECULE MATERIALS

The main strength of 3D ED is the ability to perform single crystal diffraction on sub-micrometric areas. Therefore, this technique can be used for structure determination when crystal size is the limiting factor for single crystal X-ray diffraction. The 3D ED is the technique of choice especially in the analysis of nanocrystalline mixtures, because it allows a selective screening and analysis of single crystal grains. Remarkably, such an analytical protocol can be performed even on extremely small sample batches, which cannot be conveniently prepared for conventional PXRD. Additionally, 3D ED can be used for spotting coherent and ordered sub-micrometric domains in cases of crystal plastic flexibility, twinning, or disorder.

4.1 | Twinning and disorder

Diffuse scattering is a well-known phenomenon and is the occurrence of diffraction intensity between the Bragg peaks. It is observed each time a deviation from the ideal crystalline model occurs, that is, a deviation from the perfectly repetitive arrangement of atoms or molecules in adjacent unit cells. Such disorder may be connected with the metastable nature of the nanocrystals, with the non-equilibrated conditions during their formation, with the existence of several polytypes that alternate at a very fine scale, with a non-stoichiometric composition or with the energetic frustration of certain elements of the structure (Mugnaioli & Gorelik, 2019). Because the related signal is generally weak and there are multiple effects that can contemporarily interplay (vacancies, dislocations, stacking faults, local modulations ...), the study of diffuse scattering has remained confined to relatively few and specific studies (Krysiak et al., 2020; Welberry & Weber, 2015).

Sometimes a seemingly single crystal may be composed of unit cells which are not related by pure translation, but compose instead a complex pattern of crystalline domains with different, but still geometrically related, orientations. Such phenomenon is known as twinning and each single coherent domain is referred as a twin domain. Twinned crystals are not obviously recognized at the macro- and microscale, because domains may be too small to be observed by light microscopy. Moreover, twinning is virtually invisible for PXRD, which is only sensitive to the scalar values of *d*-spacing. Generally, in single crystal diffraction, specific families of reflections from different twin domains overlap, while others appear to split. Merohedral twinning is a special case of twinning where all reflections systematically overlap, mimicking a higher-symmetry space group inside the same crystalline family. Advanced programs exist, which can selectively take care of twinning during reflection integration or structure refinement. Still, twinning can easily introduce uncertainty in space group determination and instability in the refinement procedure.

In cases of disorder or twinning, 3D ED may allow probing single nanometric volumes that are internally ordered and coherent. The resulting structure can be subsequently used for building a disorder or twinning model that complies with the observed diffuse scattering or reflection splitting. This protocol has been effective for several small-molecule organic materials. For example, the α^{II} phase of unsubstituted quinacridone showed diffuse scattering along one of the main crystallographic axes. The 3D ED experiments performed on single domains of few hundreds of nanometers allowed to determine the average unit cell and the average atomic structure, and to relate the observed diffuse

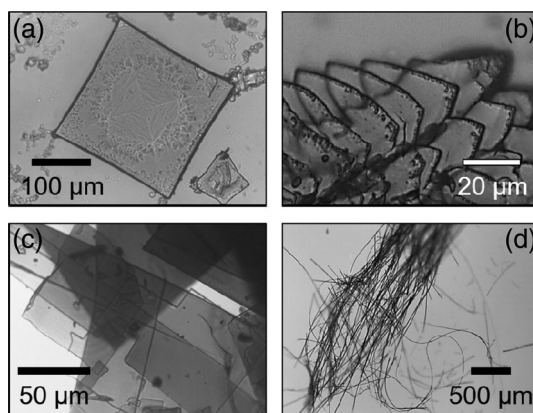


FIGURE 6 Showcases of materials that can be analyzed by 3D ED method: An orthocetamol large and apparently well-defined quadrilateral platelet (a) and a closer look of such platelet (b), revealing that it is eventually composed by a conglomeration of far smaller crystals; thin leaflet morphology of terrylene (c); hairy habit of δ -indomethacin (d).

scattering with the weakly bonded layers of the molecular packing (Gorelik et al., 2016). In the case of the organic pigment 2-monomethyl-quinacridone, the faint diffuse scattering streaks were instead connected with the presence of two twinned domains. The domains share one common axis and the diffuse scattering is always associated with the smaller and weakly scattering one (Schlesinger et al., 2020).

