Mechanical and structural studies of internal lipid-containing bacteriophage PRD1

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“I see now that the circumstances of one's birth are irrelevant. It is what you do with the gift of life that determines who you are.”

— **Takeshi Shudo**

RESUMEN

La protección del genoma viral durante el viaje extracelular es un requisito absoluto para la supervivencia y replicación del virus. Una vez que la partícula viral se une a la célula huésped, puede comenzar la eyección del material genómico. Además de las cápsides proteicas casi universales, ciertos virus poseen una capa de membrana que encierra su genoma de ADN bicatenario (ds) dentro de la cubierta proteica. Aunque las propiedades mecánicas de otros virus se han estudiado anteriormente, esta tesis investiga un virus con una membrana bajo su cápside proteica. Usando el virus enterobacterial PRD1 como prototipo de virus sin cola que contienen membrana, y una combinación de ensayos de nanoindentación mediante la microscopía de fuerza atómica y modelado de elementos finitos, mostramos que la arquitectura jerárquica de PRD1 (cubierta de proteína, vesícula proteolipidica, ds ADN) proporciona una mayor estabilidad contra el estrés mecánico que la lograda por otros virus icosaédricos ds ADN que carecen de una membrana. La combinación de un caparazón proteínico rígido y quebradizo junto con una vesícula de membrana blanda y dócil produce un nanomaterial compuesto resistente adecuado para proteger el ADN viral durante el transporte extracelular. Además, observamos la estructura y las propiedades mecánicas que implican el ensamblaje del tubo de eyección de ADN de dicho virus. Si bien los estudios han resuelto la estructura de varios virus en tubos, aquí investigamos un fago que forma el tubo de eyección de ADN al unirse. Empleamos la reconstrucción con crio-microscopía electrónica (cryo-EM) para mostrar que esta partícula viral está produciendo un tubo estructurado, no helicoidal, con una simetría axial de siete veces, que se forma utilizando la membrana proteolípica. En combinación con ensayos de nanoindentación de microscopía de fuerza atómica (AFM), visualizamos partículas individuales que producen tubos y mostramos que el tubo de PRD1 tiene una rigidez comparable a estructuras tubulares similares, como túbulos lipídicos gruesos o virus del mosaico del tabaco (TMV). Hay sugerencias sobre la capacidad del tubo de autocurarse, cuando se libera presión mecánica externa después de la compresión más allá del punto de

fluencia. Estos resultados proporcionan la primera comprensión de la relación entre la estructura, las propiedades mecánicas y la función de los virus que contienen membrana. Podrían beneficiar a los nanoingenieros que podrían incorporar el diseño compuesto de PRD1 en su búsqueda de nanopartículas más estables, pero también la búsqueda de nuevas soluciones farmacéuticas que se ocupen de la resistencia antibacteriana y las infecciones bacterianas en general, ya que este nanotubo proteolipídico puede perforar agujeros en la pared celular bacteriana.

El trabajo presentado en esta tesis implica el bacteriófago sin tubo PRD1, un virus con una membrana lipídica debajo de su capa proteica. Aquí se abordan cuestiones relacionadas con la estabilidad mecánica de la partícula, junto con las contribuciones de cada uno de sus componentes arquitectónicos. También arrojamos luz sobre el ensamblaje, la estructura y las características del tubo de eyección de ADN proteolipídico; esta información sin precedentes podría proporcionar nuevas estrategias sobre el diseño de nuevos antimicrobianos y nanopartículas.

Esta tesis tiene los siguientes objetivos generales:

• La investigación mecánica mediante microscopía de fuerza atómica (AFM) del bacteriófago PRD1 que contiene lípidos mediante el análisis de cada componente arquitectónico utilizando partículas derivadas de PRD1.

• La investigación mecánica y estructural del tubo de eyección de ADN de PRD1, utilizado para la infección de células bacterianas, mediante microscopía crioelectrónica (cryo-EM) y AFM.

La tesis se estructura de la siguiente manera:

El Capítulo 1 es el capítulo introductorio y cubre una descripción general de virus y bacteriófagos, con un enfoque específico en PRD1; así como una descripción general de las dos principales técnicas utilizadas durante el lapso de la tesis: AFM y cryo-EM.

El Capítulo 2 cubre los objetivos de este estudio.

El Capítulo 3 describe la investigación nanomecánica de los componentes arquitectónicos individuales de PRD1, y dilucida cómo estos contribuyen a la rigidez y la estabilidad mecánica del virión PRD1.

El Capítulo 4 estudia la investigación estructural y nanomecánica del tubo de eyección de la cola de PRD1. El Capítulo 5 es el capítulo final que fusiona las principales conclusiones de cada capítulo y brinda una breve perspectiva de lo que esta investigación puede proporcionar a los futuros investigadores.

El Capítulo 6 contiene la bibliografía para la tesis completa.

El apéndice tiene una lista de todas las abreviaturas presentes en este trabajo, así como una colección de artículos publicados del trabajo que se han realizado en los últimos 4 años.

CONCLUSIONES

• Los virus se pueden ver como entidades biológicas compuestas donde los ácidos nucleicos, las proteínas y los lípidos se ensamblan para producir partículas funcionales para la infección. Esta tesis ha proporcionado una primera comprensión de las propiedades nanomecánicas de los virus con una membrana interna. Se puede esperar que se convierta en una referencia para el trabajo futuro con esta clase de virus.

• Las propiedades nanomecánicas de PRD1 muestran que el genoma presurizado proporciona rigidez, pero no mejora la estabilidad, ya que los mutantes sin genoma ceden bajo la misma fuerza, incluso si son menos rígidos.

• Las cápsidas de proteínas Penton-less rinden 3 veces antes que los mutantes libres de genoma, pero que contienen vesículas.

• La vesícula proteolípica es de un orden de magnitud menos rígida que cualquier partícula PRD1 investigada, pero se comporta de manera similar a otras vesículas lipídicas.

• Proponemos que, como en un material sándwich compuesto, una matriz proteínica / polipéptido interfacial en PRD1 genera una conexión estrecha que acopla mecánicamente la cápside y la membrana. Será interesante ver si el principio de diseño de mejorar la estabilidad

mecánica mediante la formación de una doble capa compuesta de cápside-membrana es exclusivo de PRD1 o generalmente empleado para esta clase de virus.

• El tubo de eyección de ADN de PRD1 está compuesto de material proteolípido. La polimerización de la proteína formadora de tubos conduce a una serie de discos apilados, cada uno compuesto de siete subunidades, y que tiene una simetría de siete veces, pero no una helicoidal.

• Estudios previos de mutación del fago PRD1, junto con nuestros resultados actuales, sugieren que la proteína transmembrana P32 es la proteína polimerizante.

• Este enriquecimiento de proteínas durante la remodelación de la membrana conduce a una bicapa de lípidos más delgada y colapsada durante la polimerización del tubo.

• Las características nanomecánicas del tubo, además de la presencia de una estructura definida, insinúan la presencia de material proteico, ya que exhibe un carácter más rígido en comparación con la vesícula lipídica sola.

PERSPECTIVAS

Cuando respondimos algunas preguntas sobre la estructura mecánica y el ensamblaje de la cápside y el tubo de PRD1, también surgen preguntas adicionales.

1. Se debe realizar un trabajo final sobre la estructura del tubo.

2. Queda por validar si otros virus que contienen membrana muestran un comportamiento similar al que muestra PRD1. Esto podría generalizar nuestro modelo propuesto de que la arquitectura multicapa proporciona una mayor estabilidad de partículas.

3. La investigación de mutante P32, que producen tubos más cortos proporcionaría una mayor comprensión en el montaje del nanotubo.

En una perspectiva amplia, nuestros resultados pueden ayudar a los nanoingenieros en su búsqueda de nanopartículas más estables, ya que la naturaleza compuesta de PRD1 puede ser una inspiración para el diseño de nanopartículas. Además, la búsqueda de nuevos antimicrobianos podría ser asistida por el uso de máquinas de nanodrilling, como el tubo de eyección del bacteriófago PRD1.

