Structure Analysis of Nano-Size Crystals by the XtaLAB Synergy-ED: An Integrated Platform for 3D ED/MicroED

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Abstract

3D ED/microED has been attracting much attention because it enables structure analysis of crystals smaller than 1 μm. In this article, examples of crystal structure analysis and some applications of MicroED/3D ED will be presented.

1. Introduction

What is the minimum crystal size required for single crystal X-ray structure analysis? The answer to this question depends on X-ray sources and detectors. The latest laboratory systems are capable of determining the structure of 1–10 μm crystals; however, it remains difficult to measure a crystal smaller than 1 μm. Despite extensive attempts, crystallization often fails to produce crystals exceeding 1 μm, making crystallization the largest obstacle in crystal structure analysis currently. Recently, single crystal analysis using an electron beam instead of X-rays has been attracting much attention as a method to overcome this problem. Single crystal analysis using electron diffraction has been referred to by a number of different names—RED, cRED, EDT, etc.—since several groups started publishing papers actively within the past few years. In this article, we will use the term "3D ED/MicroED," and will focus on examples and applications of 3D ED/MicroED.

2. Why Electron Diffraction?

As mentioned above, an electron beam can be used to determine the structure of crystals smaller than 1 μm. This is because the scattering cross section of the electron beam is 10^5 to 10^6 times larger than that of X-rays; therefore, its interaction with materials is stronger by several orders of magnitude[1]. Meanwhile, the stronger interaction with materials also indicates that there is a possibility that samples can be readily damaged by the electron beam. For this reason, until recently, 3D ED/MicroED was limited to inorganic compounds that are relatively resistant to damage by the electron beam and was rarely used for general structure determination. However, the situation has changed dramatically. Technological innovations in detectors have made it possible to reduce the intensity of the incident electron beam to the level where damage becomes negligible. A 3D ED/MicroED system equipped with an HPC (Hybrid Photon Counting) detector can be used for structure determination of samples susceptible to electron beam damage, such as organic and protein crystals[2]. Since almost all crystalline samples are now eligible for 3D ED/MicroED measurements, this technique first developed in 1940s to 1970s has been attracting great attention once again in recent years. In fact, in 2018, a paper on structure determination by 3D ED/MicroED was selected as the "Breakthrough of the Year" in *Science*[3].

Despite increasing demand for 3D ED/MicroED, measurement and analysis with this analytical technique has not been straightforward. Until recently, electron diffraction experiments were performed mainly as a function of general-purpose transmission electron microscopes. The hurdles were higher than those for X-ray structural analysis because it required an expert in electron microscopy to determine the optimal setting of the equipment for diffraction experiments. To address this situation, Rigaku and JEOL jointly developed an electron diffraction platform dedicated to 3D ED/MicroED. The system is called XtaLAB Synergy-ED (Synergy-ED hereafter). With the Synergy-ED, anyone can easily perform electron diffraction experiments[4].

3. Examples of Structure Determination and Applications

3.1. Structure determination of rotaxane

The authors have so far determined the structures of more than 320 compounds using the Synergy-ED. Most of the crystals were smaller than 1 μm. However, 3D ED/microED is effective sometimes even when the crystal is sufficiently large for X-ray diffraction. It has been observed that the Synergy-ED is capable of determining the structure of crystals diffracting X-rays only to 3 Å resolution.

In collaboration with the University of Birmingham and the University of Nottingham in UK, the authors performed structure determination of rotaxane (ca. 3500 Da) using the Synergy-ED[5]. Initially, they attempted to determine the structure at a synchrotron beamline.

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However, diffraction was observed only to 1.25 Å resolution and initial phase determination by direct methods was difficult. Modeling of the counterion, hexafluorophosphate, was impossible. Therefore, we performed measurements with the Synergy-ED on the same batch of crystals used for the synchrotron experiment. As a result, data to 1 Å resolution were obtained and the structure was successfully determined by direct methods (Fig. 1). The difference Fourier map clearly showed peaks corresponding to the hexafluorophosphate and it was successfully included in the structure refinement. It is undisputed that single-crystal X-ray structure analysis using synchrotron radiation is very important. However, the structure determination of rotaxane is a good example of how 3D ED/MicroED is more effective for poorly diffracting crystals than X-ray diffraction.

