The Assembly and Confinement Properties of the Cowpea Chlorotic Mottle Virus

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1.1 General introduction

When discussing virus particles, generally their infectious properties are the first thing that come to mind. This is no wonder, since their general purpose is to invade other organisms and reproduce at their hosts’ expense. Viruses have a huge impact on human health, considering, for example, the devastating effects of the Ebola epidemic in 2014, the continuous struggle against HIV and its related disease AIDS, and the periodic recurrence of the flu epidemic. For this reason, virus-related research was for many years primarily focused on treating or, preferably, preventing viral infection.

However, over the years scientists have come to view viruses in a different perspective. Their highly-defined, symmetrical architecture, their monodispersity in shape and size, and the possibilities for chemical or genetic modification surpass any synthetic nano-sized structure and make them ideal for applications in various fields. Furthermore, with the huge number of viruses found on Earth, there are many shapes and sizes to choose from. Making use of these properties, viruses have already been applied in, electronics, materials science, catalysis, and medicine.

Both to advance the application of viruses, as well as for the prevention and treatment of virus-related diseases, it is important to understand their assembly and disassembly behavior, and the associated interactions between their subunits. For this reason, a lot of research is focused on studying the various facets that steer the structures that are formed from virus-based components. However, as viruses generally consist out of many subunits, and interactions between them strongly depend on environmental conditions, such as pH and ionic strength, these aspects are highly complex and not yet fully understood.

1.2 Aim and outline of this thesis

The aim of this thesis is to broaden our understanding of virus and virus-like particle (VLP) assembly and disassembly. Furthermore, we study the conditions in the confined space of a protein capsid, as clustering of molecules and charges may cause these to deviate from bulk conditions.

To address these matters, we study the cowpea chlorotic mottle virus (CCMV) as a model virus. CCMV is a well-studied virus, of which disassembly and reassembly, either in wild-type virus or in VLPs, can be controlled. Gaining
insight in the interactions that control the formation of CCMV-based structures, may help to better understand viruses in general. Moreover, the techniques we introduce here to study these aspects may be applied to other viruses as well, which would allow for comparing virus’ properties over a broad range of species.

Chapter 2 of this thesis gives a literature overview concerning the structure of viruses, and their assembly into native or non-native structures. The role of both protein-protein and protein-cargo interactions are discussed, including theoretical study of these aspects.

Chapter 3 introduces microscale thermophoresis to study capsid protein self-assembly. We use this technique to study and compare the pH-induced assembly of three types of CCMV-based capsid proteins at various ionic strengths.

In chapter 4, we apply isothermal titration calorimetry to study capsid protein-cargo interactions and VLP assembly, to obtain information on the thermodynamics that are involved in virus assembly. We extensively study polystyrene sulfonate-templated VLP assembly and make a comparison with single-stranded DNA-templated assembly.

Chapter 5 deals with the assembly of CCMV capsid protein around oligonucleotides. Here, we mix CCMV capsid protein with single-stranded DNA with varying number of nucleotides, ranging between 10 and 40, and study the assembly product. In this way, we determine a minimum number of nucleotides, and thus number of negative charges, required for VLP assembly under the applied conditions.

Chapter 6 describes a study of the physical conditions, specifically the acidity, inside a CCMV-based capsid. Using a pH-responsive fluorescent probe, we measure the pH inside a VLP and compare this to the pH of the VLP’s environment. Using a theoretical model, we are able to explain the observed differences.

In chapter 7, we develop new ways to assemble and functionalize CCMV-based capsids. We apply nickel-polyethylenimine complexes as a template for the assembly of hexahistidine-modified capsid protein. Furthermore, we use the same histidine-nickel interaction, in combination with nitrilotriacetic acid moieties, to functionalize VLPs. Lastly, we introduce a kinetic labelling technique to covalently modify the N-terminus of CCMV coat protein.
Finally, Chapter 8 shows the work that was done towards performing polymerization reactions inside a CCMV-based capsid. Here, we develop a polystyrene sulfonate-based macroinitiator for atom transfer radical polymerization reactions and use these to template VLP assembly, aiming to initiate polymerizations only inside the capsid.

1.3 References


Chapter 2

The structure and assembly of viruses and virus-like particles

The research field of physical virology is dedicated to study the physical properties of viruses and virus-like particles (VLPs), in an attempt to gain insight in reproduction, self-assembly, genome packaging and release, and structure of virus(-based) materials. Such research broadens our understanding of virus particles and may aid in the development of treatments for virus-related diseases but also in the development of virus-based materials. This chapter gives an overview of the current knowledge of the structure and assembly of viruses and VLPs.

Part of this chapter is published as:
2.1 Introduction

Over the years, scientists have used proteins for applications other than their natural purpose. This often involves modifying the protein structure, to apply them as, for example, sensors or structural materials. Furthermore, proteins have been combined with a variety of other materials to obtain hybrid structures that have interesting properties, often incorporating characteristics of both building blocks in the same material. While much research in this field focuses on the use of single proteins, protein cages like viruses and virus-like particles (VLPs) offer extra possibilities. Viruses are nanometer-sized parasitic species, which rely on their host’s biochemical machinery for replication. They are well-defined structures that occur in different shapes and sizes depending on the virus species and are highly symmetrical and monodisperse. Furthermore, many viruses possess a natural self-assembly behavior which allows for the encapsulation a variety of materials. Moreover, the proteins of these particles can be chemically and genetically modified giving them new, unique properties. As estimates suggest that millions of different viruses can be found on Earth, scientists have a huge pool of structures to choose from. Figure 2.1 shows the structure of a number of different viruses, with their dimensions and triangulation number (T, see section 2.2.3).
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Icosahedral viruses

Satellite Panicum Mosaic Virus (SPMV)
17 nm (T = 1)

Alfalfa Mosaic Virus (AMV)
22 nm (T = 1)

Bacteriophage QB
25 nm (T = 3)

Cowpea Mosaic Virus (CPMV)
27 nm (T = 3)

Cowpea Chlorotic Mottle Virus (CCMV)
28 nm (T = 3)

Brome Mosaic Virus (BMV)
30 nm (T = 3)

Hepatitis B Virus (HBV)
36 nm (T = 4)

Polyoma Virus
55 nm (T = 7)

Bacteriophage P22
64 nm (T = 7)

Rod-like viruses

Tobacco Mosaic Virus (TMV)
18 x 300 nm

Bacteriophage M13
6.6 x 880 nm

Figure 2.1: An overview of the structure of various viruses. Structures of the icosahedral viruses are reproduced from the Viper database (http://viperdb.scripps.edu/) and the protein database (https://www.wwpdb.org/), PDB ID’s: SPMV (1STM), AMV (1XOK), Qβ (1QBE), CPMV (1NY7), CCMV (1CWP), BMV (1JS9), HBV (3J2V), polyoma virus (5FUA), and P22 (2XYY). TMV is adapted with permission from Schlick et al., copyright © 2005 American Chemical Society. M13 is adapted from Yoo et al., copyright © 2014 Yoo et al. (open access).

A vast amount of research has been conducted to gain insight in the structure and assembly of viruses, with the aim of better understanding their infectious pathway, in an attempt to treat or prevent viral infection, or to use viruses and their components for non-natural applications. This chapter discusses the current knowledge on virus(-like) particle structure and assembly, including protein-protein and protein-cargo based assembly, to provide a theoretical background.
for Chapters 3, 4, 5, and 6. Furthermore, virus hybrid materials, with a focus on virus-polymer hybrids, are discussed as an introduction to Chapters 7 and 8.

2.2 Viral structure

In the simplest form viruses consist out of nucleic acids – the viral genome that can either be RNA or DNA depending on the type of virus – surrounded by a number of copies of a single type of protein, the capsid protein (CP). Besides these simple viruses, much more complex species can also be found. An example of more complex structures are the so-called enveloped viruses, whose protein capsid are surrounded by a lipid membrane. This difference in external surface leads to a difference in their infection pathway. Interestingly, despite their differences in outer surface, the core of both enveloped and non-enveloped viruses is often similar, consisting of nucleic acids and a protein capsid. For physical virology purposes commonly non-enveloped viruses are studied, therefore the remainder of the work described here focuses on these.

Due to viruses’ nanoscale size, the viral genome has a limited length which constrains the complexity of the proteins that it encodes for. The nucleic acid is too short to contain information for a single protein that, by itself, can form the entire capsid. This constraint is the reason that virus structures to consist of a single type, or only a few types, of identical proteins. Watson and Crick assumed that the identical subunits have similar interactions with each other, which causes the structure to be highly repetitive and symmetrical. To build a structure out of identical subunits with similar environments, mathematically this structure can only be either rod-like or spherical.

2.2.1 Rod-like viruses

One way to arrange identical protein subunits into a rod-like structure, is by following a helical arrangement. Work on rod-like viruses has been ongoing for a long time, and already in 1955 it was recognized by Fraenkel-Conrat et al. that in these types of virus structures, the nucleic acids are surrounded by the capsid proteins in a helical fashion. In principle, any length of rod can be formed in such a way, depending on the number of subunits. In spite of the simplicity of the helical architecture, only 10% of the viral families known today are rod-like. This is mainly due to the unfavorable surface-to-volume ratio and the reduced structural stability of long, thin structures. Well-known examples of rod-like
viruses are the tobacco mosaic virus (TMV), the Ebola virus, and the rabies virus. Although the structure of these viruses is similar, the morphology can be very different from one another. For example, TMV has a rigid rod-like structure, while Ebola has a flexible structure.

### 2.2.2 Spherical viruses

The second way to arrange identical subunits in identical environments is by placing them on the facets of polyhedrons, leading to roughly spherical structures. Theoretically only five structures – the Platonic polyhedra – allow such a symmetrical packing: the tetrahedron, the cube, the octahedron, the dodecahedron, and the icosahedron (Figure 2.2).

![Figure 2.2: a) The Platonic polyhedra: 1. the tetrahedron (4 identical triangular faces), 2. the octahedron (8 identical triangular faces), 3. the cube (6 identical square faces), 4. the dodecahedron (12 identical pentameric faces), and 5. the icosahedron (20 identical triangular faces). b) The symmetry axes of an icosahedron: 1. fivefold axis, 2. threefold axis, and 3. twofold axis. Adapted from Horne,14 Copyright © 1974 with permission from Elsevier.](image-url)
Subunits forming these structures can be placed at equivalent positions on the faces of the polyhedra, with three, four, and five equivalent positions for triangular, square, and pentameric faces, respectively. This yields structures consisting out of 12 (tetrahedron), 24 (octahedron and cube), or 60 (octahedron and icosahedron) subunits. The latter two are most efficient by enclosing the largest space with only a single repeating unit, however due to the preferred bending at the fivefold symmetry axis over the threefold symmetry axis (Figure 2.2b) most spherical viruses display icosahedral symmetry.\textsuperscript{8}

### 2.2.3 Quasi-equivalence

Using strictly equivalent subunits in icosahedral symmetry limits the structure to 60 subunits. This means that the structure size is limited and requires an increase in monomer size to form larger capsids. Viruses with a capsid consisting out of 60 proteins do exist, for example the plant satellite virus\textsuperscript{15} and the hepatitis E virus\textsuperscript{16}, however many viruses have capsids containing more than 60 proteins. These viruses rely on the quasi-equivalence theory, as described by Caspar and Klug in 1962,\textsuperscript{17} in which identical monomers adopt slightly different conformations to account for slight differences in their local environment to form an icosahedral symmetric structure.\textsuperscript{8, 17-19} According to the theory, the planes of the icosahedral particles can be subdivided in quasi-equivalent triangles. This process, called triangulation, leads to icosahedral particles that are built up out of $T \times 60$ monomers. The triangulation (T) number is defined as $T = h^2 + hk + k^2$, in which $h$ and $k$ are non-negative integers or zero. Viruses with various T numbers are shown in Figure 2.1.

Although impossible according to this theory, viruses and virus-like particles (VLPs) consisting of 120 subunits have been observed and are referred to as pseudo $T = 2$ particles. The occurrence of such structures can be explained by considering them a $T = 1$ particle built from dimers, or by allowing a small conformational distortion.\textsuperscript{20, 21}

### 2.2.4 Cowpea Chlorotic Mottle Virus

Due to their infectious properties, viruses are commonly associated with all types of diseases, such as AIDS, Ebola, or the common flu. However, more and more research is conducted towards using viruses, or their components, to form new
The structure and assembly of viruses and virus-like particles

materials with applications in, for example, the fields of medicine, catalysis, or nanotechnology.

In these studies, often model viruses are used. A commonly studied model virus is the cowpea chlorotic mottle virus (CCMV), a virus that infects the cowpea plant. CCMV is a positive sense single-stranded RNA (ssRNA) bromovirus of the Bromovidae family. The virus is built up from 180 capsid proteins (CPs, ~20 kDa) forming a T = 3 icosahedral protein shell encapsulating four different stands of ssRNA into 3 different virion structures. ssRNA1, with a length of 3,171 nucleotides (nt) and ssRNA2 (2,774 nt) are encapsulated on their own, while ssRNA3 (2,173 nt) is encapsulated together with the non-infective ssRNA4 (824 nt), leading to loading of ~3000 nt per capsid. Interestingly, both ssRNA1 and ssRNA2 are required for infection, the addition of ssRNA3 aids in the infection efficiency.