ABSTRACT

Protection of the viral genome during extracellular travel is an absolute requirement for virus survival and replication. Once the viral particle attaches to the host cell, the ejection of the genomic material can begin. In addition to the almost universal proteinaceous capsids, certain viruses possess a membrane layer that encloses their double-stranded (ds) DNA genome within the protein shell. While the mechanical properties of other viruses have been studied before, this thesis investigates a virus with a membrane under its protein capsid. Using enterobacterial virus PRD1 as a prototype of tail-less, membrane-containing viruses, and a combination of nanoindentation assays by atomic force microscopy and finite element modelling we show that the hierarchical architecture of PRD1 (protein shell, proteo-lipidic vesicle, dsDNA) provides greater stability against mechanical stress than achieved by other dsDNA icosahedral viruses that lack a membrane. The combination of a stiff and brittle proteinaceous shell coupled with a soft and compliant membrane vesicle yields a tough composite nanomaterial well-suited to protect the viral DNA during extracellular transport. Furthermore, we look at the structure and mechanical properties involving the assembly of the DNA ejection tube of such a virus. While studies have solved the structure of various tailed viruses, here we investigate a phage that forms the DNA ejection tube upon attachment. We employed cryo electron microscopy (cryo-EM) reconstruction to show that this viral particle is producing a structured, non-helical tube, with a seven-fold axial symmetry, that is formed using the proteo-lipidic membrane. In combination with atomic force microscopy (AFM) nanoindentation assays, we visualize individual tube producing particles, and showed that the PRD1's tube has a stiffness comparable to similar tubular structures, such as thick lipid tubules, or tubular-shaped tobacco mosaic virus (TMV). Hints regarding the tube's ability to self-heal are present, when external mechanical pressure is released after compression beyond the yield point. These results provide the first insight into the relationship between structure, mechanical properties and function of membrane-containing viruses. They could benefit nanoengineers that could incorporate the

composite design of PRD1 in their quest for more stable nanoparticles, but also the quest for novel pharmaceutical solutions that deal with anti-bacterial resistance, and bacterial infections in general, as this proteo-lipidic nanotube can drill holes into the bacterial envelope.

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AIMS AND OUTLINE OF THE THESIS

The work presented in this thesis involves tail-less bacteriophage PRD1, a virus with a lipid membrane under its proteinaceous shell. Questions regarding the mechanical stability of the particle, along with the contributions of each of its architectural constituents are being addressed here. We also shed light on the assembly, structure, and characteristics of the proteo-lipidic DNA ejection tube; this unprecedented information could provide new strategies insights into the design of new antimicrobials, and nanoparticles.

This thesis has the following ***general aims*** and *i*s structured as follows:

- The mechanical investigation via atomic force microscopy (AFM) of lipid-containing bacteriophage PRD1 by analysis of each architectural component using PRD1-derived particles.
- The mechanical and structural investigation of PRD1's DNA ejection tube, used for bacterial cell infection, by cryo-electron microscopy (cryo-EM) and AFM.

Chapter 1 is the introductory chapter and covers a general overview of viruses and bacteriophages, with a specific focus on PRD1; as well as an overview on the two major techniques used during the span of the thesis: AFM and cryo-EM.

Chapter 2 covers the objectives of this study.

Chapter 3 describes the nanomechanical investigation of the individual architectural constituents of PRD1, and elucidates how these contribute to the stiffness and mechanical stability of the PRD1 virion.

Chapter 4 studies the structural and nanomechanical investigation of PRD1's ejection tail tube.

Chapter 5 is the concluding chapter which merges the main conclusions from each chapter and provides brief perspectives on what this investigation can provide to future researchers.

Chapter 6 contains the bibliography for the full thesis.

Appendix has a list of all abbreviations present in this work, as well a collection of published articles from work that has been performed that past 4 years.

Chapter 1: Introduction

1. INTRODUCTION

1.1. VIRUSES

Viruses are found infecting all living organisms, ranging from bacteria, yeast, plants to animals. The life cycle of a virus is relatively simple: the only mission in its life is to replicate itself. Viruses cannot do this on their own, as compared to organisms; they need the aid of the host-cells. Viruses use different strategies to shuttle their genome into the cell, where thousands of copies are synthesized for the new virus progeny that finally leave the host cell ready to infect other cells. The infection usually leads to an inflammatory response by the host-cell with consequent infection signs, and possibly to cell apoptosis.

Viruses are also masterpieces of nano-bioengineering. They use their shells (a protein capsid, or a lipid envelope fenestrated by glycoproteins in the case of enveloped viruses) to protect the genome from being damaged during their extracellular travel. For some viruses, their proteinaceous capsids reveal minimalistic and highly symmetric architecture. Icosahedral particles with a 5-, 3-, and 2-fold rotational symmetry allow placement of 60 identical units with equivalent contacts between them. Despite this simplicity, this design allows for genome protection, viral infection and replication. The shell must fulfil “contradictory” roles: it shall be strong and flexible enough against internal and/or external pressure, mechanical deformation and certain environmental changes, such as temperature and pH. As an example of the mechanical strain the capsid suffers, the internal genome pressure of bacteriophages and many other double-stranded (ds) DNA viruses reaches tens of atmospheres: so that their genome can be ejected into the cell after attaching to the cell surface; yet the pressure has to be sustained by the shells before infection (Ivanovska et al., 2004, Santos-Perez et al., 2017). These properties, as well as their bio-compatible nature, render these structures efficient nano-containers for potential biomedical uses.

In addition to the structural studies, which reveal the molecular organization of viruses, nanomechanical studies of viruses have recently gained much attention. Such mechanical

studies can clarify the organization of the viral shells and how the molecular structure relates to mechanical properties, their responses to environmental changes (pH, temperature, osmotic effects), and the correlation between the viral mechanics and viral functions in their life cycle. Viruses during their evolution, have developed different techniques for efficiently delivering their genomic material in the infected cell. The most studied and best described systems are those devised by the tailed dsDNA bacteriophages, which generally package their genome at very high densities within a rigid proteinaceous capsid shell. The elastic, electrostatic, entropic, osmotic, and hydration energetic costs incurred by constraining the dsDNA at very high densities within the fixed volume of these phage capsids generate a significant internal pressure (Kindt et al., 2001, Ghosal, 2012, Purohit et al., 2005, Panja and Molineux, 2010, Evilevitch et al., 2003, Gelbart and Knobler, 2009). This pressure is thought to provide the initial force driving genome ejection into the cell, while DNA-binding proteins, DNA condensation or enzymes may help during the final stages of the transfer (Molineux, 2001, Jeembaeva et al., 2010, Inamdar et al., 2006, Purohit et al., 2005, Zandi et al., 2003).

A common trait for all tailed dsDNA phages, e.g. *Myoviridae*, *Podoviridae*, and *Siphoviridae*, is that their proteinaceous DNA injecting machineries are always assembled or completed prior to the time of infection. Likewise, for this group the DNA exits the capsid through the tail. For members of the *Myoviridae* family, energy stored in the conformation of the proteins of their long contractile phage tails, is critical for the penetration of the outer membrane and the peptidoglycan cell wall, and thus to reach the inner cell membrane (Leiman et al., 2004). For bacteriophages with non-contractile tails, neither the short (*Podoviridae* family, Figure 1.1A, left) nor the long non-contractile tail (*Siphoviridae* family, Figure 1.1A, right) are known to provide energy for the infection process.

In contrast to the wealth of experimental and theoretical information that has been acquired about the genome entry of tailed dsDNA bacteriophages (Figure 1.1A), very little is known

about this process for viruses with an internal membrane vesicle such as bacteriophage PRD1 (Figure 1.1B).