3.2 Structure analysis of microcrystals in a tablet

Crystal structure is related to physical properties such as solubility and elution of active ingredients in a tablet. Since the active ingredients of small molecule drugs on the market are often in the form of crystals, it is expected that the molecular structure can be determined by single crystal structure analysis. However, crystals in a tablet are often smaller than 1 μm, and it is difficult to determine the structure by X-ray diffraction. One way to circumvent the problem is to recrystallize the active ingredient. However, there is no assurance that the obtained crystal structure represents the form present in the tablet. In such cases, the Synergy-ED becomes a powerful tool because it does not require recrystallization and can directly analyze the crystal in the tablet as it is. The authors performed measurements on an over-the-counter drug for allergic rhinitis. We first gently crushed the tablet to extract some powder from the tablet. Then we measured and analyzed the fine powder using the Synergy-ED. The structure of the active ingredient was successfully determined within 5 minutes. The short measurement time is an additional advantage of 3D ED/MicroED. In addition, the structure of a compound thought to be an additive (Fig. 2) and the diffraction pattern possibly belonging to a crystalline polymer were also obtained.

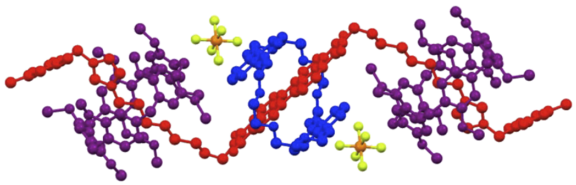

Fig. 1. The structure of rotaxane determined with the Synergy-ED.

Microcrystal measurements with the Synergy-ED enabled us to determine the structures of the active ingredients of nearly all the other commercially available drugs we tested. The structure determination process by the Synergy-ED is much faster than conventional structure determination using X-rays. 3D ED/microED can eliminate the recrystallization of the

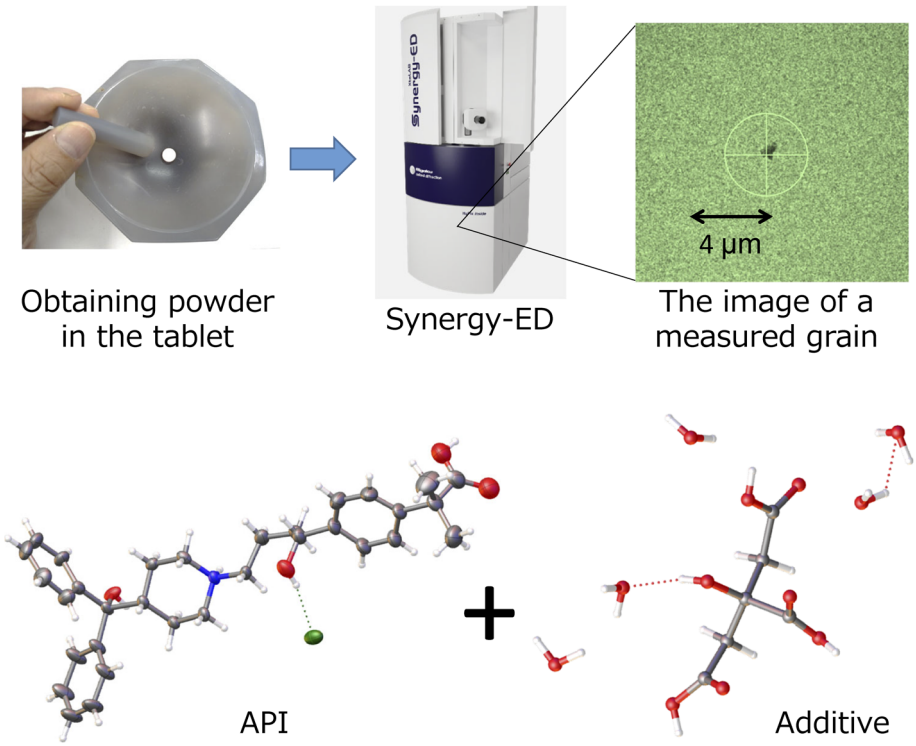

Fig. 2. Structure determination of compounds in a medicine tablet. The tablet was crushed (upper left) and the fine powder in the tablet was measured (upper middle and right). The thickness of the measured crystals was approximately 500 nm. The structure of the active ingredient (lower left) and a compound considered to be an additive is shown (lower right).