The capsid of wild-type (WT) CCMV particle, i.e. including viral RNA, has an outer diameter of 28 nm and an inner volume of $5.5 \times 10^3$ nm$^3$. The coat proteins are assembled in 12 pentameric faces, showing fivefold axis of symmetry, and 20 hexameric faces, forming a capsid that is stable at pH 3 - 6 and low ionic strength (~0.1 M). Upon raising the pH above 6.5 the outer shell swells up to 10% along the quasi-threefold symmetry axis at pH 7.0. This swelling is caused by deprotonation of carboxylic acid groups at the threefold symmetry axis, causing deformation due to repulsion. This effect can be prevented by the addition of divalent metal ions, such as Ca$^{2+}$ and Mg$^{2+}$, which bind to carboxylic acids. The swelling induces the formation of 60 pores of about 2 nm, allowing transport of larger molecules between the virus cavity and the medium surrounding the virus (Figure 2.3).
Figure 2.3: Cryo TEM and image reconstruction of CCMV at a) unswollen state (pH < 6.5, in the presence of metal ions) and b) in the swollen state (pH > 6.5, no metal ions present). Reprinted from Liu et al.,47 Copyright © 2003, with permission from Elsevier.

This swelling under influence of pH and ionic strength was also confirmed by Comellas Aragonès et al. using small-angle neutron scattering (SANS). This technique also revealed that the RNA is bound close to the protein shell.50

Since 1967 it has been known that the virus can be disassembled into viral RNA and 90 CP dimers by increasing the pH to 7.5 and increasing the ionic strength (I > 0.5 M).44, 51-53 It was shown that this disassembly is reversible, reforming infectious particles from the isolated components.53 After removal of the viral RNA, it is possible to reassemble the CP dimers into VLPs. Two different strategies can be used to this end, both allowing encapsulation of non-natural cargo (Figure 2.4).
The structure and assembly of viruses and virus-like particles

**Figure 2.4:** Schematic representation of CP isolation from WT-CCMV and the formation of VLPs from the isolated CPs. 1. WT-CCMV at pH 5 and I ≤ 0.1 M; 2. Raising the pH to 7.5 and I ≥ 0.5 M causes the virus to disassemble and allows for the isolation of the CP dimers; 3. Lowering the pH to 5 at I ≥ 0.5 M causes the CPs to self-assemble into 28 nm T = 3 capsids. Cargo present in the solution is statistically encapsulated leading to 4. Empty and (partially) filled VLPs; 5 Addition of a polyanionic species can induce particle assembly at pH 7.5 and I < 0.3 M leading to 6. VLPs loaded with the polyanionic cargo.

By lowering the pH to approximately 5.0 at high ionic strength (I > 0.5 M) the CP dimers self-assemble into T = 3 capsids, allowing random encapsulation of cargo in the medium. The second approach relies on the assembly of CP dimers around polyanionic species – e.g. DNA, anionic polymers, or negatively-charged nanoparticles – in solution at pH 7.5. Depending on the cargo size T = 1 (~18 nm, 60 CPs), T = 2 (~22 nm, 120 CPs), and T = 3 (~28 nm, 180 CPs) have been formed.42, 52, 54

Besides using electrostatic or random encapsulation, other approaches for loading cargo into CCMV have been shown in literature, which often involve coupling of the cargo to the CP’s N-terminus. To this end, cargo was attached non-covalently by means of coiled-coil peptides which were connected to the CPs N-terminus and to the cargo.55, 56 Another non-covalent approach involves the interaction between Ni²⁺ and a hexa histidine group on both the CP’s N-terminus and the cargo.57 Covalent attachment of cargo to CCMV CP’s N-terminus has been used for capsid loading as well, either by means of protein engineering,56 or through a sortase A-mediated enzymatic coupling of a cargo to engineered CCMV CP.58 A disadvantage of these approaches is that they require laborious
modification of the CP through protein engineering, in order to introduce the required functionalities at the CP’s N-terminus.

## 2.3 Virus assembly: Protein-protein interactions

To form a protein capsid, the interactions between the protein subunits – *e.g.*, electrostatic, Van der Waals, and hydrogen bonding interactions – play a crucial role. Different studies suggest that capsid assembly is primarily entropy driven, involving the burying of hydrophobic patches on the protein by interacting with a neighboring protein. This is entropically favorable due to the release of water molecules from these surfaces.\(^{59-61}\) By altering the solution conditions, subunit interactions can be steered towards either free subunits, or fully formed capsids. Often assembly also requires interactions with cargo – *in vivo* this would be the viral genome – however a number of cases exist where the isolated protein subunits can reassemble into empty particles. This was shown for the first time using the CPs of CCMV by Bancroft *et al.* in 1967.\(^{62}\) It was shown that the assembly products of the isolated CPs were directed by the pH and ionic strength of the solution, yielding empty capsids or other structures such as double-shelled particles, rosette-like structures, or tubes. The assembly was further defined by Adolph and Butler, who developed a phase diagram for the assembly of CCMV CPs at various pH and ionic strength conditions.\(^{44}\) This work was further extended by Lavelle *et al.*, studying a wider range of conditions and the effect of different types of buffer.\(^{63}\) Both phase diagrams are presented in Figure 2.5.
The assembly and disassembly processes of virus capsids is, however, not as straightforward as these diagrams suggest. Studies involving assembly and disassembly of such structures by changing the pH from high to low or from low to high have shown clear hysteresis in the assembly and disassembly curves: the particles assembled at a pH ~5.5 when titration was performed from high to low, while they disassembled at pH ~6.0 when titration was performed from low to high.64, 65 Similarly, hysteresis in the disassembly was shown to occur in dilution studies, where upon dilution formed particles were stable at much lower concentration than the concentration at which assembly could occur.66

Assembly of CCMV CP into empty capsids is induced by a change in pH, however other viruses can form empty protein capsids under other conditions. For example, the core proteins of the hepatitis B virus (HBV) can spontaneously assemble when the ionic strength is raised above 0.6 M,67 the CPs of the turnip crinkle virus (TCV) can assemble into empty particles when their RNA binding domain is removed,68 and the CP of the bacteriophage P22 requires a scaffolding protein to form a capsid.69

### 2.4 Virus assembly: Protein-cargo interactions

Besides the protein-protein interactions, also the cargo strongly influences the assembly and the properties of viruses and VLPs. In wild-type viruses, this cargo
consists of the virus’ native genome, but also numerous examples of other types of cargo, such as non-native nucleic acids, synthetic polymers, proteins, and nanoparticles, can be found in literature, creating all types of hybrid materials with unique properties.

### 2.4.1 Polymer-virus hybrid materials

In recent years proteins and polymers have been combined in a variety of hybrid materials that have interesting properties, often incorporating characteristics of both building blocks in the same material. While much research in this field focuses on the use of single proteins, protein cages like viruses and virus-like particles (VLPs) offer extra possibilities. Viruses’ well-defined highly symmetrical and monodisperse structure, great variety in shape and size, (Figure 2.1) and their natural self-assembly behavior make them ideal candidates for the encapsulation a variety of materials. Furthermore, the proteins of these particles can be chemically and genetically modified giving them new, unique properties.

**Packaging of (bio)polymers**

One of the interactions stabilizing viruses are the electrostatic interactions between the negatively charged DNA or RNA and the positively charged capsid interior. In this regard, the polynucleotide can be considered as a (bio)polymer template for virus particles. Initial research focused on capsid assembly addressed the question whether virus capsid can be disassembled and reassembled from their isolated components,\textsuperscript{12, 53, 70} and whether capsids can also form with different RNA templates such as homologous RNAs and RNAs from different viruses.\textsuperscript{71-73} It was shown that the capsids of BMV and CCMV formed particles similar in size to the native viruses upon interaction with non-native RNA and did not have preferences for particular sequences. Interestingly, by mixing CPs of BMV, CCMV, and broad bean mottle virus (BBMV), VLPs with capsids containing a mix of the different CPs could be formed.\textsuperscript{74}

RNA’s ability to adopt different topologies by base pairing strongly influences its templating behavior during capsid formation and in the packaging of DNA into preformed capsids.\textsuperscript{75} An increasing number of branch points on the RNA leads to increasing packaging efficiency. This phenomenon was observed experimentally when comparing packaging efficiencies of BMV RNA and CCMV RNA in CCMV CP\textsuperscript{76} and was explained by modelling of free energies for varying RNA.\textsuperscript{77, 78} Similarly, molecular dynamic simulations by Zhang \textit{et al.} showed that
hyper-branched polyanions are more efficient at VLP formation. In contrast with these results, it was shown in a competition study between CCMV’s native ssRNA and comparable polyuracil (polyU) strand, which lacks all secondary structures, that preferentially polyU filled particles were formed. These unexpected results may be explained by a difference in assembly kinetics between the CP-ssRNA and the CP-polyU system, leading to kinetic traps. These results underline the complexity of virus assembly, and show that the process is currently not yet fully understood.

More studies focus on understanding the packaging of the RNA templates into viral capsids. Upon increasing the RNA length it was observed that larger particles were formed and also the packaging of one RNA template in multiple capsids was observed. This is likely due to multiple nucleating centers forming on the same template. This partial encapsulation of long RNA strands has been applied by Garmann et al. to develop a method for monofunctionalization of CCMV. By partially encapsulating a sufficiently long RNA strand one end extended out of the capsid which was available for functionalization. They suggest that this technique may be used for monofunctionalization of icosahedral viruses in general.

Garmann et al. also investigated the formation of VLPs around ssRNA to elucidate the roles of subunit interactions during VLP formation. In their work they vary the CP-RNA interaction by changing the ionic strength of the solution, while the CP-CP interaction was tuned by varying the pH. They suggest a two-step approach, in which first the ionic strength is lowered to induce CP-RNA interactions, and secondly the pH is lowered to induce CP-CP interactions, which gives the best yield of spherical VLPs.

The use of biopolymers as templates for CCMV capsid assembly has also been extended to include DNA, DNA-containing materials, and DNA-origami. Because of comparable charge distribution of DNA and RNA, these molecules will interact in a similar way with the positively charged protein interior. Double stranded (ds)DNA, ssDNA, and DNA micelles have been studied as templates. Depending on the rigidity of the template, viral coat proteins were assembled into tubes, in the case of dsDNA, and into spherical particles, comparable to native capsids, for ssDNA and DNA micelles. It was shown by De la Escosura et al. that by combining ssDNA with appropriate guest molecules such as naphthalene and stilbene derivatives the rigidity of the template could...
be altered, allowing for a transition of the capsid assembly from spheres to tubes. Mikkilä et al. have shown that viral coat protein can also self-assemble onto DNA origami structures. It was shown that the protein coating enhanced transfection of DNA origami structures into human cells.

In the cases discussed so far the biopolymer acts as a template for the assembly of protein cages, however the opposite case also occurs when the capsids template the structure of the genetic cargo. The packaging of genetic material of several viruses and bacteriophages occurs by translocation of the genetic material into a preformed capsid using a molecular motor. Both theoretical and experimental studies have shown that large forces are involved in the packaging of the DNA. The confinement forces the DNA into an out-of-equilibrium, glassy state and relaxation of the DNA is slowed significantly. The conformational changes of the DNA are suggested to enhance DNA release during infection.

Using a conceptually different approach neutral biopolymers have also been used as a template for capsid assembly. Elastin-like polypeptides (ELPs) were fused to the CCMV coat protein. This fusion product retained the pH responsive capsid formation of the CCMV coat protein, but capsid formation could also be triggered by a salt- and temperature-response of the ELP part (Figure 2.6). Well-defined spherical particles of different sizes were observed for the two assembly pathways. Later research showed that the addition of metal ions enhanced the stability of these particles allowing them to be used under conditions at which an enzymatic cargo was active. This opens up a new approach for the use of non-charged (bio) polymers as templates for capsid formation and allows for the formation of new responsive materials.
The structure and assembly of viruses and virus-like particles

Using the same principle as with polynucleotides, synthetic polymers such as polyanetholesulphonic acid,\textsuperscript{102} poly(styrene sulfonate) (PSS),\textsuperscript{54, 72, 103-106} and poly(acrylic acid) (PAA)\textsuperscript{105} have been incorporated in VLPs. In fact, the self-assembly of CCMV coat protein with PSS is a widely studied model for capsid assembly.\textsuperscript{54, 103-105} It has been shown that depending on the molecular weight of the PSS template spherical particles with varying sizes are formed. Sikkema \textit{et al.} showed the formation of 16 nm (T = 1) icosahedral particles when using low molecular weight PSS (average 9900 Da),\textsuperscript{54} while experiments by Hu \textit{et al.} utilizing high molecular weight polymers (400 kDa to 3.4 MDa) demonstrated the formation of 22 nm (T = 2) and 27 nm (T = 3) particles.\textsuperscript{103} It has been observed that larger polymer templates induce larger assemblies, indicating that the size of the polymer cargo is an important factor in directing the capsid size of the formed assemblies.

