EUROPEAN SYNCHROTRON RADIATION FACILITY

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Experiment Report Form

	Experiment title: High resolution powder diffraction of proteins associated with pharmaceutical interest	**Experiment number**: LS-3012
Beamline: ID22	**Date of experiment**: from: 30 November 2021 to: 4 December 2021	**Date of report**:
Shifts: 9	**Local contact(s)**: Andrew N. Fitch	*Received at ESRF:*

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Report

In the present experiment we collected X-Ray Powder Diffraction (XRPD) data from macromolecular samples on ID22, the high-resolution powder diffraction beamline at ESRF. Approximately 200 samples of human insulin co-crystallized with various small organic ligands at different pH levels were measured during this experiment. Human insulin is a highly polymorphic protein, with 3 distinct molecular conformations and crystallizing in over 30 crystal forms (*i.e.* crystal polymorphs) by variation of its physicochemical environment. Our team has been systematically exploring the phase map of human insulin upon ligand binding and pH variation, for the development of novel, more effective, crystalline, anti-diabetic formulations (**Figure 1**; Spiliopoulou *et al.*, 2020).

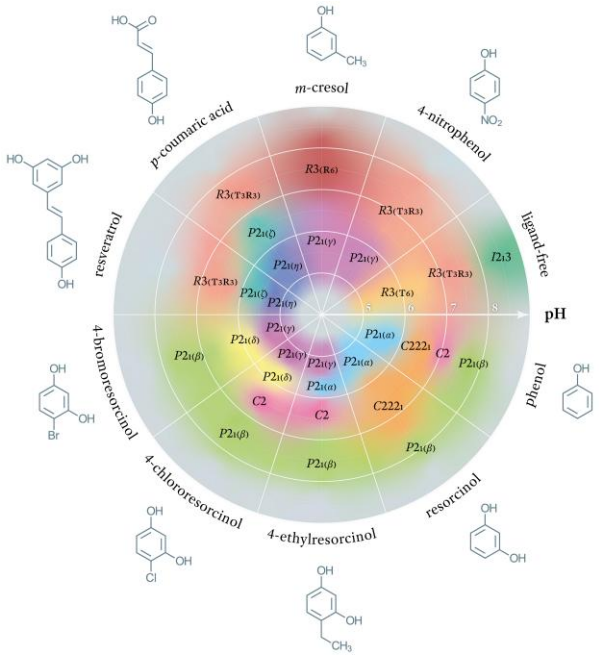

Figure 1: The ever-expanding phase map of human insulin co-crystallized with various ligands and at varying pH levels, as determined by our team using ID22 data over the years. Different colors correspond to distinct crystal polymorphs.

Using an already established crystallization protocol, we prepared insulin samples with 11 different ligands, namely derivatives of phenol (phenol, 4-nitrophenol, *p*-coumaric acid, resveratrol), resorcinol (resorcinol, α-resorcylic acid, β-resorcylic acid) and benzoic acid (benzoic acid, 4-hydroxybenzoic acid, gentisic acid, gallic acid). Data were collected from borosilicate glass capillaries loaded with the polycrystalline slurries, employing the new 13-channel crystal analyzer stage and the EIGER 2M detector. The data were then indexed, and Pawley refinements were performed, enabling the extraction of accurate unit-cell parameters for each phase present per samples. Multi-phase refinements were performed for datasets with co-existing crystal polymorphs.

Contrary to single-crystal techniques, the ability to characterize the entire crystalline sample with powder diffraction has proven to be crucial in our case. While the high polymorphicity of human insulin constitutes it an ideal target for polymorph screening experiments, at the same time, slight variations on the crystallization protocols can lead to vastly different polymorphs. For the past two years we have been trying to grow single crystals for several polymorphs of interest, especially for phenol and resorcinol complexes. By adapting the batch protocols for single crystal growth, we have introduced additional variation to the crystallization condition, resulting in our inability to grow crystals of certain polymorphs. By collecting data at ID22 from samples grown with multiple crystallization protocols, we were finally able to identify the factors affecting polymorph selection

on our samples. Using the insights from the high-resolution XRPD data, we managed to grow single crystals for the polymorphs of interest and collect diffraction data at other synchrotron facilities. Structure solution and refinement is still ongoing.