Another showcase of the potential of the 3D ED method related to twinned crystalline domains is the structure determination of orthocetamol. This regioisomer of the well-known paracetamol was first reported more than one century ago, but its structure could not be obtained by X-ray diffraction due to a pervasive twinning. Orthocetamol apparently crystallizes in relatively large and well-defined quadrilateral platelets (Figure 6a), but a closer look reveals that in fact each platelet is composed by a conglomeration of far smaller crystals (Figure 6b). The 3D ED was used for acquiring data from areas of few hundreds of nanometers, which are supposedly less affected by twinning or disorder. Indeed, the structure of orthocetamol was solved *ab initio* with a fully kinematical approximation, up to the localization of some of the hydrogen atoms. The subsequent least-squares refinement confirmed the presence of pseudo-merohedral twinning, related with the occurrence of two cell parameters with almost equivalent length (Andrusenko et al., 2019).

4.2 | Handling of thin platelets and crystal with high plasticity

Recently, 3D ED has also been applied to the structure determination of complex polyaromatic hydrocarbons (PAHs). The crystal structure of terrylene was determined by direct methods (Hall et al., 2021), an achievement that could not be performed by single crystal X-ray diffraction due to limited crystal size and the thin leaflet morphology (Figure 6c). Terrylene has found many uses in pigments, dyes, and small-molecule organic devices (Weil et al., 2010), in particular, it is a very capable fluorophore, potentially usable as label for single molecule spectroscopy (Białkowska et al., 2017). A better understanding of the structure–property correlation of PAHs could provide an effective design for small-molecule devices for high-performance bioanalytical measurement systems.

Terrylene is such an example among the many small-molecule and macromolecular organic compounds that appear in the form of thin platelets, unsuitable for single crystal X-ray measurements even when their lateral dimensions are larger than the critical size of 1 μm . The 3D ED can instead be efficiently used for the structure analysis of such tiny samples, whose relatively constant thickness is even an advantage for continuous data acquisitions because it makes the experiment insensitive to small sample drift (Das et al., 2021). The main issue in case of flat crystals is the occurrence of a strong preferential orientation. In order to increase the chance of obtaining crystals in various orientations and improve completeness by merging multiple data sets from different crystals, three-dimensional support grids can be used, or flat support carbon layer grids can be coiled by gently stroking with a brush (Wennmacher et al., 2019).

Single crystal X-ray diffraction can rarely be applied to samples with high plastic flexibility. In these cases, the sample typically appears with a hairy habit (Figure 6d), due to continuous occurrence of defects that distort the lattice continuity. Recently, two very plastic polymorphs of indomethacin, the δ -polymorph formed via desolvation and the θ -polymorph obtained by microdroplet melt crystallization, were solved on the basis of 3D ED data (Andrusenko

et al., 2021; Lightowler et al., 2022). Both samples appear as long, flexible, and intertwined wires, a shape that has hindered any attempt of single crystal X-ray analysis. In fact, until recently, the two polymorphs were confused due to their similar infrared spectra.

4.3 | Chirality

The determination of the chirality of a compound is essential in drug development and manufacturing for medical applications, since one isomer can have beneficial bioactivity, whereas the other can be useless or even harmful. While kinematical diffraction is insensitive to chirality, dynamical scattering retains information on the absolute structure, therefore 3D ED appears particularly suitable for determining the chirality of target compounds. The determination of chirality is indeed based on the comparison of dynamic scattering effects for two enantiomeric crystals. Comparing the match between observed and predicted ED patterns for both enantiomeric models allows one to determine the absolute configuration of chiral species. This approach was originally proposed for inorganic compounds (Jansen et al., 1998), but recently found a straightforward application also for small-molecule organic compounds. Brázda et al. (2019) analyzed the diffraction of a pharmaceutical co-crystal of enantiopure sofosbuvir and L-proline, showing that dynamical diffraction effects are sufficiently strong even for highly beam sensitive and light atom structures. The determination of the absolute structure of nanocrystals is one of the most important perspectives of 3D ED.