By fluorescent labeling of a PSS template, Cadena-Nava \textit{et al.} were able to address the question how many polymer chains are packaged inside different sized capsids.\textsuperscript{104} Their experiments demonstrated that larger capsids can accommodate more polymer chains of the same molecular weight, indicating that not only the charge ratio but also the molar ratio between template and coat proteins plays an

\textbf{Figure 2.6:} Schematic representation of the self-assembly of the CCMV CP-ELP product (upper) and TEM images of the two different assemblies (lower). Adapted with permission from Van Eldijk \textit{et al.},\textsuperscript{99} Copyright © 2012 American Chemical Society.
important role in the formation of different sizes of capsids. Theoretical studies into these observations are discussed in section 2.5.

The possibilities that controlled polymerization techniques like ATRP and RAFT offer, such as control over polymer length and polydispersity, and different ways of modifying polymers, enables the design of specific polymeric structures. This can be used to create templates to address questions about capsid assembly that remain. For instance the influence of polymer topology on the assembly, as for example observed by Setaro et al. when encapsulating various dendrimers in CCMV VLPs, could be investigated. However, no examples that exploit these possibilities in literature are known to our knowledge, except for the fluorescent labeling of the template.

By using functional polymers as template, large materials can be loaded inside virus-like particles. Polymers that have been encapsulated as functional cargo include the fluorescent poly(5-methoxy-2-(3-sulfopropoxy)-1,4-phenylenevinylene) (MPS-PPV), the redox-active polyferrocyenylsilane (PFS) and supramolecular polymers of zinc phthalocyanine (ZnPc), a photosensitizer. The effect of encapsulation on the properties of the functional material is of great interest. As with the native virus, the protein shell often provides protection to its cargo, i.e. the encapsulated polymer. For example, Brasch et al. showed that MPS-PPV inside spherical particles could not be quenched by methyl viologen present in solution. Interaction with the protein shell can interfere with the original properties as in the case of encapsulated PFS. Minten et al. observed that encapsulated PFS could only be oxidized and not reversibly reduced. Finally, the shape of the formed structures, and the conformations the polymer is able to adopt inside, have consequences for the properties of the new material when these properties are conformation-dependent. Besides spherical particles, MPS-PPV can induce the formation of tubes when in its stretched form. Ng et al. showed that both the spherical particles and the tubes, both based on the same protein and polymer, possessed different optical properties.

As polymers can act as templates for viral coat proteins, like-wise the empty virus capsid can be envisioned as a scaffold for polymer growth. By functionalization of amino acids, either naturally occurring or recombinantly introduced, with a suitable initiating group polymerization can be induced. Using this approach, Abedin et al. constructed a branched polymeric networks inside the small heat
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shock protein, a protein cage of approximately 12 nm, via stepwise growth employing the Cu(I)-catalyzed azide-alkyne cycloaddition.\textsuperscript{112} Also, the P22 capsid, a 60 nm $T = 7$ protein cage, was changed in to a macroinitiator for ATRP in this manner and linear polymers and networks of cross-linked polymer of 2-aminoethyl acrylate (AEMA) were polymerized in its interior (Figure 2.7). Lucon \textit{et al.} showed that the polymers can be modified with functional molecules, yielding a MRI contrast agent (using Gd-diethylenetriamine pentaacacetate) or a photocatalytic active particle (using $[\text{Ru}(5$-methacrylamido-phenanthroline)]\textsuperscript{2+}).\textsuperscript{113, 114}

\textbf{Figure 2.7:} Representation of the confined ATRP polymerization inside the P22 capsid and subsequent labeling with a dye or Gd-DTPA complex. Reprinted from Lucon \textit{et al.},\textsuperscript{113} with permission from Springer Nature, Copyright © 2012.

Enzymatic polymerization of 3,3-diaminobenzidine (DAB) by an engineered ascorbate peroxidase APEX2 inside a variant of capsid-forming enzyme lumazine synthase, AaLS, was shown by Frey \textit{et al.}, resulting in Poly(DAB)-capsid nanoparticles.\textsuperscript{115} Hovlid \textit{et al.} performed an experiment in which 2-dimethylaminoethyl methacrylate (DMAEMA) was polymerized inside the 25 nm $T = 3$ bacteriophage Q$\beta$ VLPs.\textsuperscript{116} Furthermore, cellular uptake of these VLPs was studied, with and without modification of the outer surface, and showed greater internalization for cationic polymer-filled VLPs compared to similar VLPs lacking this polymer cargo. These results show the potential of the polymer-protein hybrids for biomaterial and biomedical applications.
Some capsids contain natural occurring motifs to anchor the necessary moieties for polymerization to the interior without the need for chemical functionalization. For example, apo-ferritin, a protein cage of approximately 12 nm, possesses metal-binding sites which can be used for other metals than iron. Abe et al. introduced rhodium(II)-catalysts for the polymerization of phenyl acetylene at the interior of apo-ferritin and subsequently used the inorganic-virus hybrids for formation of poly(phenyl acetylene).117 Another example of a polymerization with the catalyst inside a protein cage was presented by Rengli et al. who performed ATRP inside the cavities of a 16 nm chaperonin by confining a copper catalyst.118 This system was shown to yield polymer chains with a very low polydispersity.

So far, in all examples of confined polymerization using protein cages it has been observed that the cage limits polymer growth, for both linear chains and branched networks.112, 113, 116-118 The confinement of the polymer growth in some cases also creates products with narrower polydispersities compared to the same molecules created in solution.117, 118 However, details of the exact mechanism for polymerization and the influence of the confinement remain unknown. Theoretical simulations of for example catalytic reaction sites provide more details,119 yet experimental data investigating these mechanisms further are currently not available.

The capsid shell itself allows for certain selectivity in monomers for the confined polymerization. Monomers must pass through the pores of the protein shell, restricting the size of the molecules. When a high concentration of charged groups is present at the pore interior, selection may occur based on charge. Indeed, Abe et al. demonstrated positively charged phenyl acetylene derivatives could not be polymerized inside rhodium-containing apo-ferritin.117

From a materials perspective, the confined polymers inside capsids offer possible advantages for the introduction of functionality. This was demonstrated by Lucon et al. by the insertion of metal complexes to branched networks inside the small heat shock protein.120 When the confined polymers possess free moieties, these are amenable for post-polymerization modification. It has been shown that in this manner a variety of small molecules, such as fluorescent dyes and imaging agents, can be incorporated with a dramatically increased loading compared to functionalization of interior amino acids only.113, 114, 116
Exterior modification

The surface functionalization of viruses and VLPs with polymers has mainly been focused on the development of hybrid materials for biomedical applications. To this end, poly(ethylene glycol) (PEG) and oligo(ethylene glycol) methacrylate (OEGMA) functionalized with carbohydrates have been attached to different viruses via the grafting-to approach employing standard bio-ligation techniques, such as oxime ligation, \(^3\)\(^{,}1^{2}_{1}\) activated esters, \(^{10}_{6},1^{2}_{2}-1^{2}_{4}\) thiol-maleimide couplings, \(^{12}_{5}\) and the Cu(I)-catalyzed azide-alkyne cycloaddition. \(^{12}_{6}-1^{2}_{8}\) PEG is biocompatible, soluble in aqueous solutions and, most importantly, it reduces the immunogenic response. Indeed, reduced immunogenic response has been observed for PEG-covered virus-like particles compared to normal viruses. \(^{12}_{2}-1^{2}_{5},1^{2}_{9}\) For biomedical applications, addition of other surface functionalities, such as cell-targeting moieties for cell specific uptake, to these particles may improve their properties as well. Functional groups can be introduced at the end of polymer chains attached to the capsid surface or to functional monomer side groups prior to attachment to the capsid. In this manner fluorescent dyes \(^{12}_{2}\) and carbohydrates for tumor cell targeting \(^{12}_{6}\) have been introduced. Additionally, the number of attached polymer chains can be decreased, leaving non-functionalized amino acids for modification with other molecules. However, it should be noted that in this approach the effective shielding of the PEG chains will be lowered, altering the immunogenic response to these particles, depending on polymer length and conformation. \(^{12}_{3},1^{2}_{9}\)

To a lesser extend the grafting-from approach has been explored for the creation of virus-like particles with biomedical applications. Hu et al. coupled an ATRP initiator to a horse spleen ferritin protein cage, and polymerized both 2-methacryloyloxyethyl phosphorylcholine and PEG methacrylate onto the surface. \(^{13}_{0}\) Pokorski et al. modified the surface of the Qβ capsid with initiating groups for ATRP and used this macroinitiator for the polymerization of OEGMAs with and without pendant azide-moieties. \(^{13}_{1}\) The great advantage of these virus-like particles is that they can act as a scaffold for many different functionalities by simply changing the molecules that can be attached to the monomer units.

Attachment of polymer chains on the surface of a protein cage can induce the dissociation of the protein shell as was observed by Comellas Aragonès et al. in the case of the CCMV virus. \(^{10}_{6}\) However, the PEG-functionalized protein subunits
could be reassembled using PSS as a template, resulting in VLPs with polymers on the interior and the exterior (Figure 2.8).

Figure 2.8: The controlled incorporation of polymers at the surface and the interior of the CCMV capsid. Reprinted with the permission from Comellas Aragonèes et al., 106 Copyright © 2009 American Chemical Society.

One of the greatest obstacles in the development of virus-based materials is the limited compatibility of many viruses with organic solvents. However, polymer-virus hybrids are a potential solution to this problem. For both PEG-functionalized TMV121 and Cowpea Mosaic Virus (CPMV)132 their solubility in organic solvents have been studied. PEG-TMV could be transferred into chloroform and even less polar solvents or solid polystyrene.121 PEG-CPMV was freeze-dried before successful introduction into organic solvents.132 Interestingly, thermal annealing of the freeze-dried PEG-CPMV yielded a solvent-free liquid state of the polymer-virus hybrid. In all cases, the viruses remained intact. Polar organic solvents remain a problem because the viruses fall apart, likely due to hydrogen bonding between solvent and the proteins subunits, disrupting their structure. However, the compatibility of polymer-virus hybrids with organic solvents opens up new possibilities for other virus-based materials in non-aqueous conditions.

The way a polymer is attached to the virus capsid can increase the stability of the particles. Manzenrieder et al. showed that multi-point attachment of poly(oxazolines) to the Qβ capsid effectively cross-linked the particle.133 This yielded particles that were thermally stable upon heating to 100 °C. In contrast, when the polymer was attached monovalently the capsids were disassembled at these temperatures, even though the protein subunits retained their secondary
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structure. Control over the size of these polymer-virus hybrids was obtained by changing polymer length and attachment density.

As described above, polymer-virus hybrids offer a facile way to introduce different properties into virus-like particles by changing the polymer type attached to the surface or by adding functional groups to an attached polymer. For example, stimuli-responsive behavior could be inferred by a responsive polymer.

Higher order assemblies

Assembly of individual virus(-like) particles into larger, multi-particle, assemblies opens the way to more complex materials. For example, studies have shown that virus-polymer complexes can be used to improve gene delivery and allow for easier large-scale processing of viruses.134

Anisotropic particles, such as the TMV, can crystallize into ordered structures through depletion interactions.135-137 It was even shown that from filamentous bacteriophages M13 and fd, both having a diameter of 6.6 nm and a length of 800-900 nm, 3D structures can be formed using 3D guided extrusion.138

Virus particles that possess a negatively charged surface can complex with positively charged macromolecules, which will induce clustering. Kostiainen et al. investigated the assembly of CCMV with cationic linear polymers, dendrons, and dendrimers, and found that the branched cationic templates were more efficient in the assembly of virus-like particles, indicating the need for multivalency.139 This method can be extended to empty and loaded VLPs and several other protein cages, such as ferritin.140 The size, and the corresponding icosahedral symmetry, of CCMV-based VLPs seems to affect the organization of the formed structures when it is clustered with linear poly-A-lysine or dendritic poly(aminoamine).141 Even more control over the assembly product can be obtained by using amphiphilic polymer structures with viral capsids.142

Stimuli-responsive assemblies between virus-like particles and polymers can be made introducing responsive groups in the employed polymers. Temperature-switchable assemblies have been made by using a thermoresponsive block-copolymer. This system could reversibly be assembled and disassembled several times simply by increasing or lowering the temperature.143, 144 Furthermore, it is
possible to create assemblies with optically triggered disassembly by using dendrons with a photo-cleavable group (Figure 2.9).\textsuperscript{145}

![Figure 2.9: The assembly of CCMV with photocleavable dendrons and its optically triggered disassembly (above) and TEM images of the different stages of assembly and disassembly (below). Reprinted from Kostiainen et al.,\textsuperscript{145} with permission from Sprinter Nature, Copyright © (2010).](image)

The properties of free particles and assembled particles can differ as was shown by Kostiainen \textit{et al.}, who investigated the difference in magnetic properties of free and assembled magnetoferritin.\textsuperscript{146} Therefore, it may be interesting to study assemblies formed by co-aggregation of virus-like particles and polymers in order to form new functional materials. Co-assembly of VLPs with different cargos may yield materials with interesting optical or magnetic properties.

