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PNNL-29546

MicroED for rapid and comprehensive characterization of the unknown small molecule composition of crude plant and soil extracts

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U.S. DEPARTMENT OF ENERGY
Prepared for the U.S. Department of Energy
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Summary of LDRD project, April-September 2019:

JR Cort, IV Novikova, JE Evans

Introduction

This project implemented at PNNL a method introduced recently [1] for *de novo* structure elucidation of small organic molecules: Micro-Electron Diffraction (henceforth microED). Micro-ED is an alternative to X-ray diffraction (XRD) or Nuclear Magnetic Resonance (NMR) for atomic-resolution structure elucidation of small organic or biological molecules, and it can succeed in solving structures when these well-established methods fail. In particular, microED offers the possibility of determining structures from far smaller quantities of material than would be required for NMR or even XRD, or from substances that fail to form crystals suitable (i.e., large enough) for X-ray crystallography, because microED requires only microcrystalline samples: in principle, a single crystal with ca. 100 nm dimensions would be suitable. MicroED is performed typically with standard electron microscopy (EM) instrumentation. The substantial potential of microED has drawn significant attention and examples of its use to solve difficult structure elucidation problems where X-ray or NMR methods failed has been described recently, e.g. [2].

The project had two aims 1) implement microED at PNNL at a functional level, on “ideal” samples, and 2) adapt the method for increasingly complex samples—for example, two or more compounds together with impurities, multiple compounds in widely varying quantities, and crude solvent extracts of plants or soil that have been minimally or partially fractionated. The complex sample cases are particularly relevant to the EBSD “decoding the molecular universe” directorate objective. Achieving these aims could enable future efforts where microED of microcrystals is used to solve structures organic compounds isolated by ion-mobility separation with soft landing, a potentially transformative combination for large scale, high throughput identification of all components of a complex mixture. Establishment of routine microED and automation of certain steps could dramatically change the pace of unknown metabolite identification and dereplication, particularly from challenging systems of soil isolates.

The scope of the project was constrained at the outset by the short duration (approximately 6 months) of the project. Significant instrumentation outages (such as unexpected 3 months ETEM downtown) delayed the start of data collection and thereby constrained the scope, in terms of the number and variety of samples that could be run. Nonetheless, some important progress was made in the sample preparation and screening aspect of microED, and some high-quality data was collected that will enable science questions to be answered and published once the data is processed and analyzed.

Outcomes

1) Preparation of Suitable Crystals. One of the potential advantages of micoED is organic compounds in the solid state often are microcrystalline. Thus, extensive crystallization trials are not necessary—usually a reasonably pure sample in solution can simply be dried down. However, crystals can easily be too large, clumpy, or amorphous for microED. After testing a few published approaches, we narrowed our sample preparation protocol to ultratip micro-sonication methods for production of crystals of suitable size for microED data collection. We found that this sonication procedure was the quickest, the most consistent, gentle and applicable to a diverse range of samples. The prodedure is dry a sample dissolved in volatile solvent, resuspend solid in a solvent in which it is insoluble, sonicate, and then dry the solicited material. It has been tested successfully for a number of crystalline organic samples in our laboratory. Samples prepared this way are then introduced onto the cryo-EM grid. Because they are more consistently suitably sized for microED, the screening step goes faster.

2) Screening for suitable crystals. We have determined that the most time-consuming step in the microED data collection is actual screening for suitable crystals. While microED can work on very small single

crystals, the crystal must be produced, identified and determined to be suitable for data collection. Right now, that can only take place in a cryo-EM instrument, and screening a grid for suitable crystals is very slow and occupies valuable instrument time. The screening process will probably accelerate when new cryo-EM instrumentation scheduled for installation in EMSL is operational. Other approaches for pre-screening should be investigated.

3) MicroED workflow at PNNL. A successful microED data collection routine was established on ETEM using a direct electron detection camera and SerialEM/script software at cryo-EM conditions. Full datasets for multiple crystals from several organic compounds have been collected (see Fig. 1, below). One of the original aims was to address impure samples isolated and partially fractionated from natural sources, such as plants and peat bogs, that had been shown to contain novel secondary metabolites. Because of the challenges in screening crystals, we opted to focus on somewhat more purified samples for which there were unanswered scientific questions.

Results

The data shown (right) is from an idiosyncratic isoflavonoid compound of interest, wrightiadione [3] (below), that may exist as an unusual type of isomer. Characterization of wrightiadione by NMR and MS previously showed that two species of equal mass and identical atom connectivity were present. Our hypothesis is that two species differing only in their electronic configuration are present and inverconvert slowly if at all, in solution, probably because the destabilizing antiaromatic (4n pi electrons) indenone ring system on the right side of the species shown below at left likely exists in a triplet state, as is typical for antiaromatic systems. The species shown below on the right is fully aromatic (4n+2 pi electron systems), but would itself be somewhat destabilized due to charge separation. Because this species almost certainly is a singlet, a characteristically slow singlet-triplet conversion could enable simultaneous existence of the two distinct “electroisomeric” forms.