Investigating the structural and material properties of proteins, complexes, and viruses, via different methodologies, we can explain the correlation between the molecular structure and its biological function. As such, structural virology can provide high-resolution molecular structures (Rossmann, 2013): capsid-protein arrangements such as inter-subunit and intra-subunit organization, as well as genome–capsid interactions that are established during or after the assembly process are revealed. Meanwhile, multidisciplinary studies via mechanical virology, can quantify capsid mechanical and material properties and investigate their relation to structural changes and the virus self-assembly process (Roos et al., 2010, Mateu, 2012).

These two physical virology branches, together with biological, biochemical and chemical approaches, create a deeper understanding of how viral proteins and capsomers self-assemble into highly ordered shells, and how conformational changes inside the capsid relate to genome packaging or release (Mateu, 2012). These key insights not only elucidate the biology of viruses pivotal for harnessing viral pathologies, but also allow to manipulate viruses and to create virus-like particles with numerous applications in nanomedicine and nanotechnology (Singh et al., 2006, Wen et al., 2013).

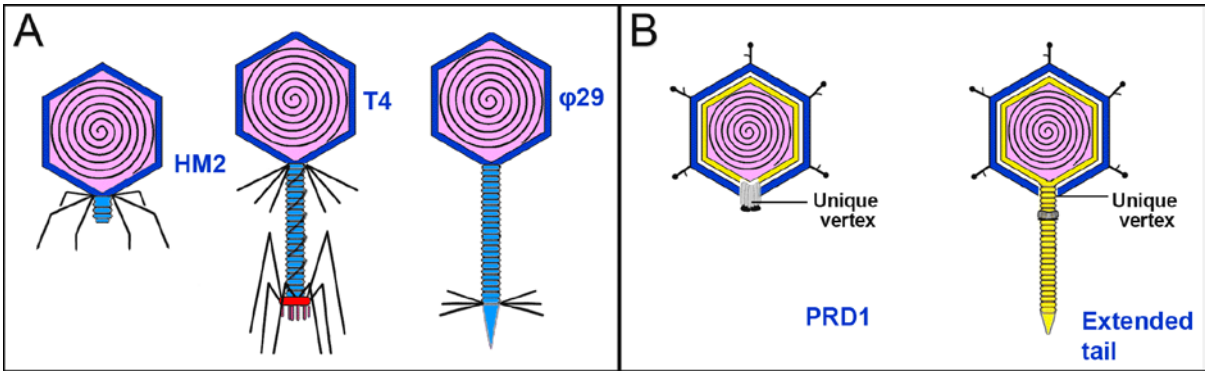

Figure 1.1: Morphology comparison between (A) various tailed phage types (podovirus HM2, myovirus T4, syphovirus φ29) and (B) a tail-less phage with an internal vesicle that forms a tube upon infection (PRD1).

This thesis targets bacteriophage PRD1, a prototype for membrane-containing viruses (Abrescia et al., 2004, Bamford and Mindich, 1982, Bamford and Bamford, 1990, Butcher et al., 2012, Cockburn et al., 2004, Grahn et al., 2002b, Hong et al., 2014, Huiskonen et al., 2007, Martin et al., 2001, Mindich et al., 1982, San Martin et al., 2002, Santos-Perez et al., 2017, Stromsten et al., 2003). The thesis investigates the mechanical properties of PRD1 and provides novel insights into such a type of viral particles. Then, as PRD1 and other recently discovered bacteriophages infect the host-cell via the formation of a nanotube (Scholl, 2017, Nilsson, 2014, Parisien et al., 2008, Qadir, 2015, Waters et al., 2017), we determined the structure and mechanical properties of the PRD1 DNA ejection tube by high-resolution (HR) cryo-EM and AFM.

In conclusion, this thesis is the result of an integrative structural approach that finally allows (*i*) to understand the nanomechanical properties of membrane-containing viruses and (*ii*) to propose a mechanism of the tube self-assembly and correlate its mechanical properties with its DNA shuttling function at infection.

1.1.1. Bacteriophage PRD1

PRD1 is a tailless, icosahedral virus that contains an internal lipid membrane underneath its protein capsid. The virus is roughly 65 nm in diameter, and extensive efforts have been made into solving the atomic structure of this system (Abrescia et al., 2004, Cockburn et al., 2004, Huiskonen et al., 2007, Mindich et al., 1982, Bamford and Mindich, 1982, San Martin et al., 2002). It is a member of the *Tectiviridae* family which typically infects gram-negative or gram-positive bacteria, with their spike-decorated capsid. The virion contains about 18 distinct protein species, and is roughly 66 MDa in mass (Figure 1.2). Within 60 minutes between infection and cell lysis the virion is able to replicate itself (Figure 1.3). The linear dsDNA genome and covalently attached terminal proteins are delivered, via formation of a proteo-lipidic tube, into the cell where replication occurs via a protein-primed mechanism.

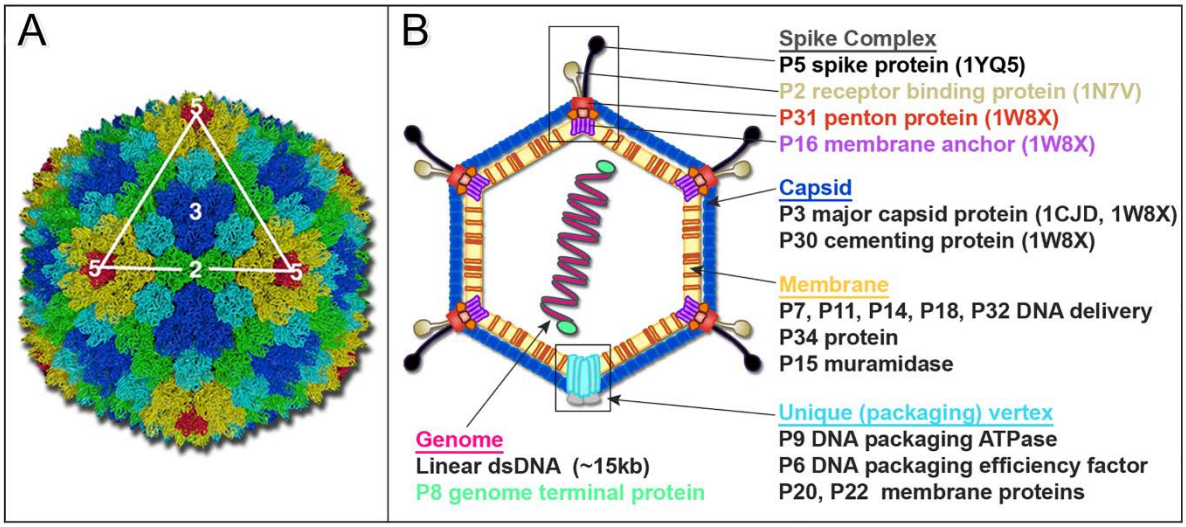

Figure 1.2: (A) PRD1 capsid organization represented as in (Abrescia et al., 2004) with each of the 4 P3 MCPs trimers composing the asymmetric unit coloured in green, cyan, blue, and yellow; in red is the vertex penton protein P31. The white triangle marks a virus facet, and 2, 3, and 5, respectively, the 2-, 3-, and 5-fold icosahedral symmetry axes. (B) Schematic presentation of wt PRD1 architecture according to the current knowledge (numbers in parentheses identify the corresponding protein structures in the Protein Data Bank).