In the examples above both the virus-like particles and the polymeric template are hydrophilic and therefore form homogeneous assemblies. Li \textit{et al.} developed a method to assemble both spherical and rod-shaped viruses and polymers in large core-shell assemblies using an amphiphilic template, poly(4-vinylpyridine) (P4VP).\textsuperscript{147-150} Investigation of the formed particles revealed a virus shell and polymer core. Varying the mass ratio virus/polymer allows control over the size of these colloidal assemblies. Furthermore, Suthiwangcharoen \textit{et al.} reported on virus-polymer hybrid materials that could be used as nano-sized drug delivery vehicles by loading the core with a small drug molecule and placing a cell-targeting group on the virus shell.\textsuperscript{151} Inclusion of a pH-sensitive block in the polymer allows for the assemblies, which are stable at neutral pH, to disassemble at acidic pH.
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Assembly of virus-like particles into multi-layer films has been achieved by using Layer-by-Layer (LbL) assembly with polyelectrolytes. Due to the overall negative surface charge, the virus-like particles can be used as negatively charged component instead of the polyanion. Steinmetz et al. showed that the spherical viruses are readily incorporated into a multilayer system, while rod-shaped viruses assemble in an ordered manner on top. The virus-based films have mainly been developed as scaffolds for cell adhesion because of the biocompatibility due to the presence of viruses. By surface functionalization of viruses with, for example, cell-targeting peptides additional properties can easily be introduced into the thin films. However, virus-based LbL assemblies are not restricted to biological purposes, also virus-based battery anodes and porous imide films are presented in literature. Furthermore, Li et al. showed that the incorporation of CPMV into oligo(9,9'-dioctylfluorene-co-bithiophene) substrates enhanced the amplified spontaneous emission (ASE) performance of these thin films. Another method for creating virus-covered surfaces was shown by Azucena et al., who showed the growth of protein nanotubes at various surfaces using the self-assembly of TMV-derived coat proteins on immobilized RNA. This technique also allows for patterning of the surface with these nanotubes using lithography techniques.

Rod-shaped viruses like TMV and bacteriophage M13 have been able to template polymeric wires of poly(aniline) of several micrometers in length. The rod-shape viruses assemble in a head-to-tail fashion and provide a scaffold for the aniline monomer. Upon addition of an initiator, the monomer is polymerized around the virus template. Depending on the pH conditions, single wires (near neutral pH) and bundles of wires (acidic pH) could be made. Addition of PSS in the wires increased their conductivity, which makes these materials interesting for electronic materials. Rong et al. have explored the conductive properties of the virus-polymer wires combined with titanium oxide in LPG gas sensors films. TMV was also included into polyvinylalcohol (PVA) nanofibers as a universal method for including functionalities into such fibers.

2.4.2 Non-polymeric virus hybrids

Besides polymers, many other materials have been used in combination with viruses, to create new hybrid materials. Various non-polymeric virus hybrids are described in literature. One way to create such hybrids is to introduce metal ions to functionalize viral capsids. For example, binding Gd³⁺ to either natural or
synthetic binding sites on a viral capsid renders paramagnetic nanoparticles that may be used as magnetic resonance imaging (MRI) contrast agent.\textsuperscript{168, 169} Besides introducing new functional properties, metal ions can also be used for the stabilization of VLPs\textsuperscript{170} and the loading of cargo into them.\textsuperscript{57, 171}

Another interesting group of virus hybrid materials involves inorganic nanoparticles. The cavity of viruses capsid have been used to templated mineralization of various materials, often yielding inorganic nanoparticles with well-defined dimensions.\textsuperscript{102, 172-176} Likewise, the outer surface of viruses can be applied for the templated synthesis of inorganic nanostructures.\textsuperscript{157, 177-180}

A different approach of combining viruses and inorganic nanoparticles is to use pre-formed nanoparticles rather than forming the nanoparticles \textit{in situ}. Chen \textit{et al.} showed that tetraethylene glycol (TEG) coated gold nanoparticles (AuNPs) can be encapsulated efficiently into BMV CP-based viral capsids.\textsuperscript{181} In line with the cargo-controlled VLP size observed when encapsulating different lengths of (bio)polymers, research showed that the size of the VLPs that are formed when encapsulating AuNPs can be controlled by the size of the bare AuNPs, yielding T = 1, T =2, or T= 3 VLPs depending on the AuNP size (Figure 2.10).\textsuperscript{182, 183} The efficiency of encapsulation was strongly influenced by the charge density on the AuNPs.\textsuperscript{183, 184}
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**Figure 2.10:** a) 3D reconstructions of T = 1, T = 2, and T = 3 particles, b) T = 1 VLP with a 6 nm AuNP core, c) T = 2 VLP with a 9 nm AuNP core, and d) T = 3 VLP with a 12 nm AuNP core. Reprinted from Sun et al., Copyright © 2007 National Academy of Sciences.

Using comparable assembly pathways, quantum dots were encapsulated into VLPs, yielding hybrids with unique properties and improved biocompatibility.

**Higher order assemblies**

Like with polymers, viruses have been combined with various types of nanoparticles to create higher order assemblies. This can yield unstructured aggregates, however by making use of the patchiness – the clustering of charges – of virus capsids and tuning the solution conditions, superlattice structures can be formed. For example, CCMV and TMV were combined with AuNPs forming superlattice structures, but also two differently modified protein cages were shown to assemble into superlattices yielding micrometer-sized, free-standing crystals.

**Molecular stacking templated assembly**

In most cases, the assembly pathway strongly influences the structure of the final product. This was specifically shown in cases where virus capsids were combined with stacking molecules. When mixing CCMV CP with zinc-phthalocyanines (ZnPc) at pH 7.5, where the CPs are in dimeric state, T = 1 VLPs were formed in
which the ZnPc’s were densely stacked. These particles were stable at pH 7.5 and the cargo was not lost upon dialysis. However, when the same components were mixed at pH 5, were the CPs are assembled in T = 3 capsids, a much lower loading of the ZnPc’s into the VLPs was observed. Furthermore, the cargo diffused out of the VLPs upon dialysis, and upon changing the pH to 7.5 the T = 3 structures were transformed into T = 1 structures and free CP.51,193 Other research showed that Pt(II) complex amphiphiles, upon mixing with CCMV CP, formed either spherical or rod-like VLPs, depending on the assembly procedure and molecular structure of the amphiphiles.194 These types of structures, as well as the other polymeric and non-polymeric virus hybrid structures described in this chapter, show the great diversity in which viruses are being applied and underline their huge potential for applications any many different fields.

2.5 Theoretical study

The large number of species involved in capsid assembly, and the strong influence of various environmental parameters on the assembly process, make understanding the processes involved highly complex. In attempt to gain insight into the interactions involved, various research groups are performing theoretical studies of CP assembly. These studies can be subdivided into those involving only protein-protein interactions, and those that include protein-cargo interactions.

2.5.1 Protein-protein interactions

Models have been developed to describe the formation of the simplest VLPs, those involving only the self-assembly of the capsid protein subunits. Several theoretical studies use structural data to estimate CP-CP interactions for all possible protein structures that can be formed during assembly.195-197 Using such an approach it was possible to determine the substructures and assembly pathway of a number of viruses, and the formation of several intermediate states, which were also observed experimentally. These models are limited by the fact that they use the association energies of the CPs in the fully formed capsid, and do not account for conformational changes during assembly which alter the interactions. Various other approaches have been used to model virus assembly, with varying results.67,198-202
Zlotnick *et al.* developed a model based on equilibria between free subunits, intermediates, and fully formed structures. In this model, modest subunit interactions lead to the formation of stable capsids in a cascade of equilibrium reactions. At equilibrium, mainly free CP and fully formed capsid are found, with few intermediate structures. Building on this model, they developed a more robust and broader applicable, so-called kinetically limited, model which allows for high association energies and non-uniform steps during assembly. In particular, this model includes a rate-limiting nucleation step, in which the slow formation of a nucleus, build up out of a small number of CPs, leads to formation full capsids. This model is also less susceptible to kinetic traps caused by high CP concentrations or high association energies. Predictions made using these models were confirmed experimentally, for example by determining that the assembly nucleus of HBV is a trimer of dimers and that CCMV nucleates from a pentamer of dimers. Furthermore, subunit association energies were determined for HBV and CCMV based on these models showing low energies of -3 to -4 kcal/mol subunit.

Hysteresis between assembly and disassembly, which was observed experimentally, are explained by this model due to kinetic stabilization of the capsid. When a capsid loses a single subunit further disassembly is in competition with reassociation to reform a full capsid, creating a barrier against disassembly. Another explanation is given by an assembly model based on nucleation theory, which suggests the phenomenon to be a direct result of the law of mass action, rather than a free-energy barrier.

Simulations of capsid assembly have been performed by several groups to further elucidate the influence of various parameters on the assembly process. Using such an approach, driving forces for assembly could be distinguished, and their impact on assembly pathways and intermediate states could be determined.

### 2.5.2 Protein-cargo interactions

Besides studies that only focus on the interactions between the capsid subunits, a lot of research is also done towards the influence of a cargo on the assembly of the capsid. Besides extensive experimental study (see section 2.4) also significant theoretical work in this direction has been performed.
Theoretical studies into the assembly of coat proteins with a polymeric template have highlighted the influence of polymer length on capsid assembly. Encapsulation is most efficient at polymer lengths that scale with the inner surface area of the capsid. Increased polymer length can cause the formation of malformed capsids where the polymer sticks out or may induce the encapsulation of one template by multiple capsids. Additionally, these studies have elucidated the contributions of the polymer template in the assembly mechanism. The template lowers the nucleation barrier due to stabilization of assembly intermediates by the polymer and by increasing the local concentration of capsid protein due to absorption onto the template. Furthermore, the electrostatic attraction between template and coat proteins enhances the growth rate of the capsid.

Modeling of polyelectrolyte packaging has shown that such cargo is preferentially arranged near the inner surface of the capsid, which is in line with experimental observations. Other aspects, such as RNA organization, the effect of packaging signals, and overcharging of capsids can also be explained using various models. Simulations have also predicted the influence of various parameters, such as ionic strength and subunit interactions, on the assembly pathway and the products obtained (Figure 2.11).

Figure 2.11: a) Phase diagram correlating the prevalent assembly product after simulation to the subunit-subunit attraction and the ionic strength of the solution. b) Structures formed during assembly simulations. Reprinted from Perlmutter et al., Copyright © 2014, with permission from Elsevier.

Studies involving the assembly around polyelectrolytes, such as ssRNA, are very relevant to understand the assembly of viruses. For the development of hybrid
materials, the assembly of viral proteins around non-native cargo, such as nanoparticles, has been modeled as well.\textsuperscript{224-228} These models show that a surface charge density threshold exists for assembly to occur,\textsuperscript{226} which is in line with experimental results.\textsuperscript{184} In further research it was shown that the gradual increase in encapsulation efficiency with increasing surface density could be explained by the presence of surface charge variations or small variations in the size of the nanoparticles.\textsuperscript{227, 228}

2.6 Concluding remarks

Viruses are very diverse structures that have great potential for a wide range of applications. To reach this potential, understanding their assembly and disassembly behavior, their interaction with various materials, and the physical properties at their interior is of crucial importance. A lot of research has been done to broaden our knowledge in these directions. As mentioned above, various experimental studies of the assembly of viral CP around native or non-native cargo have been carried out, and interesting new applications for viruses and their capsids are being developed. Furthermore, theoretical studies and \textit{in silico} simulations of virus assembly, of which a few are mentioned above, help us to get a better understanding of the various processes and pathways that are involved. Such research helps to gain insight in the influence of assembly parameters, such as subunit concentrations, pH, temperature, and ionic strength, on the assembly processes. Although none of the current models is able to describe virus assembly perfectly, they do allow for a fast analysis of assembly behavior over a wide range of conditions. The results of these studies can in turn be verified by comparing them to relevant experimental studies.

By combining theoretical and experimental results we obtain more and more information concerning virus structures, which hopefully will lead to a thorough understanding of all interactions involved. From a medical perspective, this will allow for the development of better anti-viral treatments. In the field of material science such knowledge is crucial for the development of new virus-based structures and to be able to predict the properties of these materials.

