

# Deciphering the role of mycobacterial secreted proteins through structural biology

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# Summary

Proteins are the most sophisticated biomolecules in nature, and their involvement in every biological process makes them essential for the proper maintenance and performance of life. Structure determination of these elements represents invaluable information to understand their function, correct a medical problem, or use proteins for biotechnological purposes. Technological developments have made the structure determination a routine activity; however, characteristics intrinsic to proteins challenge current methods. In the last decade, electron microscopy in cryogenic conditions has gone through a resolution revolution, now comparable to other high-resolution techniques. Only limited by protein size ( $> 50$  kDa), cryo-EM has the potential to determine the structure at (near-) atomic resolution while giving mechanistic information on the protein function. These advantages have attracted many users, explaining the exponential growth over the years. Unfortunately, this trend will slow down due to the different bottlenecks or difficulties found in the current workflow, which are the subject of intense work.

This thesis describes the development of a new instrument that could replace the semi-manual preparation of samples embedded in vitreous ice (vitrification) for cryo-EM to a fully automatic process (**chapter 2**). This new machine replaces the sample application via pipetting and excess removal with filter paper to a pin-printing method, reducing sample waste and artefacts caused by the filter paper. The incorporation of cryogen jets allows the use of premounted autogrids (metal-edged grids for sturdy handling) ready to be inserted into the microscope, avoiding manual handling of the grids post-vitrification that could lead to ice contamination or grid deformation.

During the development of the VitroJet, we were confronted with the lack of a standard protein available in large amounts to characterise the instrument. Other techniques have easily accessed proteins for training or maintenance purposes, like the lysozyme for MX or BSA for

SAXS. In cryo-EM, ferritin is the protein of choice; its symmetry, solubility, and size allow structure determination with a few micrographs enabling prompt characterisation. Unfortunately, the commercially available ferritin (from horse spleen) lacks stability, while stable human ferritin has a complicated purification protocol that will limit its access to laboratories with specific biochemical expertise. With this in mind, we established a high-yielded and straightforward purification protocol for mycobacterial ferritin BfrB (**chapter 3**). As a proof of concept, Bachelor students with limited biochemical expertise carried out the protocol leading to >50 mg of protein with good purity. Structure determination led to a high-resolution model where conformation changes of amino acids and flexible regions were visible, valuable information that could aid to understand how the function is carried out. This chapter had a double intention: to provide the cryo-EM community a standard protein that could serve as a workhorse for instrument maintenance and development and structural characterisation of a mycobacterial protein essential for the pathogenesis of *M. tuberculosis*, which can serve as a therapeutic target.

In the following chapters, I used structural techniques, particularly cryo-EM, to describe virulence factors of *M. tuberculosis* and understand their role in the functioning of the type VII secretion system and possibly in the pathogenesis of this microorganism. One big mystery that surrounds T7SS is the elements that compose the outer-membrane portion, critical information for the proper understanding of the system. EspB protein has been proposed to be one of these elements. **Chapter 4** describes the conditions that trigger the oligomerisation of EspB and the characteristics that support the hypothesis that this protein is part of the missing components. To further understand the function of EspB and its regulation mechanisms, we studied its binding partner, EspK, another virulence factor of *M. tuberculosis*. Low-resolution studies by SAXS, complemented with CD and sequence alignments, described the presence of two structured domains linked by an unstructured region, characteristic of all the EspK proteins

from the *Mycobacterium* genus (**chapter 5**). Previous studies described the interaction between the C-terminal domain and EspB; for this reason, it was further characterised at high resolution by X-ray crystallography in complex with EspB (**chapter 6**). Such structure resembled the PE-PPE bound to their chaperone EspG, suggesting a possible role of EspK as a chaperone of EspB. This hypothesis was supported by the fact that EspK disrupted the EspB oligomer, ensuring the latter to be in a secretion-competent state.

Overall, this work intended to demonstrate the power of structural visualisation applied in a specific health problem, tuberculosis, to close the knowledge gap that will lead to efficient therapy one day. Likewise, it is imperative to continue the development of structural techniques to expand our understanding of the mechanisms underlying nature.