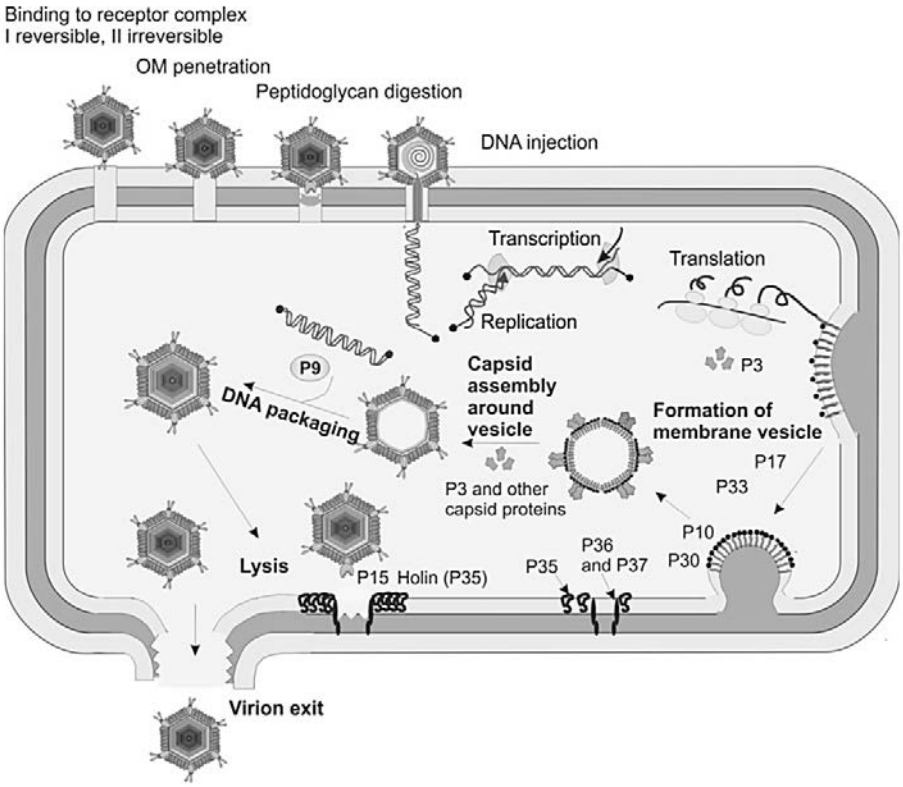

Figure 1.3: Life-cycle of bacteriophage PRD1. Adapted from (Butcher et al., 2012).

Little is known regarding the structural role of the inner-membrane in the assembly of PRD1. While there is evidence to support that it serves as an assembly platform, by close interaction with the dsDNA and the major capsid protein (MCP) P3 (Cockburn et al., 2004, San Martin et al., 2002), in this thesis we investigate whether the membrane vesicle also offers a mechanical advantage, *e.g.* a mechanical contribution to the stability of the virion. Furthermore, the membrane is used during the formation of the ejection tail tube, independently of the presence or absence of the packaged DNA (Peralta et al., 2013, Santos-Perez et al., 2017). It is proposed that the deflation of the internal vesicle, as well as loss of unique-vertex proteins can initiate the self-assembly of the tube. In **Chapter 3** of this study, we investigate both structurally, and mechanically, the mechanism behind the formation of the PRD1 DNA ejection tube.

1.2. METHODS TO STUDY VIRUS MECHANICAL PROPERTIES

There are multiple ways to study the mechanics of viruses and nanomaterials. One example is the osmotic shock method, by which bacteriophages were first incubated in high salt solutions and quickly diluted. Upon dilution, phages with capsids which are more permeable to water than to metal ions will burst. The T2, T4 and T6 phages were found to be easier to break, whereas T1, T3, T5 and T7 phages remained intact when exposed to the same treatment (Roos et al., 2007), revealing that osmotic shock method applies pressure isotropically. The aforementioned method was performed on samples with a large number of particles and provided information on the collective behaviour of virions, but the individual mechanical information is lost.

Other mechanical methods used for single particle analysis are optical tweezers. This single-molecule manipulation studies have a wide range of applications (Ashkin and Dziedzic, 1987, Heller et al., 2014, Moffitt et al., 2008, Neuman and Block, 2004, Wuite et al., 2000), and work by “trapping” the biomolecules via optically trapped microbeads that act as handles. In a study by (Moffitt et al., 2009), one bead bound to a DNA molecule and another bead to a

bacteriophage Φ29 prohead were trapped by two laser beams. By approaching the beads, the packing motion and force of DNA by the viral motor was recorded by the optical tweezers. The motor can work against a force of up to 57 pN on average, with a step size of 10 bp, which is one of the strongest molecular motors reported (Smith et al., 2001). One of the drawbacks, however, of this technique is the limited resolution of the optical microscopy, making optical trapping difficult, even when the forces and displacements can be deduced from the light diffracted by the trapped beads (Gittes and Schmidt, 1998).

Around the same period that optical tweezers were evolving, atomic force microscopy jumped from providing topographical and mechanical information of large conductive material, to providing a direct way to image and probe biological material, like individual virus particles (and other biological macromolecules), as further described in detail in the following section.

1.2.1. Introduction to atomic force microscopy (AFM)

The AFM was first described in 1986 (Binnig et al., 1986). The AFM is based on a previous technique, called scanning probe microscopy, which originally allowed imaging of solid surfaces with subnanometer resolution, and the measurement of forces as low as a few tens of pN. This ability to investigate surfaces at nanometre resolution triggered the development of a range of AFM-related techniques, which used a variety of probes to locally sense interactions and manipulate matter (Binnig and Rohrer, 1999). Imaging viruses (or any other microscopic or nanoscopic object) by AFM is often compared to the procedure a blind person may use to feel the shape of an object, by touching it with his/her fingertips. The ability to “see” depends on many factors, including proper immobilization of the sample, the sample properties (softness, adhesiveness, fragility), and the sensitivity and properties of the instrument itself. The atomic force microscope works under the following principle: a nanometer-sharp AFM tip is suspended over a sample. The primary mode of collecting topographic data was using “contact mode” by which the selected probe would scan the sample surface very closely. By

collecting the reflection of a focused laser beam, we can "feel" our sample, and by measuring the deflection of said laser, we can reconstruct a 3D topographic image of our sample (Figure 1.4A).

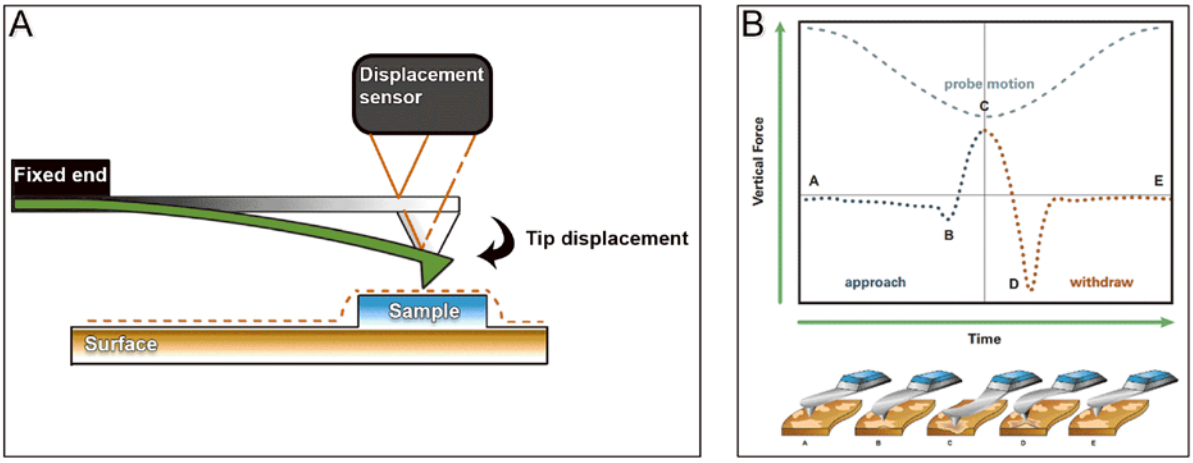

*Figure 1.4: (A) Diagram of the basic AFM components and working principle. Whilst scanning a defined area of our sample, the tip (green) displacement can be measured within nanometer precision, allowing for reconstruction of sample topography (red dashed line) based on a combination of tip and sample properties and interactions. (B) Knowing the material properties of our tip, we can also extract the mechanical and/or electrochemical information of a sample. In Peakforce tapping, the oscillation of the cantilever (with the tip) is driven by a sinusoidal wave and a force curve is simultaneously collected and displayed here as force versus time graph. The idealized sample would exhibit the indicated characteristics when approaching the surface (*A-B*), during compression/force loading* (B-C*), unloading (*C-D*), and retracting from the sample (*D-E*). Deviations from the ideal curve reveal properties of the investigated sample. Image adapted from (Kaemmer, 2011).*