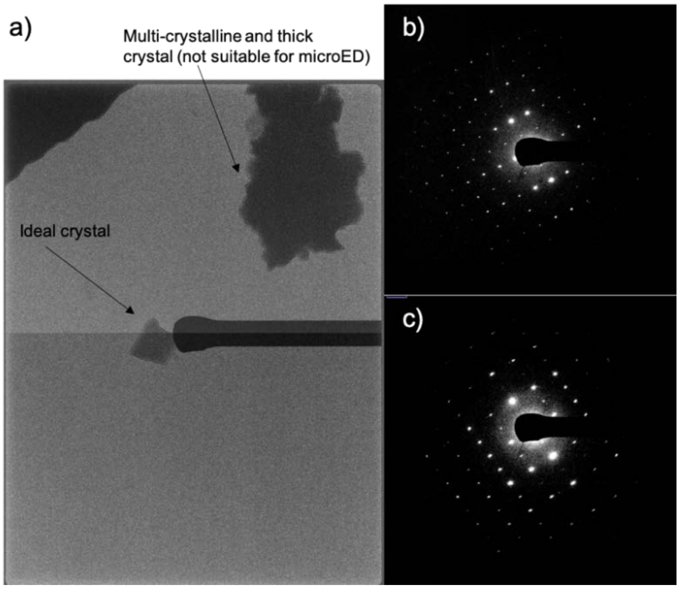

Fig. 1. Wrightiadione crystals. a) Low magnification image of two of the compound’s crystals. It is a good representative image that shows the differences between suitable and not suitable crystals for microED, b) and c) representative micro electron diffraction patterns (extracted from microED movies)

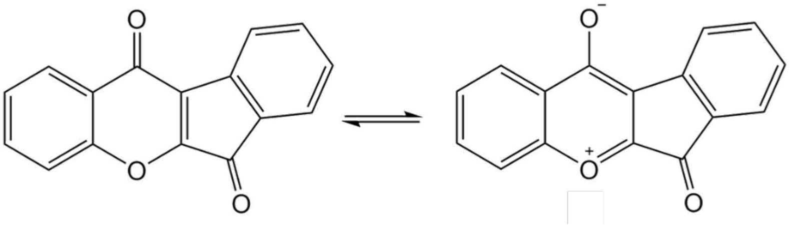

Fig. 2. Wrightiadione structure (left) and a hypothesized alternative electronic configuration.

Crystal structures of wrightiadione would give information about structure(s) present in the solid state, as the two forms shown above would have significant differences in several bond lengths. Our microED data obtained for wrightiadione crystals showed that different microcrystals gave different diffraction patterns, an exiting result suggesting both proposed species are present in the solid state. Intermolecular interactions in the solid state could further increase the energetic barrier to species interconversion relative to that in solution, and allow the two species to crystallize separately. Alternatively, either there could be a single species that crystallizes in two different forms, or the original hypothesis could be wrong and the isomeric species could be a constitutional isomer, although NMR data argue against this possibility. The diffraction data collected for wrightiadione is currently being analyzed; if the data confirm our hypothesis, this will be a notable discovery that would have broad interest when published.

Conclusions

1) Efficient screening for good microcrystals, and/or generating good microcrystals in the first place are essential—we cannot waste time on the instrument searching for the occasional good crystal in a mostly poor sample. For instance, in ETEM, we can work only with one sample grid at a time. A new cryoEM instrument (Krios) that is coming to EMSL at the beginning of 2020, allows batch screening of 12 sample grids and diminishes this obstacle. Nevertheless, the challenges of screening will be multiplied for complex samples, such as the crude soil extracts such as were envisioned initially. For this reason, we elected to pursue samples that had a higher probability of yielding good data in the limited instrument time available. It is worth thinking about additional purifications and isolation of single HPLC peaks for microcrystallization. Also, it is worth considering some kind of optical pre-screening of crystalline material.

2) The indexing of microED data is performed by adapting software tools developed for X-ray crystallography such as iMosflm, XRD and DIALS. However, microED diffraction patterns host a number of unique challenges. In particular, due to smaller scattering angles, the Ewald sphere's is less curved than in the X-ray data which causes autoindexing failures using standard X-ray auto-processing approaches. To account for that, a lot of parameters are tested manually, and these steps are not straightforward and not automated. Additional training in this regard is being pursued.

3) Overall, microED has substantial potential that our short project has hinted at, for example in the wrightiadione example shown above, and with one other sample that never yielded crystals suitable for X-ray crystallography. While we were not able to test complex crude samples as originally envisioned, new equipment, improved pre-screening approaches, and additional purification of crude samples may enable such samples to be evaluated productively. We still currently have on hand dozens of such samples (plant and soil extracts) from collaborators in the natural products field.

References

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