2.7 References


The structure and assembly of viruses and virus-like particles


The structure and assembly of viruses and virus-like particles


Chapter 3
Using microscale thermophoresis to study pH-induced capsid assembly

The (self-)assembly of virus capsid proteins into virus-like particles, and their application in various fields, ranging from materials science to medicine, has been studied extensively. Commonly used analysis techniques for this multi-protein particle formation are size exclusion chromatography, gel electrophoresis, light scattering, and electron microscopy. Here, we introduce microscale thermophoresis as a new technique to study virus protein assembly, having a high throughput while requiring small sample volumes. After proving its applicability, we use microscale thermophoresis to study and compare the pH-induced assembly and disassembly of the cowpea chlorotic mottle virus capsid protein and two modified versions of this protein, at various salt concentrations. We find similar assembly behavior for all studied proteins, except for the elastin-like peptide modified protein in 2 M NaCl solution, which is assembled over the full pH range that was investigated. The disassembly of wild-type protein and hexahistidine-modified protein was similar, but particles formed from the protein modified with an elastin-like peptide show higher stability, disassembling at higher pH values. We show that microscale thermophoresis is very effective in studying virus assembly and suggest that its application may be extended for the study of other (self-)assembling systems.
3.1 Introduction

Viruses, from either animals, plants, or bacteria, are commonly highly defined and homogeneous structures. For this reason, they have increasingly become the subject of research, for example in the fields of nanotechnology, material science, and medicine.\textsuperscript{1-5} To develop new applications of viruses, and to better understand virus’ behavior and replication, it is important to understand the assembly and disassembly mechanisms of viruses and virus-like particles (VLPs).\textsuperscript{6} Both theoretical and experimental research has provided insight into the aspects that influence viral structures, such as the conformation of the subunits of a viral particle, the interactions between the various components, and their response of to environmental conditions.\textsuperscript{7-12}

In spite of this work, much is still unknown about viruses and virus assembly. Even for the simplest viruses, those consisting of many copies of only one type of protein surrounding the viral genetic material, exact assembly pathways are currently not fully understood.

A simple, commonly studied virus is the cowpea chlorotic mottle virus (CCMV). CCMV is an ssRNA positive-strand Bromovirus from the Bromoviridae family, which in its native form consists of viral ssRNA surrounded by 180 capsid proteins (CPs) forming a capsid with $T = 3$ icosahedral symmetry.\textsuperscript{8, 13, 14} It was shown that the virus can be disassembled in vitro into CP dimers and the viral RNA by increasing the pH of the virus solution from acidic to neutral, which reduces interprotein interactions, and by increasing the ionic strength (I $> 0.5$ M) to reduce electrostatic CP-RNA interactions. After isolation, the CPs can be reassembled into VLPs in two ways. Firstly, by exploiting protein-cargo interactions using a polyanionic cargo, e.g. native or non-native RNA,\textsuperscript{7, 15} synthetic polyanions,\textsuperscript{16} or nanoparticles\textsuperscript{17, 18} which template the formation cargo-filled VLPs. A second route involves lowering the pH of the CP solution from neutral to acidic, which increases protein-protein interactions, causing the formation of empty capsids when pure CP solutions are used, or random encapsulation of species present in the CP solution.\textsuperscript{10, 19} CP assembly based on environmental conditions – i.e. pH, ionic strength, and temperature – has been studied both theoretically and experimentally.\textsuperscript{12, 14, 19} This has yielded understanding of the conditions required for assembly: CCMV CPs assemble at pH $< 5.5$ and ionic strength $> 0.3$ M.
Commonly used techniques to study these systems are size exclusion chromatography (SEC), gel electrophoresis (GE), light scattering (LS), and electron microscopy (EM). This work aims to add microscale thermophoresis (MST) to this collection of techniques by investigating pH-induced CCMV CP assembly as a model. This technique is commonly used to determine binding affinities, based on movement of particles in a laser light induced temperature gradient. The movement in the heated region is detected by the fluorescence emission of the appropriately labeled species. We expect that the movement of protein assemblies, i.e. virus capsids, over a temperature gradient will differ from the movement of free proteins, in this case CCMV CP dimers, allowing the distinction of these species in solution. In this way virus capsid assembly can be studied using a high-throughput technique which requires only small sample volumes. Furthermore, if MST is effective for studying virus assembly it might also be a method to study (protein) assembly in a more general sense, e.g. to study nanoparticle formation or protein aggregation.

This study focuses on the pH-induced assembly pathway forming empty protein capsids from CP. Three types of CCMV CPs are studied and compared: (1) wild-type CP (WT-CP) which is isolated from the native virus, (2) CCMV CP which is modified with a hexahistidine group at the end of the N-terminus (His-CP), and (3) CCMV CP of which the RNA-binding domain of the N-terminus is replaced by an elastin-like peptide sequence (ELP-CP). WT-CP is extracted from plants, while His-CP and ELP-CP are expressed in bacteria. Although all three CPs show pH-induced assembly, these differences in origin may slightly affect their structure and properties. Furthermore, His-CP can bind metal ions which stabilizes the formed capsids. Possibly, the histidines at the N-terminus of the CP have other effects on assembly that have not been observed so far. The ELP-CPs has two assembly pathways: besides CCMV CP’s natural pH-induced assembly, ELP-CPs also have an ELP-induced assembly pathway. ELPs are known to show lower critical solution temperature (LCST) phase behavior, meaning that depending on the temperature they can be in an extended, hydrophilic or in a collapsed, hydrophobic state. The transition temperature for this reversible state change can be influenced by pH, peptide structure and length, and salt concentrations. For ELP-CPs it has been shown that ELP-induced assembly into 18 nm VLPs occurs at room temperature and neutral pH at ionic strengths above 2 M. After proving that MST can be used to study the assembly of CCMV CP, the effects of both N-terminal modifications of the CP on
the protein’s self-assembly is studied using this technique, and the results are verified using conventional techniques such as SEC, LS, and EM.

3.2 Results and discussion

3.2.1 Microscale thermophoresis for assembly studies

To test whether MST can be used to study virus assembly, isolated CCMV CP solutions were prepared at pH ranging from ~5 to 7.5. Typical data from an MST measurement is shown in Figure 3.1.

Figure 3.1: (a) Typical MST curves for WT-CP (20 µM CP in a buffer containing 50 mM sodium citrate and 0.5 M NaCl) at pH ranging from 4.8 (red) to 7.9 (blue). (b) Fluorescence signal after 30 s laser exposure plotted against the pH of CP solution, average of three sets of samples.

Figure 3.1a shows the MST curves of WT-CP at pH 4.8 to 7.9. The fluorescence signal of the sample until the laser is switched on (T = -5 to 0 s) is stable and the average signal measured during this period is used to normalize the entire measurement, giving a relative fluorescence of 1 before the laser is switched on. Upon switching on the laser, at T = 0 s, the sample is heated locally causing a change in the fluorescence signal in this area due to thermophoresis. Under the sample conditions (Figure 3.1), the fluorescence decreases over the 30 s while the laser is switched on. However, the degree in which the signal decreases varies with pH, which is indicative for a difference in mobility or structure of the fluorescent moiety. This is shown in Figure 3.1b, in which the fluorescence intensity just before the laser is switched off, at T = 30 s, is plotted against the pH.
The fluorescence intensity at $T = 30$ s decreases with decreasing pH, but stabilizes at pH ~5.5. The stabilization of the signal at pH 5.5 can be explained by the expected incorporation of all CPs in capsids at pH < 5.5,$^{13,14}$ causing them to react similarly to the temperature increase caused by the laser. The relatively linear signal change at pH > 5.5, however, was unanticipated, since CPs are expected to be in the dimer form at pH > 5.5, resulting in a similar signal for all these samples. An explanation for the gradual pH change at pH > 5.5 could be that at any given pH an equilibrium exists between assembled and disassembled CPs. At pH < 5.5 this equilibrium is shifted to having almost only CPs assembled into capsids, whereas at pH > 7 it is shifted to full disassembly of the CPs into dimers. At a pH between 5.5 and 7, this equilibrium gradually shifts to the fully assembled system as the pH of the solution decreases, causing a gradual change of the fluorescent signal. This is in agreement to literature measurements on the assembly behavior of CCMV CP.$^{26}$ We separately tested whether the dye that was coupled to the CP was affected by the pH, and observed this was not the case (Figure 3.2).

![Figure 3.2](image_url)

**Figure 3.2:** (a) MST curves of free dye at pH ranging from 4.8 (red) to 7.9 (blue). (b) Fluorescence signal after 30 s laser exposure plotted against the pH of dye solution, average of three sets of samples.

To confirm the results of the MST measurements a similar set of samples was measured using dynamic light scattering (DLS), to determine the particle size at a pH ranging from 4.8 to 7.9 (Figure 3.3).
Figure 3.3: (a) DLS curves at pH ranging from 4.8 (red) to 7.9 (blue). (b) Number average (Mn) diameter of WT-CP containing samples at pH ranging from 4.8 to 7.9, average of three sets of samples.

Figure 3.3 shows that at pH < 5.5 particles with a number average diameter (Mn) of Mn~24 nm are measured by DLS. This corresponds to the Mn measured for WT-CCMV at pH 5. At pH > 5.5 the measured Mn ranges between 2 and 6.5 nm, suggesting no capsids are formed under these conditions. The small number of capsids that are likely present, based on literature data and as observed in the MST measurements, contribute little to the Mn until the solution mainly contains capsids. The measured values at which assembly occurs correspond to those expected for the CCMV CP assembly behavior described in literature.10, 13, 14

3.2.2 Assembly of modified CPs at various salt concentrations

The data measured by MST is in agreement with both the DLS measurements and the data shown in literature, suggesting that this technique can indeed be used to study virus assembly. Therefore, we extended our study to the disassembly process, by going from pH 5 to pH 7.5. Furthermore, a comparison is made between the WT-CP, His-CP, and ELP-CP, to see how modification of the N-terminus of the protein influences this assembly and disassembly behavior. Lastly, the influence of the ionic strength was studied by varying the NaCl concentration from 0.5 M to 2 M. The results of the MST measurements at these conditions are reported in Figure 3.4.
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Figure 3.4: Fluorescence signal after 30 s laser exposure plotted against the pH of the (a) WT-CP solution, (b) His-CP solution, and (c) ELP solution, with protein concentrations of 20 µM in a 50 mM citrate buffer. Samples were prepared going from pH 7.5 to pH 5 (dashed lines) and from pH 5 to pH 7.5 (solid lines) at NaCl concentrations of 0.5 M (black lines), 1 M (red lines), and 2 M (blue lines). All curves are the average of three sets of samples.

A number of observations can be made from Figure 3.4. First of all, the thermophoretic response when looking at the assembly of the CPs to VLPs (indicated by the broken lines) stabilizes at pH < 5.5 in the cases of WT-CP and ELP-CP, however this is not the case for His-CP. Possibly, His-CP requires a lower pH to be fully assembled due to the presence of the histidines, with a pKa of ~6, at the N-terminus. A second option is that the assembly of His-CP occurs much more slowly than the assembly of WT- and ELP-CP. However, this is not very likely since the samples are incubated for more than 24 h before measurement; generally this should be sufficient for assembly to be complete.
Secondly, for all CPs hysteresis is observed between the assembly and the disassembly processes: i.e. the disassembly of the capsids into CP dimers occurs at a higher pH than the assembly of CP into capsids. Where for all CPs assembly occurs at pH < 5.5, disassembly occurs at pH > 6.2. These values are in agreement with data of the pH-induced assembly of CCMV CP into capsids shown in literature.26, 27 Similarly, hysteresis in capsid disassembly has been shown to occur in dilution studies.28 Being able to identify hysteresis between assembly and disassembly, that matches literature data, again underlines the suitability of MST for the study of CP self-assembly. The hysteresis is even more extreme for the ELP-CP which is likely due to the favorable aggregation of the ELPs inside the capsids and the absence of repulsive forces because of the replacement of the positively charged N-terminus with the ELP.