1.2.2. The development of complementary imaging modes

Likewise, AFM can be used in probing the mechanical features of viruses (or any other object) since we can fine-tune force parameters and probing materials. In one particular imaging technique called "Peakforce tapping" (JPK Instruments manual. 2011. Nanowizard 4 – the next benchmark for BioAFM, Berlin), "Jumping mode" or "Quantitative imaging", force curves are collected across each pixel (Kaemmer, 2011, De Pablo et al., 1998). In Peakforce tapping, technique used in this thesis, the tip moves laterally at a constant rate irrespective of the tip being in contact with the sample or not. Then the tip moves laterally when it is not in contact with the sample. The unique versatility of AFM to image, probe and manipulate materials makes it attractive to use in nanoscience and nanotechnology. Furthermore, the possibility to

operate in liquid environments and at ambient temperature moved AFM towards biology, and led to the analysis of biomolecules and cells at (sub-) nanometre resolution. To address the wide complexity of biological systems, which can range from nucleic acids and proteins to cells and tissues, a variety of AFM modes have been developed over the years (Figure 1.5) to avoid issues with "contact mode" (CM). One of the main issues with CM are the occurrence of shear forces. These forces are present due to the friction between AFM tip and sample, potentially deforming or modifying the sample surface. Additionally, the AFM tip can be modified by adhering particulates, or damaged by these interactions, therefore affecting the measurements. Thus, new ways to minimize imaging forces were investigated, in particular when imaging soft biological material in liquid medium.

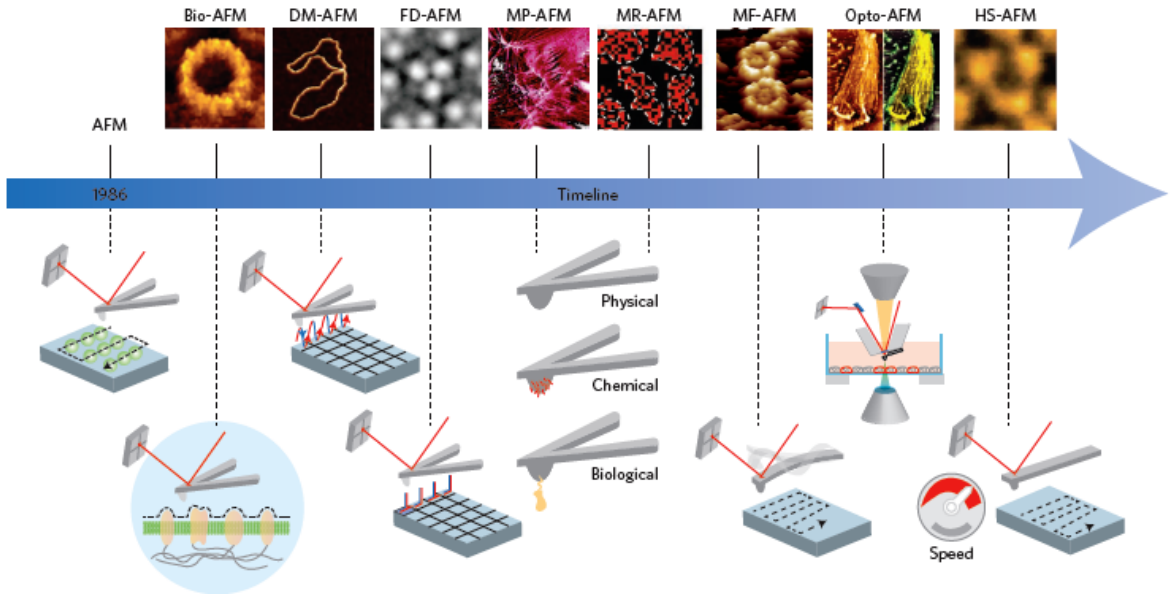

Figure 1.5: Timeline image of major developments in AFM modes for imaging biological material since the development of contact mode AFM in 1986. These include: bio-AFM that allowed operation in aqueous solutions; dynamic mode (DM-AFM) which allowed tip oscillation, reducing friction effects on the sample; force-distance AFM (FD-AFM) which records a pixel-by-pixel full distance curve while providing with a surface contour; up to high-speed AFM (HS-AFM) which increased imaging acquisition times up to a factor of 1000, allowing for investigation of dynamic biological processes. Other imaging modes include multiparametric AFM (MP-AFM), which contours the sample while mapping multiple physical or chemical properties; molecular recognition AFM (MR-AFM), which images and maps specific interactions of biological samples; multifrequency AFM (MF-AFM), which contours the sample while oscillating the cantilever tip at multiple frequencies, thus mapping various physical parameters; correlating advanced optical imaging and AFM (Opto-AFM) for the imaging of complex biological systems (Image from Dufrêne et al, 2017).

Major advances in high-resolution imaging have also been achieved in complementary methods, including super-resolution optical microscopy and cryo-electron microscopy, which enrich the imaging toolbox now available to molecular and cell biologists. DNA helices, proteins, and capsomer organization on the surface of the virus can be visualized by AFM, as was shown on brome mosaic virus crystals, mimivirus, and minute virus of mice among numerous others, including work presented in this thesis (Figure 1.6) (Carrasco et al., 2006, Kuznetsov et al., 2010, Lucas et al., 2001).

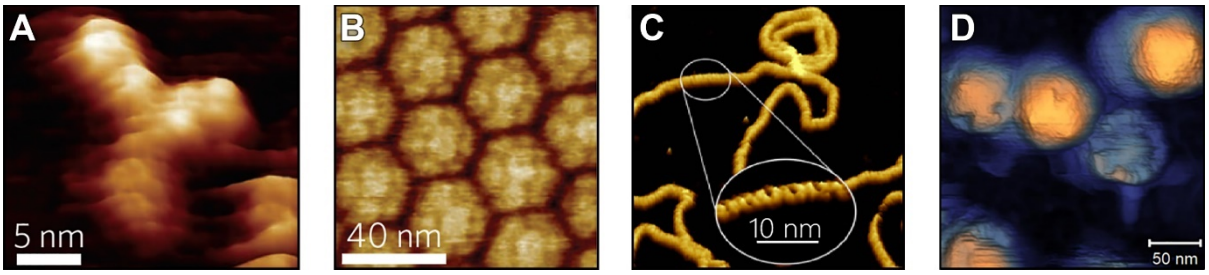

Figure 1.6: AFM images showcasing the resolution thresholds of the technique. (A) An IgG antibody visualized via FM-AFM in aqueous solution adsorbed on a mica surface. Image from (Ido et al., 2014). (B) Capsomer organization on individual brome mosaic viruses (BMV) visualized by tapping mode on BMV crystal. Image from (Lucas et al., 2001). (C) AFM topography of plasmid DNA showing major and minor grooves, obtained via PeakForce tapping. Image from (Pyne et al., 2014). (D) wt PRD1 particles showing protein organization on full and damaged particles, missing peripentonal vertices among other features (as discussed later in this thesis).