5 | FUTURE PERSPECTIVES

5.1 | Software and hardware improvements

Many current limitations in electron crystallography derive from the lack of dedicated instrumentations, which are not optimized for 3D ED experiments. So far, different set ups for 3D ED experiments have used a TEM, a multifunctional machine that was originally designed as an instrument for imaging. Only recently, dedicated electron diffractometers started to appear on the market. In 2021, the first models of two commercial single crystal electron diffractometers were presented. Both RIGAKU Corporation and ELDICO Scientific instruments have optimized solutions for continuous 3D ED data collection with possibility of crystal search and image tracking (Ito et al., 2021; Lanza et al., 2021). The required sample is virtually the same as for standard TEMs, but for the moment it is not possible on these dedicated instruments to work at low temperatures for cooling and cryo-transfer experiments. Additionally, unlike X-ray diffractometers, these first electron diffractometers have a single axis goniometer, thus the data completeness is still limited for low symmetry or platelet like crystals.

While single crystal electron diffractometers are operated and fully controlled through dedicated software, ED data collections performed by TEM are in most cases not automated or just semi-automated, relying on different collection of scripts that depend on the TEM manufacturer or model. More advanced and automated routines for data collections have started to appear only recently (Cichocka et al., 2018; Kodjikian & Klein, 2019; Plana-Ruiz et al., 2020).

The fast spread of the technique has also enabled researchers to develop different software suites for data analysis. Most of these routines allow the refinement of experimental parameters, the reconstruction and visualization of the 3D diffraction volume, the determination of cell parameters, and the integration of the reflection intensities, but their intermediate outputs are often not reciprocally compatible. This may complicate the whole process if more programs are involved in data analysis. Therefore, dedicated software packages for controlled data collection and precise data analysis would be of great benefit for fast and efficient structure determination especially for challenging samples.

5.2 | Serial ED and serial RED

Despite the recent advances in single particle cryo-EM, still the large majority of structures are determined by crystallographic methods. Notably, during the past few years X-ray free-electron laser (XFEL) crystallographic techniques have motivated the development of serial crystallography for poorly crystalline and very sensitive materials. Here, instead of a rotation data set, one or a few single snapshots from each crystal are acquired, reducing the beam damage while

BOX 1 X-rays, neutrons and electrons

Compared with X-rays and neutrons, electrons have much stronger interaction with matter, making it possible to acquire diffraction data from sub-micrometric domains with atomic resolution. The 3D ED approach for collecting electron diffraction drastically reduces most of drawbacks of such strong interaction, like dynamical effects and excitation error. Additionally, the possibility to work with low dose illumination and collect diffraction data in a fast way allows to use this technique also for very beam sensitive and unstable materials.

BOX 2 The importance of crystalline structure

Nanostructures can be utilized as delivery mediators for encapsulating drugs or attaching therapeutic medications and transport them to target tissues, more precisely and with a controlled release. Atomic structure determination allows understanding the ligand binding interaction and controlling the polymorphic stages in a crystallization process, which is extremely important for drug development.

sufficient completeness is achieved through merging of thousands of such snapshots. However, the scarcity and cost of XFEL beamlines limit their use for routine structure determination.