active ingredients, as well. The Synergy-ED is expected to contribute to speeding up the entire R&D process of small molecule drugs.

3.3 Quantitative analysis

Since 3D ED/MicroED can measure submicron crystals, it is possible to measure crystals as small as those used for powder X-ray diffraction. Furthermore, since the measurement time with 3D ED/MicroED is extremely short, quantitative analysis is possible by sequentially measuring hundreds of crystals scattered on a grid. Additionally, since the crystal structure can be determined, the individual features of each crystal, such as differences in crystal packing and lattice constants, can also be captured.

Acetaminophen, which is widely prescribed as an antipyretic analgesic for coronavirus infections, is known to have several crystal polymorphs[6]. At room temperature, almost all crystals exist as Form I. It is known that recrystallization by heating, melting, and cooling produces Form II. Additionally, two additional forms (Form III and Form IV) have also been identified. For quantitative analysis, acetaminophen that had been recrystallized by heating, melting and cooling with an XRD-DSC attachment was used. The measurement was performed using "queue mode", a function of CrysAlis^{Pro} for ED, the control and analysis software integrated in the Synergy-ED. Queue mode provides semi-automatic measurement, collecting data sets for a list of crystals submitted prior to the measurement. In addition to measurement, data processing and structure determination can also be performed simultaneously using the scripting function of CrysAlis^{Pro} for ED. Quantitative analysis results are shown in Fig. 3. A total of 200 crystals were measured and structures were obtained for 100 crystals. As expected from powder X-ray diffraction results, recrystallization reduced the population of Form I to 10%. In contrast, Form II and Form III were found to be more abundant than Form I. In addition to the ratio of each polymorph, the structure of each crystalline polymorph was automatically determined (Fig. 4).

Here, quantitative analysis with simultaneous structure determination was performed on a medicine. This capability may also be useful in other fields such as materials science.

3.4 Determination of absolute structure

It is important to determine the absolute configuration of optically active compounds because each conformer has different biological effects on living organisms. For example, L-leucine is bitter while D-leucine is sweet.

In X-ray crystallography, the absolute structure is determined by utilizing the discrepancy between the Friedel pairs; i.e., $I(h\ k\ l) \neq I(\bar{h}\ \bar{k}\ \bar{l})$. Meanwhile, in 3D ED/MicroED, Friedel's law is broken not by anomalous dispersion but by multiple scattering. It

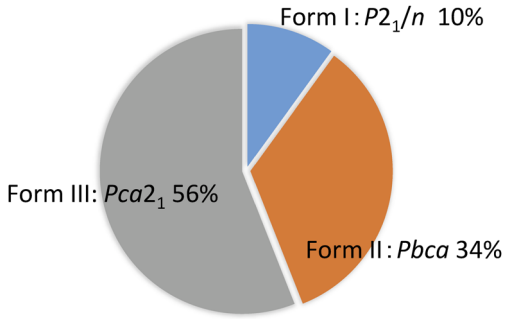

Fig. 3. Results of quantitative analysis of acetaminophen. 100 structures were determined automatically out of 200 measurements.

