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SMALLER crystals, FASTER experiments, BRIGHTER beams: Drug screening & innovation via X-ray powder diffraction.

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Knowledge of the 3D structures of proteins is a key element for understanding functions and mechanisms necessary for the conception of drugs. Drugs can be proteins, as insulin, or small molecules that interact with biological targets. To date, more than 100 proteins are approved for clinical use in the European Union and the USA. The need for a large number of experimental structural data is common in all drug-related projects and demands continuous improvement of methods for determination of protein structures. Until now, the majority of protein structures are determined by X-ray diffraction on single crystals with typical sizes > 5 - 10 μm at micro-focus synchrotron beam lines. Despite significant progress there are still limitations of the research on single crystals. Difficulties in protein crystallization are the major bottleneck for single crystal diffraction. Polymorph screening - critical for drug innovation – is not possible and last but not least single crystal diffraction is not ideal for time-resolved studies (dynamics). Already in 1999 the power of powder diffraction for revealing protein structures was demonstrated for hen egg white lysozyme by Bob Von Dreele. Since 2003, our team at the ESRF and later at UPAT, have proved that protein structures can also be obtained from sub-micron crystals with typical sizes > 0.1 μm via X-ray powder diffraction using synchrotron and laboratory sources[i, ii]. This approach provides medium-resolution structural models (3 - 10 Å) and allows for the study of low-quality crystals; polymorph screening is a routine practice and time-resolved studies are also possible[iii, iv]. In addition, powder data reveal characteristics of the microcrystalline samples such as purity, sample homogeneity, highly accurate cell dimensions and lattice strains induced by sample preparation; critical parameters for the development of therapeutic formulations. In 2013, the UPAT team installed a modern laboratory diffractometer which is routinely employed for structural studies of proteins. Data collection in the lab prior to synchrotron measurements is a major advance. The lab instrument also allows for high-throughput crystal screening and optimization, polymorph identification and delivers high statistics due to the much slower radiation damage of the biological samples in the lab. Currently, we study Human Insulin (HI) complexes with phenol-based ligands at different crystallization conditions. They could for example show that phenol-based molecules bind on HI and affect both HI conformation and the crystal forms adopted; they revealed very high polymorphism upon variation of crystallization pH and type of ligand and disclosed 4 novel polymorphs with enhanced characteristics as potential drug targets [v, vi, vii]. In collaboration with the R&D scientists at PANalytical, preliminary variable temperature and relative humidity studies indicated rapid phase transitions and previously unidentified polymorphs associated with distinct biological activity. Finally, combined data collected using laboratory and synchrotron instruments, allowed for the detailed structural characterization of several HI-ligand polymorphs determining not only the protein structure but also the ligand binding sites necessary for rational drug design (ESRFnews, Cover & article p. 18). These studies manifest that powder diffraction is moving 'beyond demonstration experiments' and is ready to become a strategic technique for routine characterization of micro-crystalline proteins.

[i] Margiolaki, I. & Wright J. P. (2008). *Acta Cryst.*, A**64**, 169-180, [ii] Karavassili, F. & Margiolaki, I. (2016). *Protein Pept. Lett.*, **23**, 23(3):232-41, [iii] Margiolaki, I. *et al.* (2005). *Acta Cryst*., D**61**, 423-432., [iv] Beckers, D. *et al.* (2015). *Acta Cryst.,* A**71** (a1), s510-s510., [v] Karavassili, F. *et al.* (2012). *Acta Cryst*., D**68**, 1632–1641., [vi] Valmas, A. *et al.* (2015). *Acta Cryst*., D**71**, 819-828., [vii] Fili, S. *et al.* (2015). *IUCrJ*., **2**, 534-544 (open access).

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