1.2.3. AFM nano-manipulation

AFM is not only capable of imaging, but also of nano-manipulating viruses. The first viral mechanical investigation with AFM appeared in 1997, when Falvo *et al.* manipulated tomato mosaic virus (TMV), a virus that is ~300 nm in length with an AFM (Falvo et al., 1997). The TMV was rotated, moved and dissected by an AFM tip. A few years later, the mechanical properties of viruses were determined by performing force nanoindentation on the bacteriophage Φ29 using an AFM tip (Ivanovska et al., 2004). By nanoindentation on virus particles, several direct mechanical properties can be extracted: (a) stiffness, which examines the elastic deformability of a particle; (b) yield force point, which examines the forces required to mechanically destroy or damage a particle; (c) resistance to mechanical fatigue, which

examines the durability of a particle when subjected to continuous forces. Since then, the method has been applied to study different viruses, including herpes simplex virus (HSV), various bacteriophages, cowpea chlorotic mottle virus, influenza virus, Norovirus, and human immunodeficiency virus, among others (Roos et al., 2009, Kol et al., 2007, Cuellar et al., 2010, Eghiaian et al., 2009, Schaap et al., 2012, Roos et al., 2012, Ivanovska et al., 2004, Carrasco et al., 2011, Michel et al., 2006). The stiffness can be directly measured from nanoindentation force curves, and represents the property of the virus particle as a whole. The majority of viruses, enveloped or not, were found to be as stiff as 0.2-1 N/m (Mateu, 2012). It is determined by the properties of the various shells (geometry and mechanical properties) from which the virus is made. Using appropriate models (analytical or finite element) and structural information, it is possible to determine the mechanical properties of individual shells (e.g. the Young's modulus of the proteinaceous capsid). The Young's moduli of most virus capsids were found to be around 1 GPa (Mateu, 2012), comparable to that of polypropylene (soft plastic material), or about 14 times less compared to that of a bone (Rho et al., 1993).

1.2.4. Understanding mechanical properties of nano-materials

AFM studies on viral mechanics help to investigate and understand the material properties of the viral particles and to unravel how it achieves the protection of the viral genome against mechanical stress. For example, one the first of such studies measured the distribution of the elastic constant over a bacteriophage Φ29 capsid. This was correlated with the protein distribution on the surface, and by intentionally rupturing the capsid, the limits of the genome protection were determined (Ivanovska et al., 2004). In other studies, the viral stiffness was measured at different physiochemical conditions correlated to viral activities. For example, stiffnening of HIV when it develops from the immature to the mature state was related to a major conformational change (the thinning of the inner protein shell) (Kol et al., 2007). Following a similar approach, we can deconstruct the mechanical contributions of the various

viral components (such as capsid, internal vesicle, and genome) to the mechanical stability of the PRD1 particle.

1.2.5. Introduction to transmission electron microscopy (TEM)

More than 100,000 proteins deposited in the Protein Data Bank have been solved using X-ray crystallography, a technique that allowed solving the atomic structure of many small and large molecules, including the atomic structure of the B-DNA back in 1953 (Watson and Crick, 1953). However, its versatility is limited by sample preparation methods and requirements, as crystals of the target nanomolecule are then blasted with X-rays and the diffraction data allow for the 3D structure determination.

Recent developments in transmission electron microscopy (TEM) has opened the way for the investigation of more complicated and biologically challenging systems that are impossible to tackle using crystallography [Figure 1.7, (Callaway, 2015, Blow, 2002)].

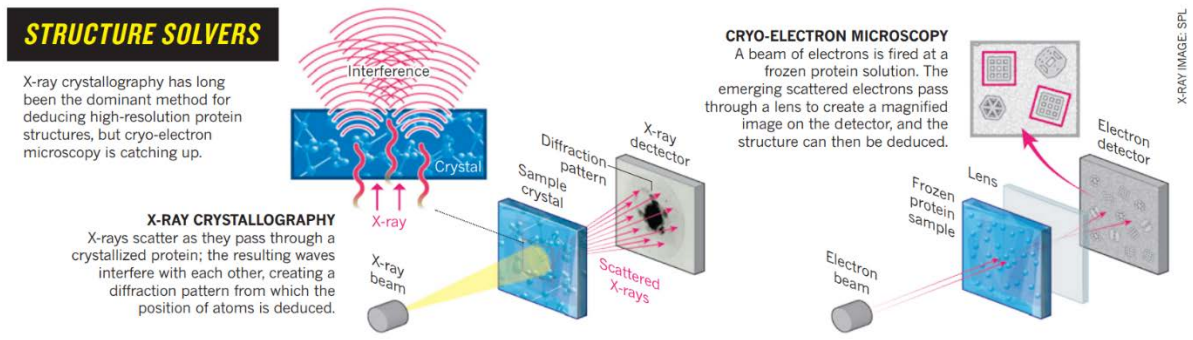

Figure 1.7: Comparison of the two major techniques used for structure solving of proteins, viruses, and other biological molecules. Image taken from Callaway, 2015.

Transmission electron microscopes were developed in the 1930's, as a consequence of the limitations that light microscopy (LM) posed. In fact, the resolution of LM has been limited by the wavelength of the light being used to form the image. Conventional light microscopes are unable to achieve better than 200 nm resolution or about half the wavelength of visible light, although in the past ten years, advances in super-resolution LM have changed this paradigm.

TEM can achieve superior resolution, due to the smaller wavelength of the electron relative to the photons from visible light. 3D structure reconstruction of viruses by TEM started via simple negative staining imaging, a technique that uses a heavy atom solution (uranyl acetate) to stain the sample (Crowther et al., 1970). At the time, resolution was limited by sample preparation methodology, radiation damage, and optical distortions. On of the cryo-EM revolution milestones was when flash-freezing the samples was introduced, enabling "close-to-native" imaging of viruses back in 1984 (Adrian et al., 1984). Since then, by implementation of automation systems (Gatan Cryoplunge, FEI Vitrobot), that helped improve the sample preparation methodology, as well as the more recent development of direct-detection cameras that allowed fast and highly-sensitive data collection, there are plenty more viruses and biological molecules structures determined using this technique.

Briefly, in cryo-EM, specimens are preserved in thin layers of vitrified ice, which eliminates the need for negative staining, reduces radiation damage and allows them to be imaged much closer to their native state, albeit at very low contrast. However, biological samples vitrified without any staining have very little contrast and can be difficult to see. Additionally, high energy electron beam can damage samples frozen in vitreous ice and result in more pronounced beam induced sample drifting (Henderson, 2015). Thus, although the 3D structure of biological macromolecules can now be solved at high resolution (3-4 Å) by cryo-EM, the low-dose data collection procedure always requires thousands of images with randomly oriented two-dimensional (2D) projections of the target molecule to be collected. Nowadays, state-of-the-art microscopes with autoloader and advanced software allow to perform data collection in automatic mode.

Advances in vitrification, in detection cameras, and implementation of Bayesian-Dyer algorithms have led to the so-called "cryo-EM resolution revolution" which has finally being recognised with the Nobel prize in Chemistry in 2017 to Richard Anderson, Joaquin Frank and Francois Dubuchet (Cressey and Callaway, 2017).

1.2.6. cryo-EM: brief overview

After sample preparation and data collection, individually selected particles are used to create a 3D structure of the desired molecule, derived from randomly oriented 2D particle projections. The single particle reconstruction is achieved by taking images of several thousand particles. Ideally, these particles will be random in orientation and the data set will contain two dimensional projections of every view of the sample. Using the central projection theorem, which states that, for a 3D object, the Fourier transform of each 2D projection is a central slice through the 3D Fourier transform of the object. The relative orientation is assigned (Euler angles) across all prior projections of the object and, by direct Fourier inversion, the 3D volume is reconstructed (Milne et al., 2013).

The Fourier transforms of the two-dimensional particles are combined computationally in three-dimensional Fourier space. The combined Fourier transform data is then inversely Fourier transformed back into real space in three dimensions to produce an electron density map of the target particles that was imaged. In general, the workflow for single particle cryo electron microscopy is as follows: sample optimization and freezing, data collection, motion correction, CTF correction, two-dimensional class averaging, three-dimensional class averaging, refinement, postprocessing of the final electron density map, and building an atomic model and model refinement [Figure 1.8, (Doerr, 2016)].