Taking inspiration from the XFEL concept, serial electron diffraction (serial ED) has been recently proposed as an alternative method for collecting 3D ED data (Bücker et al., 2020; Smeets et al., 2018). The serial ED technique combines computer-controlled screens of hundreds of crystals per hour with fully automatic ED data collection from all suitable crystals. However, indexing of randomly oriented diffraction patterns can be a challenging problem that may be mitigated with rotation (serial RED), that is, acquiring a short diffraction tilt from each crystal instead of a single snapshot. Combined with cluster analysis, serial RED appears an efficient multi-crystal workflow for structure determination and especially promising for extremely beam sensitive materials or phase mixtures (Luo et al., 2022).

5.3 | Perspectives for pharmaceutical science

The recent tremendous improvements of 3D ED for beam sensitive samples (Bruhn et al., 2021; Gruene et al., 2018; Jones et al., 2018; van Genderen et al., 2016), together with the appearance in the market of dedicated instruments that can be operated as proper electron diffractometers (Ito et al., 2021; Lanza et al., 2021), will possibly change the configuration and the expertise of pharmaceutical laboratories. There are already several examples of pharmaceutical molecules, whose crystal structure have been revealed only thanks to 3D ED years after their discovery. Previously presented orthocetamol (Andrusenko et al., 2019) and indomethacine (Andrusenko et al., 2021; Lightowler et al., 2022) are representative case studies. The even more recent structure determination of bismuth subsalicylate (Svensson Grape et al., 2022), a compound already commercialized since last century, suggests once more that the reported examples are just the beginning of a long list. For the early future, we expect that when unexpected diffraction peaks are detected after a preliminary powder X-ray screening of novel drugs, the sample will be routinely analyzed by electron diffraction, avoiding the long and difficult growth of large grains needed for single crystal X-ray diffraction. The same approach will also provide absolute structure information, giving insights about the handedness of the pharmaceutical samples (Brázda et al., 2019; Boxes 1 and 2).

6 | CONCLUSION

Loosening the size limitations imposed by the sample, in the past 15 years, 3D ED has been applied to many inorganic materials (nanoparticles, alloys, oxides, minerals, zeolites), hybrid compounds, macromolecules and pharmaceuticals,

and proved to be a reliable routine technique not only for atomic structure determination, but also for understanding ligand binding interactions (Clabbers et al., 2020) or unveiling polymorphic stages in a crystallization process (Broadhurst et al., 2021). For these and others scientific applications, like the study of nanoparticles and charge oxidation states, 3D ED may not only complement, but essentially extend existing methods in structural nanomedicine. Unquestionably, the quality of collected ED patterns and the accuracy of determined structures will further improve with the dedicated sensitive detectors that can reach higher resolution in a shorter time. With 3D ED the size of the crystal is not the main issue and this will have a strong impact for chemical synthesis. Indeed, it will be possible to investigate with a crystallographic method all those chemical synthesis routes where the output products are nanocrystalline and multi-phases, with the possibility to discover a number of new unknown metastable compounds and polymorphs. It can be expected that in the years to come 3D ED will become the first technique to consider when uninterpretable powder synthesis occurs, and a lot of electron diffraction instruments will possibly start to populate the structure characterization facilities of private and public research laboratories.

AUTHOR CONTRIBUTIONS

Iryna Andrusenko: Conceptualization (equal); methodology (equal); resources (equal); supervision (lead); writing – original draft (equal); writing – review and editing (equal). **Mauro Gemmi:** Conceptualization (equal); methodology (equal); resources (equal); writing – original draft (equal); writing – review and editing (equal).

ACKNOWLEDGMENTS

Iryna Andrusenko and Mauro Gemmi acknowledge the Regione Toscana for funding the purchase of the Timepix. Open Access Funding provided by Istituto Italiano di Tecnologia within the CRUI-CARE Agreement.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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FURTHER READING

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How to cite this article: Andrusenko, I., & Gemmi, M. (2022). 3D electron diffraction for structure determination of small-molecule nanocrystals: A possible breakthrough for the pharmaceutical industry. *WIREs Nanomedicine and Nanobiotechnology*, 14(5), e1810. <https://doi.org/10.1002/wnan.1810>