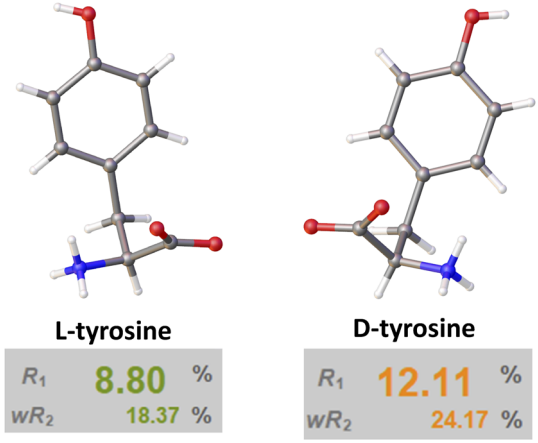

Fig. 5. Determination of the absolute structure of L-tyrosine by 3D ED/MicroED. Data collection and data processing were performed by the Synergy-ED system and the structures were refined by Jana2020.

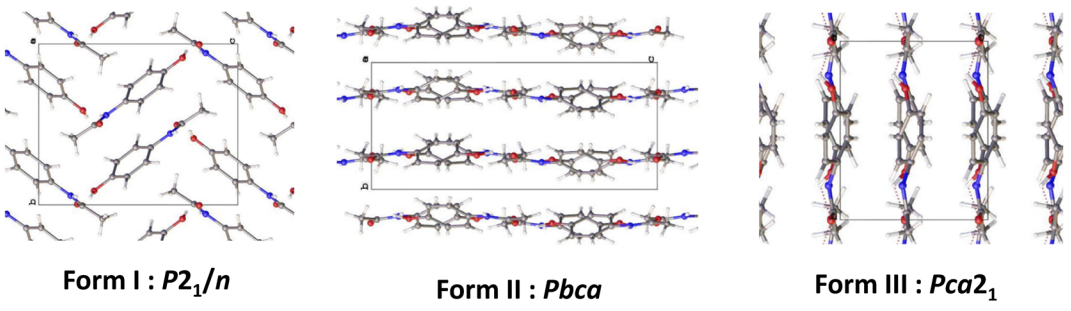

Fig. 4. Structure of each crystal polymorph of acetaminophen determined by automated analysis. The packing of each crystalline polymorph in the a-axis projection is shown. Information on the ratio of polymorphs in the sample and structural information can be obtained simultaneously.

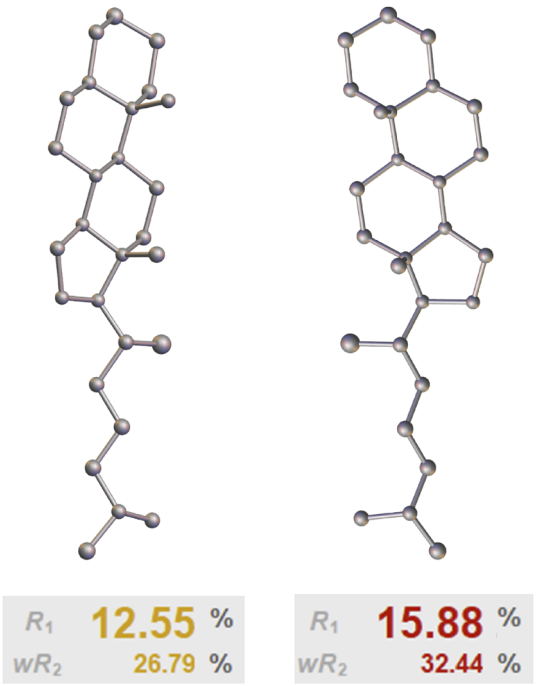

Fig. 6. Determination of absolute structure of 5α-cholestane by 3D ED/MicroED. Structural refinement was performed using only non-hydrogen atoms and isotropic temperature factors.

has been reported that the absolute structure can be determined using this phenomenon[7]. As a test case, the authors attempted to determine the absolute structure of L-tyrosine using the Synergy-ED. The flow of the absolute structure determination is as follows:

(1) Collect diffraction data with the Synergy-ED.
(2) Process the data with CrysAlis^{Pro} for ED. CrysAlis^{Pro} for ED creates a diffraction file for dynamical refinement considering multiple scattering.
(3) Import the diffraction file into Jana2020[8] to perform the dynamical refinement.
(4) Invert the structure and repeat the refinement. The enantiomorphs giving the lower *R1* and *wR2* is expected to be the one having the correct absolute configuration.