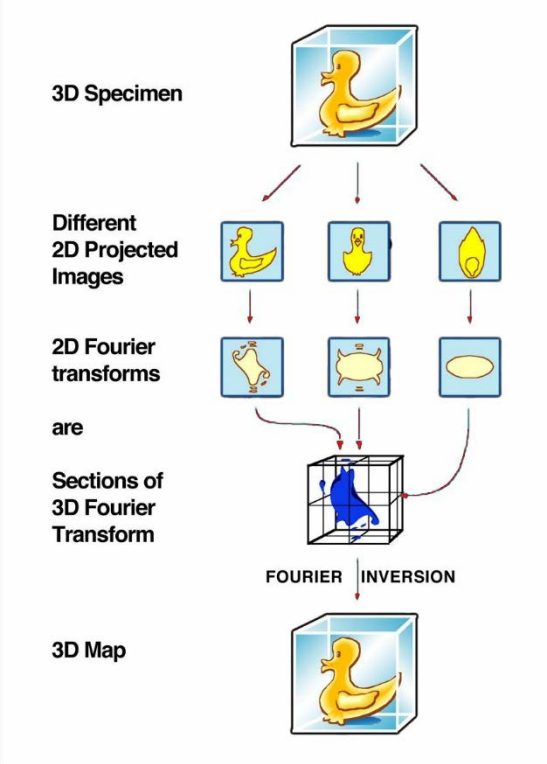

Figure 1.8: Fourier duck represents the principles of 3D structure reconstruction by Fourier inversion. The schematic illustrates that projection images of the object, each with a different orientation, have 2D Fourier transforms that correspond to sections (indicated by red arrows) through the 3D Fourier transform of the original object. Thus, once the 3D Fourier transform is built up from a collection of 2D images spanning a complete range of orientations, Fourier inversion enables recovery of the 3D structure. Adapted from (Milne et al., 2013).

1.2.7. Single-particle reconstruction methods

The most commonly used cryo-EM technique for the 3D structure determination of macromolecules, is the single-particle method. In this technique, data from a large number of 2D projection images, featuring identical copies of a protein or of a viral particle in random orientations, are processed to generate a 3D reconstruction of the macromolecule. Starting with images of fields containing many molecular complexes, individual particles are selected by hand or by automated algorithms. Once selected, a number of computer programs such as EMAN (Ludtke et al., 1999), SPIDER (Shaikh et al., 2008) and recently RELION (He and Scheres, 2017), which stood out by introducing the Bayesian maximum likelihood algorithm can be used for image processing. The degree of relatedness, whether using cross-correlation or likelihood criteria, between individual “particle” images is used to identify clusters of

similar images within the data set. Related images are then averaged to obtain characteristic projection views of the complex at much higher signal-to-noise ratios than in the original images. Iteration of this classification process, using characteristic views of the newly generated class averages as alignment references, improves the accuracy of alignment and permits visualization of finer structural features.

Statistical methods, such as principal component analysis, multivariate analysis or covariance analysis, can be used to sort images based on variations in their structural features. There are two main routes to determine the 3D structure of the target macromolecule:

1) "*Ab initio"* reconstruction assumes no prior knowledge of the structure and by applying the central projection theorem, the initial 3D structure is reconstructed.

2) Projection matching technique is used when a 3D model of a similar molecule or structure already exists (or the initial model from *ab initio* reconstruction can be used). By comparison with the 2D re-projections of the initial model, Euler angles are assigned to the raw 2D images. The raw data, with the assigned Euler angles, are then back projected to generate the 3D structure.

When X-ray atomic models are available for some or all of the sub-components of the complex, they can be placed or fitted into the density map to provide pseudo-atomic models, considerably extending the information obtained by electron microscopy (Orlova and Saibil, 2011). However, nowadays the achieved high-resolution cryo-EM maps allow modelling from scratch, of the corresponding atomic models (eg. by using COOT) and their structure refinement via Phenix software and/or Rosetta (Emsley et al., 2010, DiMaio et al., 2013).

1.2.8. Single-particle icosahedral reconstruction methods

Icosahedral viruses have been used in the past to push the limits of cryo-EM (Jiang et al., 2008). Two factors account for this:

(1) The presence of high symmetry (eg. 60-fold) confers a powerful advantage for averaging the icosahedral asymmetric unit within each virion, and

(2) The large size of the virions (tens of megadaltons in most instances) provides good contrast, in principle, for accurately determining particle orientations in projection images.

The ordered packing arrangement also lowers conformational heterogeneity of viral protein components. Icosahedral EM processing uses the same work-flow as single-particle methodology, but it imposes the icosahedral symmetry during classification and 3D particles refinement (Scheres, 2016). Because of the above characteristics, structures of numerous icosahedral viruses have been determined to resolutions better than 4 Å (Grigorieff and Harrison, 2011). An important step in the field was when the cryo-EM structure of adenovirus was solved at 3.8 Å, matching the simultaneously solved X-ray resolution (Reddy et al., 2010, Liu et al., 2010).

1.2.9. Helical reconstruction methods

Helical reconstruction has been used to determine the 3D structure of a variety of bacterial and archaeal pili, bacteriophage tubes among other systems (Craig et al., 2006, Trachtenberg et al., 2005, Leiman et al., 2010, Kahn et al., 1991). The ability of cryo-EM to deterimene helical structures was proven when the tube of the T4 bacteriophage (Leiman et al., 2010, Leiman et al., 2004) was solved. The overall workflow is similar to that of single particle reconstruction, with the additional and important step of determining the helical parameters (pitch, rise, number of subunits). These parameters are usually extracted by calculating the Fourier transform of the best 2D class averages (red star on Figure 1.9). Briefly, after obtaining the desired 2D class, the fourier transform is calculated, leading to an optical diffraction pattern. The spots and layer lines of this pattern can be interpreted in terms of a Bessel function.

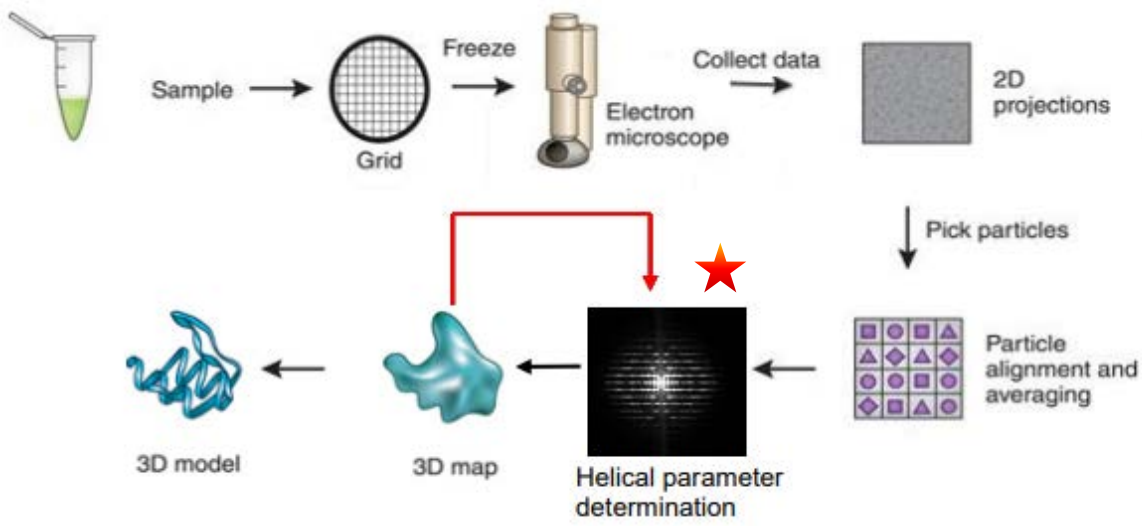

Figure 1.9: An overview of the workflow for helical data processing. While it is very similar to single particle reconstruction workflow, there is an extra and crucial helical parameter determination step (red arrow). Figure adapted from (Doerr, 2016).