Finally, Figure 3.4a and b show that the ionic strength of the solution has little influence on the assembly and disassembly behavior of WT-CP and His-CP. The assembly curves at 0.5 M, 1 M, and 2 M NaCl overlap for these CPs. Only a slight shift in the disassembly curves of WT-CP and His-CP is observed with increasing ionic strength, indicating that the capsids are slightly more stable under these conditions. On the other hand, the effect of salt concentration on the assembly and disassembly of the ELP-CPs is significant. The assembly curves at 0.5 M and 1 M of ELP-CPs are similar, showing assembly of the CPs at pH < 5.5. The disassembly curves at 0.5 M and 1 M, however, show significant differences. At 0.5 M NaCl disassembly occurs at pH > 7.0. This is higher than the pH at which the WT-CP and His-CP capsids disassemble (pH > 6.2). At 1 M NaCl, the fluorescent response in MST is almost stable over the full pH range, suggesting that, once formed, ELP-CP capsids are stable up to pH 7.5 at NaCl concentrations > 1 M, or at least that disassembly is slowed down dramatically requiring over 24 h to show significant capsid disassembly. At 2 M NaCl the ELP-CPs are assembled over the full pH range of 5 to 7.5, which was expected since previous research has shown that high salt concentrations cause ELP-induced capsid formation of these CPs. Based on this study, it is expected that the ELP-induced particles will have a T = 1 rather than T = 3 symmetric structure.23

To confirm the results observed by MST using more conventional techniques, we prepared samples by dialyzing the WT-, His-, and ELP-CP to pH 7.5, to pH 6.0 coming from pH 7.5, to pH 5.0, and to pH 6.0 coming from pH 5.0, all with 0.5 M NaCl. These samples were analyzed using SEC, DLS, and TEM (Figure 3.5).
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Figure 3.5: (a), (c), and (e) SEC traces and (b), (d), and (f) DLS data of (a-b) WT-CP, (c-d) His-CP, and (e-f) ELP-CP at pH 7.5, pH 6 coming from pH 7.5, pH 6 coming from pH 5, and pH 5. (g-i) show TEM images of (g) WT-CP, (h) His-CP, and (i) ELP-CP at pH 6 coming from pH 5. All samples have a protein concentration of 20 µM in a buffer containing 50 mM sodium citrate and 0.5 M NaCl.

The data shown in Figure 3.5 confirms the trend observed by MST. At pH 7.5 and 0.5 M NaCl all CPs are in dimeric form, as is concluded by the peak at V~18 mL in SEC which is typical for CP dimers (Figure 3.5a, c, and e). When the CPs are
dialyzed from pH 7.5 to pH 6.0 again only CP dimers are observed. Next, the samples were dialyzed to pH 5.0, which induced assembly of the CPs as can be observed from the peak at V ~10 mL in SEC. When dialyzing the samples from pH 5.0 to pH 6.0 the particles remain stable. DLS data (Figure 3.5b, d, and f) support these results, showing well defined peaks of $M_n$ ~25 nm at pH 5.0 and at pH 6.0 when coming from pH 5.0, while no well-defined particles are observed for the CPs at pH 7.5 and pH 6.0 when coming from pH 7.5. Figures 3.5g, h, and i show TEM images of the particles obtained when dialyzing the CPs from pH 5.0 to pH 6.0. TEM images were also taken from the samples at pH 6.0 coming from pH 7.5, however no particles were found in these samples.

### 3.3 Conclusions

Here, we have applied MST as a new method to study the assembly behavior of CCMV CP (WT-CP). The data obtained using MST is in agreement with data obtained using commonly applied techniques such as SEC, DLS, and EM, and is confirmed by assembly data shown in literature. Moreover, the technique shows clear hysteresis between assembly and disassembly of capsids, which has also been observed in literature using other techniques. In further research, MST was used to study and compare the assembly of two modified version of the CCMV CP: one having an hexahistidine sequence at the N-terminus (His-CP), and one of which the RNA-binding domain at the N-terminus is replaced by an elastin like peptide sequence (ELP-CP). WT- and His-CP show similar assembly behavior, while ELP-CP showed higher stability leading to disassembly of ELP-CP capsids at higher pH. This phenomenon is even more pronounced when increasing the salt concentration of the CP solutions. Overall, increasing the salt concentration has little effect on the assembly behavior, but does stabilize the formed capsid, causing them to disassemble at higher pH. The ELP-CP, however is more strongly affected by this parameter, showing little to no disassembly at I > 1 M, and being assembled over the full pH range from 5 to 7.5 at I = 2 M. To be able to distinguish between the three CPs at various pH values and three salt concentrations requires many different samples, however, due to the small sample size and the high-throughput of the technique, MST is a highly efficient technique to study the assembly processes.

Furthermore, MST proves to be a suitable methodology to monitor the pH dependent assembly behavior of CCMV CP and its derivatives. This does, for
example, allow the observation of hysteresis, but also gives hints towards the cooperativity in the assembly process. While for WT- and His-CP an almost linear response to an increasing pH is observed, in case of ELP-CP a more sigmoidal curve is observed (Figure 3c). This is in line with the previously suggested stronger protein-protein interactions for this modified CCMV CP. This highlights that MST can be a valuable addition to study virus assembly, and its use may be extended to study other (self-)assembly processes as well.

3.4 Acknowledgements

We gratefully acknowledge Dr. Lise Schoonen, Prof. Jan van Hest and Prof. Roeland Nolte for the fruitful discussion and for providing the modified CPs.

3.5 Experimental section

3.5.1 Materials

All chemicals were purchased from Sigma Aldrich and used without further purification. The wild-type CCMV virus is obtained according to literature procedures. Hexahistidine- and elastin-like peptide-modified CPs were kindly provided by Dr. Lise Schoonen from the Bio-organic chemistry research group at the Eindhoven University of Technology. Solutions were prepared using Milli-Q water (MQ, Millipore, 18.2 mΩ).

3.5.2 Wild-type capsid protein isolation

The CP of CCMV was isolated according to procedures described in the literature. Wild-type CCMV is disassembled by dialyzing against protein isolation buffer (50 mM Tris-HCl, 500 mM CaCl₂, 1 mM DTT, pH 7.5) using a 12-14 k MWCO dialysis membrane with 2 times buffer replacement. The high Ca²⁺ concentration causes the viral RNA to precipitate, and it is removed by 2 h centrifugation at 40,000 RPM (179,200 ×g) and 4°C using a Sorvall WX80 ultracentrifuge. The supernatant containing CP dimers was dialyzed against cleaning buffer (50 mM Tris-HCl, 500 mM NaCl, pH 7.5), followed by dialysis, with 3 times buffer replacement, against capsid storage buffer (50 mM NaOAc, 1 M NaCl, 1 mM NaN₃, pH 5) and stored at 4 °C for a maximum of 2 weeks until further use. To ensure the purity of the protein, only CP solutions with a 280/260 nm absorbance ratio of at least 1.5 were used.
3.5.3 Capsid protein labeling

To be able to measure a fluorescence response of the CPs in an MST measurement, all CPs were labeled with an Atto-610 fluorescent dye. For labeling, CP (~5 mg/mL = 250 µM) is dialyzed to a pH 7.5 phosphate buffer (50 mM sodium phosphate, 0.5 M NaCl, pH 7.5), after which a volume of 2% of the CP-solution’s volume of Atto-610-NHS ester (1 mg/mL = 1.7 mM in DMSO) is added. The mixture is incubated for 1 h at 20 °C under stirring. Subsequently the CP is purified using SEC, concentrated, and stored at 4 °C for a maximum of one week until use.

3.5.4 Microscale thermophoresis measurements

For MST measurements from CP to VLP, *i.e.* from high pH to low pH, WT-CP, His-CP, and ELP-CP at a concentration of 0.8 mg/mL (40 µM) are dialyzed to a 2× citrate buffer (100 mM sodium citrate; 1 M NaCl; 20 mM MgCl₂; pH (not adjusted) ~8). To obtain CP at a final concentration of 0.4 mg/mL (20 µM) in a 1× citrate buffer (50 mM sodium citrate, 20 mM MgCl₂) with the desired NaCl concentrations and pH, the CP solutions are mixed with varying amounts of MQ and HCl (for the samples with a final NaCl concentration of 0.5 M), 1 M NaCl in MQ and in HCl (for the samples with a final NaCl concentration of 1 M), and 3 M NaCl in MQ and in HCl (the samples with a final NaCl concentration of 2 M). During mixing, the CP solution is added to the MQ-HCl solution, to ensure the CP is not temporarily at a too low pH (*e.g.* if HCl is added to the CP before MQ is added).

For MST measurements from VLP to CP, *i.e.* from low pH to high pH, WT-CP, His-CP, and ELP-CP at a concentration of 2 mg/mL (~100 µM) are dialyzed to a pH 5 citrate buffer (5 mM sodium citrate; 1 M NaCl; 20 mM MgCl₂; pH 5) to ensure the samples are in capsid form. These are mixed with mixture of a high concentration citrate buffer (163 mM sodium citrate; 1 M NaCl; 20 mM MgCl₂; pH (not adjusted) ~8), MQ, and HCl (or MQ and HCl containing NaCl for the higher NaCl concentrations) to obtain CP solution of 0.4 mg/mL (20 µM) in 1× citrate buffer (50 mM sodium citrate, 20 mM MgCl₂) with the desired NaCl concentrations and pH. Again CP is mixed into the solution last, to prevent it from being temporarily at the wrong pH.
MST measurements were performed using a Monolith NT.115 setup from Nanotemper, using MO-AK002 capillaries. The system was operated with its green fluorescence channel, at a LED power of 2% and a temperature of 22 °C. Each sample was measured 3×: once with 20% MST power, once with 40% MST power, and once with 80% MST power. A typical measurement involves 5 s fluorescence measurement before switching on the laser, 30 sec measurement with the laser turned on, and 5 s fluorescence measurement after switching off the laser.

3.5.5 Size-exclusion chromatography

Analysis and purification by SEC was performed using a Superose 6 10/100 GL column on an FPLC-system (GE), eluting at 0.5 mL/min with 1x assembly buffer. Absorption was monitored at 260 nm and 280 nm.

3.5.6 Dynamic light scattering

The size distribution of the CPs at the various conditions was measured with DLS using a Microtrac Nanotrac Wave W3043. Viscosity and refractive index of water and the refractive index of native CCMV (1.54) were used.

3.5.7 Transmission electron microscopy (TEM) analysis

Samples (5 µL) were applied onto Formvar-carbon coated grids. After 1 min, the excess of liquid was drained using filter paper. Uranyl acetate (5 µL, 1% w/v) was added and the excess of liquid was drained after 15 sec and dried for 30 min at room temperature. The samples were examined on a FEG-TEM (Phillips CM 30) operated at 300 kV acceleration voltages.

3.6 References


Using microscale thermophoresis to study pH-induced capsid assembly


Chapter 4

The thermodynamics of polyanion-templated virus-like particle assembly

The basic structure of viruses comprises a protein capsid that surrounds and protects the viral genome. Previous research has shown that the (electrostatic) interaction between the capsid proteins (CPs) and the genome aids in the self-assembly of viruses. Scientists have used the same electrostatic interactions to introduce non-native cargo’s in virus capsids, forming virus-like particles (VLPs). Here, we study the assembly of CPs of the cowpea chlorotic mottle virus (CCMV) templated by polyanionic species, single-stranded DNA (ssDNA) and polystyrene sulfonate (PSS). We apply isothermal titration calorimetry to obtain information on the thermodynamics that are involved in these assembly processes, and show a three times higher affinity between CP and PSS compared to CP and ssDNA under the conditions applied here. We suggest that this higher affinity is the reason that CCMV dissembles at neutral pH in the presence of PSS to form PSS-filled VLPs.
4.1 Introduction

By combining proteins and synthetic materials, such as polymers or nanoparticles, new materials have been developed with unique properties.\textsuperscript{1-3} In this respect, a specific group of proteins, those derived from viruses, has gained interest due to the ability of viral proteins to form highly symmetrical and homogenous structures.\textsuperscript{4-8} Research in this direction has already shown the use of viruses and their components in a wide range of fields with applications, for example, in electronics and energy storage,\textsuperscript{9, 10} in drug delivery and medicine,\textsuperscript{11-14} and as catalytic particles.\textsuperscript{15-18}

To advance in these fields we need to understand the interactions taking place between the various components of such materials. Chapter 3 of this thesis describes work on virus capsid formation, involving cowpea chlorotic mottle virus (CCMV) capsid protein (CP) self-assembly into empty spheres, and shows how the protein-protein interactions can be exploited as a function of pH. However, under native conditions, i.e. involving the wild-type virus, as well as for many applications, capsids are generally filled with a cargo.\textsuperscript{19-22} In order to fully understand the interactions between the structure’s subunits, also CP-cargo interactions should be studied. Previous research in this direction has already shown that the cargo can aid in virus-like particle (VLP) formation, specifically in the early stages of assembly.\textsuperscript{23, 24} Association of CP with cargo, often involving electrostatic interactions, places CPs in close proximity. This close contact between the proteins allows for maturation of the unstructured CP-cargo aggregates into virus-like particles.\textsuperscript{24}

In this chapter, we describe a study on CP-cargo interactions, and the energies involved. To this end, we have employed isothermal titration calorimetry (ITC) to monitor heat changes caused by the interaction between CP and cargo upon mixing. ITC has been used in viral research before, for example to study membrane fusion events for the influenza virus,\textsuperscript{25} DNA ejection from bacteriophages,\textsuperscript{26} and the binding of inhibitors to viruses for the development of new antiviral treatments.\textsuperscript{27, 28} Moreover, ITC has been applied by De Souza et al. to qualitatively study the assembly of hepatitis C virus core proteins around a nucleic acid.\textsuperscript{29} Although they did not quantify the binding or assembly energies, they suggested that the assembly is mainly driven by electrostatic interactions between the protein and the nucleic acid. In contrast to this work, we attempt to
understand the CP-cargo interactions not only qualitatively but also quantitatively, to gain understanding on the cargo-related aspects that stabilize or destabilize viruses and VLPs.