During our experiment, we also collected a few datasets which were then reprocessed to reduce the axial divergence, that is especially pronounced at the low 2θ range, by employing the 2D segments recorded by the EIGER detector (Fitch and Dejoie, 2021). We chose to collect data from certain polymorphs with unit-cell volumes ranging from 1,000,000 to 3,000,000 Å^3, which have several peaks at the low angle region. The resulting reprocessed data exhibited a remarkable improvement in peak asymmetry, further reducing the heavy peak overlap observed in macromolecular XRPD (**Figure 2**). These data have enabled us to attempt Rietveld refinement on these polymorphs, for the first after 10 years, when we first identified them at ID22 (Karavassili *et al.*, 2012).

Overall, the data collected at ID22 have enriched the ever-expanding phase map of human insulin, guided subsequent single-crystal experiments and enabled structure refinement attempts of polymorphs that were previously considered too hard to tackle *via* powder diffraction.

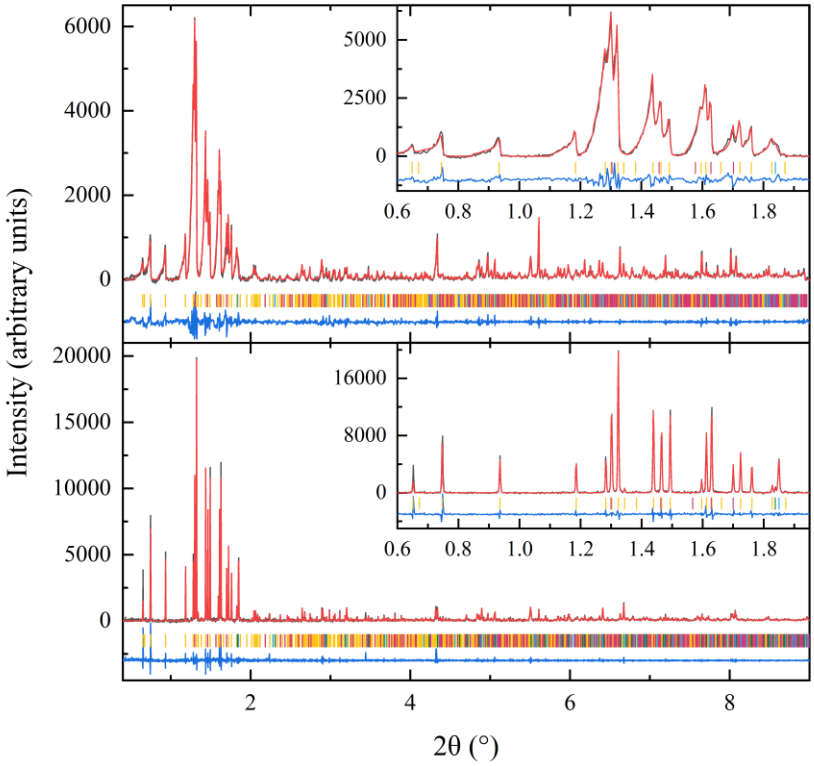

Figure 2: Multiphase Pawley refinement of polycrystalline human insulin sample co-crystallized with phenol. Data collected at ID22 with the 13-channel crystal analyzer stage, with (**bottom**) and without (**top**) axial divergence reprocessing. An additional polymorph (green ticks) could be identified from the reprocessed data. Vertical ticks correspond to Bragg peaks of the different polymorphs: $C222_1$ (orange), $C2$ (magenta), $R3_{T3R3}$ (blue), $R3_{T3R3'}$ (green).

References

Fitch, A., Dejoie, C., 2021. Combining a multi-analyzer stage with a two-dimensional detector for high-resolution powder X-ray diffraction: correcting the angular scale. *J Appl Crystallogr* **54**, 1088–1099. https://doi.org/10.1107/S1600576721005288

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