A Bessel function is a cylindrical harmonic function where the Bessel order (n) is an integer value. When the solutions to a cylindrical harmonic function are plotted in three dimensions, the result is a wave. The arrangement of subunits in a helical polymer can be described by a series of characteristic cylindrical harmonic functions which are unique to that tube or complex. This allows extraction of the preliminary helical parameters that can be applied to the first round of processing and 3D reconstruction.

Molecular complexes with helical symmetry are a unique challenge for cryo-EM reconstructions. We can reconstruct correctly the corresponding 3D structure by imposing the existence of a helical symmetry via the assignment of estimated rise and pitch values during 3D model refinement (Egelman, 2000). There is usually a limited amount of views due to the nature of the complexes; for example, top views of the complex are mostly missing. Yet applying incorrect helical parameters to the reconstruction will result in model bias, just like imposing an erroneous symmetry for single particle reconstruction, and an incorrect structure. Software like RELION recently implemented calculations that allow embedding helical symmetry operators in Fourier-space, allowing for helical structure 3D reconstructions (He and Scheres, 2017).

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Chapter 2: Objectives

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2. OBJECTIVES

The objectives of this doctoral research are:

1. **Investigation of the role of the architectural constituents in the mechanical stability of PRD1 via atomic force microscopy, to address the following questions:**

   a. What is the role of the outer protein capsid?

   b. Does the role of the inner proteo-lipidic membrane contribute to the stability?

   c. Does the dsDNA have a role in particle stability?

   d. How the conjunction of these constituents contributes to the overall virus stability and life-cycle?

2. **Investigation of PRD1's proteo-lipidic tube via atomic force microscopy and cryo-electron microscopy, to address the following questions:**

   a. Is the PRD1's tail tube structured?

   b. What are the mechanical properties of PRD1's tube?

   c. What does the combination of this information reveal about the assembly of the tube?

Chapter 3: PRD1 virion exhibits mechanics of a composite material for genome protection

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Chapter 4: Insights into the mechanics and structure of the PRD1 DNA ejection tail tube

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Chapter 5: Conclusions and perspectives

5. CONCLUSIONS AND PERSPECTIVES

5.1. CONCLUSIONS

- Viruses can be seen as composite biological entities where nucleic acids, proteins and lipids assemble to produce functional particles for infection. This thesis has provided first insight into the nanomechanical properties of viruses with an internal membrane. It can be expected that it will become a reference for future work with this class of viruses.
- PRD1 nanomechanical properties show that the pressurised genome provides stiffness, but does not enhance stability, as genome-free particles yield under the same force, even if they are less stiff.
- Particles containing the lipid vesicle yield 3 times later than the penton-less protein capsid particles.
- Proteo-lipidic vesicle is orders of magnitude less stiff than any PRD1 particle investigated, but behaves similarly to other lipid vesicles.
- We propose that, as in a composite sandwich material, an interfacial protein/polypeptide matrix in PRD1 generates a tight connection that mechanically couples capsid and membrane. It will be interesting to see, if the design principle of enhancing mechanical stability by forming of a composite membrane-capsid double shell is unique to PRD1 or generally employed for this class of viruses.
- The DNA ejection tube of PRD1 is composed of proteo-lipidic material. The polymerization of the tube-forming protein leads to a series of stacked disks, each composed of seven subunits, and having a seven-fold symmetry, but not a helical one.
- Previous PRD1 phage mutation studies, along with our current results, suggest that transmembrane protein P32 is the polymerizing protein.
- This protein enrichment during membrane remodelling leads to a thinner, collapsed lipid bilayer during the polymerization of the tube.

- The nanomechanical characteristics of the tube, in addition to the presence of a defined structure, hint on the presence of protein material, as it exhibits a stiffer character compared to the lipid vesicle alone.

5.2. PERSPECTIVES

As we answered some questions regarding the mechanical structure and assembly of the PRD1 capsid and tube, further questions also arise.

1. Concluding work on the tube structure needs to be performed.
2. It remains to validate whether other membrane-containing viruses display a similar behaviour as shown by PRD1. This could generalize our proposed model that the multi-layered architecture provides greater particle stability.
3. Investigating P32 mutants, which produce shorter tubes would provide further insight in the assembly of the nanotube.

In a broad perspective, our results can help nanoengineers in their quest for more stable nanoparticles, as the composite nature of PRD1 can be an inspiration for nanoparticle design. Furthermore, the quest for new antimicrobials, could be assisted by the use of nanodrilling machines, such as bacteriophage PRD1's ejection tube.

Chapter 6: Bibliography

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Appendix

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7. APPENDIX

7.1. ABBREVIATIONS

(**ds) DNA**: Double-stranded desoxyribonucleic acid, 2
ACN: Acetonitrile, 39
AFM: Atomic force microscopy, 2, 5, 7, 9, 15, 18, 20, 21, 22, 23, 24, 34, 37, 40, 41, 42, 45, 46, 53, 62, 64, 72, 73, 84, 85, 86, 89, 91, 96
AMP: Antimicrobial peptides, 64
APTES: 3-(2,2-aminoethylamino)-ethylaminopropyltrimethoxysilane, 40, 72, 74
BCWH: bacterial cell wall hydrolases, 64
CCMV: Cowpea chlorotic mottle virus, 24
cryo-EM: cryo-electron microscopy, 2, 7, 15, 18, 26, 27, 30, 31, 35, 62, 64, 65, 74, 84, 103
CTF: Contrast transfer function , 28, 70
DM-AFM: Dynamic mode AFM, 22
DNA: desoxyribonucleic acid, 2, 5, 7, 12, 13, 15, 17, 19, 23, 25, 34, 35, 42, 46, 48, 53, 56, 58, 61, 62, 63, 74, 94, 100, 101, 102, 103, 104, 105, 106
FD-AFM: Force-distance AFM, 22
GON: Group of nine, 34, 35, 55
HIV: Human immunodeficiency virus, 24, 25
HR: High resolution, 15
HS-AFM: High-speed AFM, 22
HSV: Herpes simplex virus, 24, 57, 58, 103
LB: Luria broth, 37
LC-MS/MS: Liquid chromatography coupled to mass spectrometry, 39
MCP: Major capsid protein, 17, 57
MF-AFM: Multifrequency AFM, 22
MP-AFM: Multiparametric AFM, 22
MR-AFM: Molecular recognition AFM, 22
MVM: Minute virus of mice, 23
Opto-AFM: Advance optical imaging AFM, 23
RNA: Ribonucleic acid, 34, 58
SDS: Sodium dodecyl sulfate, 37, 38
TFA: trifluoroacetic acid, 39
TMV: Tomato mosaic virus, 3, 23, 89

7.2. ARTICLES PUBLISHED IN THE DURATION OF THIS THESIS

7.2.1. Articles which are directly related with this thesis work

AZINAS, S., BANO, F., TORCA, I. BAMFORD, D.H., SCHWARTZ, G.A., ESNAOLA, J.A., OKSANEN, H.M., RICHTER, R.P. & ABRESCIA, N.G.A. 2018. PRD1 virion exhibits mechanics of a composite material for genome protection. *Nanoscale 10*, 7769-7779

7.2.2. Other articles

POLITAKOS, N., AZINAS, S. & MOYA, S.E., 2016. Responsive copolymer brushes of Poly [(2-(Methacryloyloxy) Ethyl) Trimethylammonium Chloride](PMETAC) and Poly (1H, 1H, 2H, 2H-Perfluorodecyl acrylate)(PPFDA) to modulate surface wetting properties. *Macromolecular Rapid Communications*, *37*(7), pp.662-667.

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