For the absolute structure determination of tyrosine, the refinement was performed on both L-tyrosine and D-tyrosine models, and *R1* and *wR2* were compared. The correct conformation, L-tyrosine, was supposed to give lower *R1* and *wR2* values than D-tyrosine. As is shown in Fig. 5, L-tyrosine indeed gave lower R1 and *wR2* values than D-tyrosine. This examination confirmed that 3D ED/microED is capable of determining the absolute structure. The fact that 3D ED/MicroED can be used to determine the absolute structure of crystals may lead to an increase in absolute structure determination of crystals smaller than 1 μm.

An additional advantage of 3D ED/MicroED is that it can determine the absolute structure of compounds consisting only of light elements such as carbon and hydrogen. The anomalous dispersion by X-ray diffraction is smaller for light elements, and it is sometimes difficult to determine the absolute structure of organic molecules. Meanwhile, in 3D ED/MicroED, Friedel's law isn't broken by the anomalous scattering but by multiple scattering. Therefore, it is expected that 3D ED/microED is less affected by the types of atoms in the compound. To verify this, absolute structure determination of 5α-cholestane, consisting only of carbon and hydrogen, was attempted using 3D ED/MicroED. The absolute structure of this compound can be determined by X-rays, but it required 10-fold or greater data redundancy and several hours of data collection. In contrast, measurement with 3D ED/MicroED took a mere 2 minutes. Structure refinement considering multiple scattering concluded the same configuration as that determined by X-ray diffraction.

It has been said that single crystal X-ray structure analysis is the sole method to determine absolute structure. However, it has been proved that 3D ED/MicroED is equally useful for absolute structure determination, as shown in the examples of L-tyrosine and 5α-cholestane. It is expected that X-ray diffraction or 3D ED/MicroED will be used as complementary techniques depending on crystal size and the type of compound, or both will be used simultaneously for verification purposes.

4. Conclusion

Crystal structure analyses by 3D ED/MicroED using the Synergy-ED were introduced in this article, focusing on measurements and application examples. The importance of X-ray crystallography is undeniable. However, in the future, complementary use of single crystal X-ray structure analysis and 3D ED/MicroED will become increasingly critical, depending on crystal size, the amount of sample, and the purpose of the experiment.

References

(1) R. Henderson: The potential and limitations of neutrons, electrons and X-rays for atomic resolution microscopy of unstained biological molecules. *Quar. Rev. Biophys.*, **28**(2), 171–193 (1995).
(2) M. Gemmi, E. Mugnaioli, T. E. Gorelik, U. Kolb, L. Palatinus, P. Boullay, S. Hovmöller, and J. P. Abrahams: 3D Electron Diffraction: The Nanocrystallography Revolution. *ACS Cent. Sci*., **28**(8), 1315–1329 (2019).
(3) R. F. Service: Molecular CT scan could speed drug discovery, *Science,* **26**, 362 (2018)
(4) S. Ito, et al.: Structure determination of small molecule compounds by an electron diffractometer for 3D ED/MicroED. *CrystEngComm.* **23**, 8622–8630 (2021).
(5) N. Pearce, K. E. A. Reynolds, S. Kayal, et al.: Selective photoinduced charge separation in perylenediimide-pillar[5] arene rotaxanes. *Nat. Commun.,* **13**, 415 (2022).
(6) A. G. Shtukenberg, M. Tan, L. Vogt-Maranto, E. J. Chan, W. Xu, J. Yang, M. E. Tuckerman, C. T. Hu, and B. Kahr: *Cryst. Growth Des.,* **19**(7), 4070–4080 (2019).
(7) P. Brázda, L. Palatinus, and M. Babor: Electron diffraction determines molecular absolute configuration in a pharmaceutical nanocrystal. *Science*, **364**(6441), 667–669 (2019).
(8) V. Petříček, M. Dušek, and L. Palatinus: Crystallographic Computing System JANA2006: General features *Zeitsch. für Kristallogr.-Cryst. Mater.*, **229**(5), 345–352 (2014).