For this purpose, we studied the cargo transfer between cowpea chlorotic mottle virus (CCMV)-based capsids and their surroundings, as well as the assembly of isolated CCMV CPs around two types of polyanionic cargo, single-stranded DNA (ssDNA) and polystyrene sulfonate (PSS). Besides using ITC to study the heat exchange, we applied analytical techniques, such as dynamic light scattering (DLS), size-exclusion chromatography (SEC), and electron microscopy (EM), as well as microscale thermophoresis (MST) for structural studies.

4.2 Results and discussion

4.2.1 Mixing CCMV and PSS

Initial experiments towards the stability of CCMV, and how well its capsids retain their cargo, were performed by mixing the wild-type virus with rhodamine labeled PSS (R-PSS). CCMV was mixed with R-PSS both at pH 5, where the interactions between CPs are strong, and at pH 7.5, where the CP-CP interactions are reduced and the particle relies on CP-cargo interaction for stability. These experiments were analyzed at various times using SEC and DLS (Figure 4.1).
Figure 4.1: SEC traces following the normalized absorbance at $\lambda = 280$ nm of a mixture of CCMV and R-PSS at various times at (a) pH 5 and (b) pH 7.5. DLS analysis of CCMV (red, dashed line), CCMV mixed with R-PSS (black, solid line), and R-PSS (blue, dash-dotted line) at (c) pH 5 and (d) pH 7.5 after 24 h. CCMV and R-PSS are mixed at a weight ratio of CCMV : R-PSS = 4.2:1 in a pH 7.5 buffer (25 mM Tris-HCl, 150 mM NaCl).

From the SEC data shown in Figure 4.1, we observe that at pH 5 no changes in size occur upon mixing; all material elutes at the same volume. The widening of the peak observed after 24 hours (Figure 4.1a, green dashed line) is, presumably, due to a smaller volume that was analyzed. At pH 7.5, the virus is in a swollen state, causing an increase in size of up to 10%.31 This effect is observed in Figure 4.1b where CCMV elutes earlier ($V \approx 8$ mL) than at pH 5 ($V \approx 10$ mL). A strong effect of mixing CCMV with PSS is observed at pH 7.5. Immediately after mixing,
we observe a second peak with an elution volume of $V \sim 12 \text{ mL}$, indicating particles of smaller size than CCMV, besides the peak of CCMV elute from the column ($V \sim 8 \text{ mL}$). From previous experiments involving assembly of CP around R-PSS (data not shown), we know that this peak is typical for $T = 1$ VLPs. Interestingly, the intensity of the peak at $V = 12 \text{ mL}$ increases over time. The measurement after 1.5 h shows the intensity of this signal is equal to that of CCMV, while after 24 h this new signal is the main peak in the SEC trace. Using DLS, we analyzed the size of the particles in the CCMV-PSS mixtures and compared this to the size of native CCMV and pure PSS at pH 5 and pH 7.5 (Figure 4.1c-d). The DLS data is in line with the SEC measurements; at pH 5, no size change is observed, showing for both CCMV and the CCMV-PSS mixture a number average ($M_n$) diameter of 25 nm (intensity average ($M_i$) = 28 nm). However, at pH 7.5, CCMV, as it is swollen, has a diameter of $M_n = 27 \text{ nm}$ ($M_i = 30 \text{ nm}$), while particles with $M_n = 17 \text{ nm}$ ($M_i = 21 \text{ nm}$) were observed in the mixture containing both CCMV and R-PSS. At both pH values, no particles larger than 5 nm were observed when measuring only R-PSS.

The rhodamine-labeled PSS ($\lambda_{max} = 548 \text{ nm}$) allowed us to monitor the co-elution of the polyelectrolyte and the CP at pH 7.5 in the $V = 12 \text{ mL}$ SEC signal (Figure 4.2a-b). Furthermore, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was applied to confirm the presence of CP in this peak (Figure 4.2c), while transmission electron microscopy (TEM) was used to visualize the particles formed at pH 7.5 (Figure 4.2d).
Figure 4.2: SEC traces of a mixture of CCMV and PSS after 24 h incubation at (a) pH 5 and (b) pH 7.5, monitoring the outflow at $\lambda = 280$ nm (red, dashed line), $\lambda = 260$ nm (black, solid line), and $\lambda = 548$ nm (blue, dash-dotted line). (c) SDS-PAGE analysis showing precision plus protein ladder (lane 1), CCMV (lane 2), the fraction eluting at V ~10 mL from the SEC measurement shown in Figure 4.2a (lane 3), and the fraction eluting at V ~12 mL from the SEC measurement shown in Figure 4.2b (lane 4). (d) TEM image of the fraction eluting at V ~12 mL from the SEC measurement shown in Figure 4.2b.

From Figure 4.2b, we observe that the fraction eluting at V ~12 mL from SEC, which forms after mixing CCMV and PSS at pH 7.5, does indeed have an absorption at $\lambda = 548$ nm, originating from the rhodamine dye on the PSS, which indicates that material corresponding to this signal indeed contains R-PSS. Interestingly, looking at the absorbance ratio at 260/280 nm we see strong variations for the different peaks. For the first peak (V ~8 mL) we measured a ratio of ~1.7, which is the expected value for pure CCMV. The second peak (~12 mL), containing the PSS-filled VLPs, had a ratio ~1.1. R-PSS does absorb at $\lambda = 260$ nm, although less strong than ssRNA, but it hardly absorbs at $\lambda = 280$ nm, whereas pure CP has a 260/280 nm ratio of ~0.6. Therefore, a ratio of ~1.1 for the second peak is in line with what is expected for PSS-filled VLPs. At elution volumes V > 15 mL, the 260/280 nm ratio was ~2. We suggest that this fraction contains the viral ssRNA that was released as CCMV CPs started binding to R-PSS, as ssRNA is known to absorb strongly at $\lambda = 260$ nm, having a 260/280 nm ratio of ~2.
At pH 5, no signal of the rhodamine dye was observed in the particle fraction (eluting at $V = 10$ mL) indicating that no R-PSS was included in this fraction (Figure 4.2a). After SEC, the fraction eluting at $V = 10$ mL at pH 5 and the fraction eluting at $V = 12$ mL at pH 7.5 were isolated and analyzed using SDS-PAGE to determine the presence of CCMV CP (Figure 4.2c). Both the particle fraction at pH 5 (SDS-PAGE gel lane 3) and the $V = 12$ mL particle fraction at pH 7.5 (lane 4) were compared to fresh CCMV (lane 2). Lanes 2, 3, and 4 all show the same protein band with a molecular weight (Mw) of ~20 kDa, which proves the presence of CP in the particles observed at pH 5 – most likely, these are CCMV particles that are unaffected by the PSS – and in the particle peak that contains R-PSS. TEM images of the PSS-containing particles show monodisperse, spherical structures with an average diameter of $17.2 \pm 1.4$ nm (Figure 4.2d). Based on these results, and earlier experiments involving the assembly of CCMV CP around PSS (not shown here), we conclude that upon mixing CCMV and R-PSS at pH 7.5 PSS-containing VLPs are formed while CCMV is disassembled releasing the viral genome. Knowing that under these conditions the CP-cargo interactions determine the stability of these particles, this suggests that CP interacts more strongly with R-PSS than with ssRNA, even though the ssRNA is much longer (~3000 nucleotides (nt)) than the R-PSS (~6700 kDa, ± 36 negative charges). At pH 5, the CP-CP interactions stabilize the particles, inhibiting PSS-induced disassembly of CCMV. All in all, these results are mostly qualitative, giving incomplete insight into the thermodynamics and kinetics, and hardly provide any information about the exchange mechanism occurring at pH 7.5. Most likely, the exchange is not yet complete after 24 h, so that the final composition does not correspond to equilibrium. The kinetics of the exchange at pH 7.5 is slow, on the timescale of days, but the intermediates by which the exchange occurs, such as possibly occurring free CP, capsid fragments, joint and partially bound RNA and PSS, etc., remain obscure. The inhibitory effect of exchange at pH 5 is most likely a kinetic effect, whereby enhanced CP-CP interactions retard the timescale of the capsid disassembly needed for release of RNA and binding of the more strongly interacting R-PSS. Therefore, more detailed investigations of the interactions between the different components are needed to shed light on parts of these processes and energies.
4.2.2 PSS-templated assembly

In an attempt to understand CP-cargo interactions, we extensively studied VLP assembly templated by PSS. We isolated the CP of CCMV by disassembling the virus and removing the viral ssRNA and subsequently performed DLS and MST measurements at various CP-PSS ratios to gain insight in PSS-templated VLP formation (Figure 4.3). In this way, the slow disassembly step of the exchange process described above can be circumvented and the, most likely fast, re-assembly step can be investigated separately and possibly at thermodynamic equilibrium.

Figure 4.3: (a) MST measurements at various PSS : CP ratios (black triangles; expressed as charge ratio, assuming one negative charge per PSS monomer unit and 10 positive charges per CP) and a trendline showing the calculated fluorescence for various ratios of encapsulated to free PSS, based on a relative fluorescence of 1 for fully encapsulated PSS and a relative fluorescence = 0.04 for PSS (average of the last 4 data points) (black line). (b) DLS data at various PSS : CP ratios, showing particle size (black triangles) and concentrations (gray squares). The black and gray lines are guides to the eye. (c) Number average DLS data of samples going from excess CP (light gray) to excess PSS (black).
The MST data shown in Figure 4.3a was obtained by following the thermophoresis of rhodamine-labeled PSS with various concentrations of CP. From low charge ratios of PSS to CP, i.e. excess CP, to a charge ratio of ~1, very similar fluorescence responses were measured. Upon increasing the charge ratio above 1, we observed a decrease in fluorescence response, which leveled off at very high charge ratios, i.e. excess PSS. We have added a trendline that plots the expected fluorescence intensity of when mixing encapsulated PSS and free PSS at different ratios, assuming quantitative encapsulation of PSS. Because of the fit of this trendline with the experimentally observed data points, we assume this decrease is merely an effect of having more free PSS in the solution at higher charge ratios, rather than having different amounts of CP binding to PSS at different charge ratios. Based on that, we conclude that at charge ratios < 1 we have solutions of bound PSS, presumably PSS-filled VLPs, and free CP, and at charge ratios > 1 we have solutions containing PSS-filled VLPs and free PSS. Thus, the assembly is practically fully formed at all charge ratios leaving the concentration of the unbound fraction of the minor component (CP or PSS) too low to measure, therefore precluding determination of the binding constant from these data.

These conclusions are supported by the DLS data shown in Figure 4.3b and c, were we titrate PSS into a solution containing CP. At low charge ratios, the number average diameter steadily increased up to $M_n \sim 16.5$ nm at a charge ratio of ~1, after which it became stable. The same trend was observed in the concentration index, a unitless indication of the particle concentration in a DLS sample as determined by the instrument. This indicates that above a charge ratio of 1 the concentration of VLPs is constant. Figure 4.3c shows an increasing peak at $M_n \sim 17$ nm when going from samples with excess CP (light gray) to samples with an excess of PSS (black).

4.2.3 The thermodynamics of PSS-templated assembly

Next, we applied ITC to observe the heat changes occurring upon mixing of the components, in an attempt to get quantitative information on the thermodynamics and/or interaction enthalpies that are associated with their self-assembly. The assembly of VLPs is highly complex, involving many components in various stoichiometries and therefore involving many interactions in a variety of stoichiometries. Yet, the process is presumed highly cooperative, leading to the absence of measurable amounts of intermediates, and allowing visualization
of the start and end situations only. The MST data shown above indicate that the assembly formation may be quantitative at any accessible concentration, so that determination of an equilibrium constant may be impossible. Yet, valuable stoichiometry and enthalpy information should be attainable.

To simplify the system, we assume that the enthalpy change ($\Delta H$) of the system consists of two contributions: the enthalpy change caused by the electrostatic interaction between the CP’s N-terminus and the PSS ($\Delta H_{CP-PSS}$), and the enthalpy change caused by the interaction between the CPs leading to VLP formation ($\Delta H_{CP-CP}$). To determine $\Delta H_{CP-PSS}$, we performed ITC measurements involving the titration of PSS into a solution of a peptide consisting of 26 amino acids corresponding to CCMV CP’s N-terminal 26 amino acids (N-term). This N-terminal section of the CP contains the ten positive charges that interact with negative cargos. In this way, we measure the electrostatic interactions without involving the CP-CP interactions. In this approach, we assume that no allosteric binding behavior occurs, meaning that $\Delta H_{CP-PSS}$ is not affected by the fact that CCMV CP occurs in dimeric form or by the VLP assembly process. Small conformational changes are expected for capsid assembly according to the quasi-equivalence theory (see Chapter 2 of this thesis), however these affect $\Delta H_{CP-CP}$ rather than $\Delta H_{CP-PSS}$. Figure 4.4 shows representative data of titrations of PSS into N-term solutions and compares them to data from similar experiments with CP instead of N-term. In these measurements, we titrated two lengths of PSS, with Mw ~6.7 kDa and ~70 kDa, into solutions of either N-term or CP while monitoring the heat supplied by the heater coil and compared the heat effects that occur. In these experiments, the concentration of CP was kept constant. For both 6.7 kDa and 70 kDa PSS, the same mass per volume ratio was used, thus keeping the number of monomer units added per injection constant. This automatically means that, due to the difference in chain length, the number of mol PSS added per injection is not equal between the two types of PSS. To prevent any effects of differences between batches of CP, e.g. protein purity, we present and compare samples that were prepared from the same batch of protein.
Figure 4.4: Heat changes per injection, normalized to heat per mol of a molecular fragment containing ten charges, of 6.7 kDa (black) and 70 kDa PSS (gray), into a solution of (a) N-term or (b) CP, as a function of the charge ratio. Heat changes per injection, in heat per mol PSS, for titrations of (c) 6.7 kDa PSS and (d) 70 kDa PSS into N-term (gray) or CP (black) solutions. The line fits presented in (c) and (d) are based on a single binding site model. In these experiments, PSS in pH 7.5 buffer (2 mg mL\(^{-1}\) PSS in 50 mM Tris-HCl, 0.3 M NaCl) is titrated into solutions of CP or N-term (0.075 mM) in the same buffer.

We assume that the CP’s N-termini, having ten positive charges, interact with a segment of ten negative charges on the PSS, leading to charge compensation. This is in line with the DLS and MST measurements discussed in Section 4.2.2, where particle formation occurs until a charge ratio of PSS to CP of ~1 is reached. When we correct the heat changes in ITC for the amount of charges added, which was kept constant between the different experiments, by calculating the heat per mol of segments of ten negative charges (10-mer) added, we see that both for the interaction of PSS with N-term, as well as PSS with CP, the difference in PSS length does not affect the thermodynamics of the system, showing similar curves in Figure 4.4a and b for 6.7 kDa PSS and 70 kDa PSS. Indeed, when looking at the
heat per mol of PSS, without correcting for the number of charges per PSS chain, significant differences in heat between titrations with 6.7 kDa PSS (Figure 4.4c) and 70 kDa PSS (Figure 4.4d) are measured, corresponding to the difference in length.

The ITC data for titrations of PSS in solutions of N-term were fitted with a simple 1:1 thermodynamic model (Figure 4.4c and d, gray solid lines). This model is based on the set of equilibrium and mass balance equations given below, where all species are assumed to be 10-mers.

\[
K = \frac{[N\text{-term}\text{-PSS}]}{[N\text{-term}] \times [PSS]} \quad \text{Eq. 4.1}
\]

\[
N\text{-term}_{\text{tot}} = [N\text{-term}] + [N\text{-term} - PSS] \quad \text{Eq. 4.2}
\]

\[
PSS_{\text{tot}} = [PSS] + [N\text{-term} - PSS] \quad \text{Eq. 4.3}
\]

Here, \( K \) is the equilibrium constant, \( N\text{-term}_{\text{tot}} \) and \( PSS_{\text{tot}} \) the total amounts of N-term and PSS in the solution, \([N\text{-term}]\) is the concentration of free N-term, \([PSS]\) is the concentration of free 10-meric PSS, and \([N\text{-term} - PSS]\) is the concentration of N-term-PSS complex. The model was used to determine \( K \), which gives the concentrations of the various components, and \( \Delta H \), which gives the amount of heat produced per mol of (10-meric) N-term-PSS complex formed.

The model assuming 1:1 complexation of 10-mers fits our data well, which suggests that one N-term interacts with a PSS 10-mer chain segment, leading to charge compensation. When viewing realistic PSS lengths, all N-term-PSS segment interactions occur according to an independent binding sites model. Since mathematically an independent binding sites model with a PSS : N-term stoichiometry of 1:3.6 (for 6.7 kDa PSS) is equal to an independent binding sites model with a stoichiometry of 1:1 with 3.6 times higher concentration of PSS with a length of 10 monomers, for data fitting the concentration of PSS is adjusted such that a binding stoichiometry of 1 is obtained.

Using this thermodynamic model, we obtained values for \( K \) and \( \Delta H \) for the N-term-PSS interactions. We assume that these thermodynamic parameters also correspond to the electrostatic interaction of CP-PSS while excluding VLP assembly (a full overview of thermodynamic parameters determined in this chapter is given in Table 4.1).
Table 4.1: Summary of thermodynamic parameters

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K$ ($\times 10^5$ M$^{-1}$)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$T\Delta S$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-term + 6.7 kDa PSS</td>
<td>2</td>
<td>-8.9</td>
<td>-1.7</td>
</tr>
<tr>
<td>N-term + 70 kDa PSS</td>
<td>2</td>
<td>-9.5</td>
<td>-2.2</td>
</tr>
<tr>
<td>CP + 6.7 kDa PSS</td>
<td>1.5</td>
<td>-11.9</td>
<td>-4.8</td>
</tr>
<tr>
<td>CP + 70 kDa PSS</td>
<td>1.5</td>
<td>-13.0</td>
<td>-6.0</td>
</tr>
<tr>
<td>N-term + PSS 0.6 M NaCl</td>
<td>0.4</td>
<td>-8.6</td>
<td>-2.2</td>
</tr>
<tr>
<td>CP + PSS 0.6 M NaCl</td>
<td>1.5</td>
<td>-9.5</td>
<td>-2.4</td>
</tr>
<tr>
<td>CP + 6.7 kDa PSS (Section 4.2.4)</td>
<td>2.8</td>
<td>-11.5</td>
<td>-4.1</td>
</tr>
<tr>
<td>CP + ssDNA</td>
<td>1.0</td>
<td>-1.8</td>
<td>5.0</td>
</tr>
</tbody>
</table>

For both 6.7 kDa PSS and 70 kDa PSS interacting with N-term, $K$ values of $\sim 2 \times 10^5$ M$^{-1}$ were found. The values for $\Delta H$, which we assume to be equal to $\Delta H_{\text{CP-PSS}}$, were -8.9 and -9.5 kcal mol$^{-1}$ for 6.7 kDa PSS and 70 kDa PSS, respectively.

Using the Equations 4.4 and 4.5, we can relate these values to the change in Gibbs free energy and calculate the entropic components of these interactions.

$$\Delta G = - R T \ln(K)$$  

Eq. 4.4

$$\Delta G = \Delta H - T \Delta S$$  

Eq. 4.5

In which $\Delta G$ is the change in Gibbs free energy, $R$ is the gas constant, $T$ is the temperature in Kelvin, and $\Delta S$ is the change in entropy. Using these equations, we calculated a $T\Delta S$ of -1.7 kcal mol$^{-1}$ and -2.2 kcal mol$^{-1}$ for 6.7 kDa and 70 kDa PSS respectively.

Knowing the contribution of the electrostatic interactions between CP and PSS, we analyzed the ITC measurements involving PSS titrations into CP solutions using a similar approach. Looking more closely into the ITC data for the interaction of CP with 6.7 kDa PSS and 70 kDa PSS, we observe a bend in both curves at a charge ratio of $\sim$1 (see Figure 4.4b). This bend suggests that multiple interactions that cause a change in heat and/or stoichiometry are involved in this system. This is not surprising, considering the complexity of VLP assembly and the large number of components making up such particles. The location of this bend suggests that this second binding has a PSS : CP stoichiometry higher than 1:1. In an attempt to determine the thermodynamic parameters accurately, we tried to model two different binding events: first, the electrostatic binding of CP to PSS (i.e. CP-PSS interactions), and second, the rearrangement of CPs to higher order structures (CP-CP interactions). Unfortunately, we were unable to develop...
a model that could accurately fit the data and give us valid information on the thermodynamic properties of the two interactions. For this reason and because of the otherwise large similarities between the data for N-term and CP, we applied the simplified model described earlier for the N-term-PSS system on the CP-PSS systems as well to get a rough estimate of the thermodynamic parameters. By assuming that the changes in heat are caused by two events – the electrostatic interactions of the CP’s N-terminus with the PSS, and the assembly of the CP-PSS complexes into VLPs due to CP-CP interactions – we can obtain information on the CP-CP interactions by comparing the thermodynamic parameters of N-term-PSS, which only involves the electrostatic interactions, with the thermodynamics of the full assembly.

For titration of PSS into CP solutions, $K$ values of $\sim 1.5 \times 10^5 \text{ M}^{-1}$ were found, while $\Delta H$ was -11.9 and -13.0 kcal mol$^{-1}$ for 6.7 kDa PSS and 70 kDa PSS, respectively. It is important to note, also underlined by the quantitative assembly formation observed by MST, that fully assembled VLPs are assumed to be formed quantitatively also under the ITC conditions. This means that the gradual curve in the heat changes, which provides the apparent values of $K$, probably reflect gradual affinity and heat changes during the progress of the titration and/or contributions from cooperative and/or multivalent behavior. The lack of proper models with such high stoichiometries and the absence of experimental observations of intermediates precludes the development of better models at this stage. Assuming $\Delta H$ in these measurements consist of $\Delta H_{\text{CP-PSS}}$ and $\Delta H_{\text{CP-CP}}$, we can calculate $\Delta H_{\text{CP-CP}}$, giving -3.0 and -3.5 kcal mol$^{-1}$ for the titrations of 6.7 kDa and 70 kDa PSS into CP solutions, respectively. Since $\Delta H_{\text{CP-CP}}$ involves CP-CP interactions, rather than CP-PSS interactions, similar values for $\Delta H_{\text{CP-CP}}$ were expected independent of the length of PSS, assuming particles of the same size are formed (as discussed below). Indeed, the values calculated for $\Delta H_{\text{CP-CP}}$ at 6.7 kDa PSS and 70 kDa PSS are comparable, giving an estimated (exothermic) heat change of approximately -3 to -4 kcal mol$^{-1}$. Interestingly, values of the same order of magnitude were found to regulate CCMV CP self-assembly at acidic pH, having a $K \sim 2.5 \times 10^5 \text{ M}^{-1}$ and association energies of $\sim 3.5$ kcal mol$^{-1}$.

The changes in entropy, $T \Delta S$, for these systems were calculated to be -4.8 kcal mol$^{-1}$ and -6.0 kcal mol$^{-1}$ for 6.7 kDa PSS and 70 kDa PSS, respectively. Comparing these values to the entropy changes for N-term PSS interactions, we determine that upon VLP assembly the system loses entropy, approximately -3 to -4 kcal
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A mol\(^{-1}\) difference is calculated between \(T\Delta S\) of the N-term-PSS interactions and \(T\Delta S\) of the CP-PSS interactions. According to literature, CP-CP interactions are driven by hydrophobic interactions\(^{35,36}\) which are commonly accompanied by favorable entropies and unfavorable enthalpy changes. We expected that burying of hydrophobic patches on the CPs, and the release of water molecules from these regions, that drive the CP-CP interactions, would cause a gain in entropy and would therefore aid VLP assembly. However, our data suggests that this effect is less than other, entropically unfavorable, events, such as possibly the restriction of CP and PSS mobility upon assembly. It should be noted that the CP-CP interactions are pH dependent and that at elevated pH (>6.5) CP-CP interactions are weak and have different molecular origin, which means different effects may be observed at different pH. Moreover, reducing mobility of ionic species in the samples possibly also reduces the systems entropy, as is discussed in Chapter 6 of this thesis where we use a Donnan equilibrium to model charge separation between the inside and the outside of the capsid.

We extended this study by looking at the effect of ionic strength on the thermodynamics of this system by performing titrations at 0.6 M NaCl, in contrast to 0.3 M in previous experiments (Figure 4.5).
Figure 4.5: (a) Integrated data of the measured temperature change per injection of 6.7 kDa PSS into a solution of CP (black triangles) or N-term (gray squares). The black and gray lines show the fitted data based for the CP and N-term, respectively. (b) DLS and (c) SEC measurement of the product after ITC.

From Figure 4.5a, we observe that at 0.6 M NaCl the interactions between PSS and CP or N-term give lower changes in heat compared to the 0.3 M NaCl samples. However, upon data fitting of the data for PSS titrated to an N-term solution, we found that mainly $K$ is affected, giving a value of $4.4 \times 10^4 \text{ M}^{-1}$ which is an order of magnitude smaller than at lower ionic strength. $\Delta H$ of the N-term-PSS interaction, which gives us $\Delta H_{\text{CP-PSS}}$, was found to be -8.6 kcal mol$^{-1}$, which is comparable to the values found at lower ionic strength. On the other hand, when looking at the data for the titration of PSS into a CP solution, we found $K$ to be $1.5 \times 10^5 \text{ M}^{-1}$, which is in the same range as similar measurements at 0.3 M NaCl. $\Delta H$ for this titration was found to be -9.5 kcal mol$^{-1}$, giving a $\Delta H_{\text{CP-CP}}$ of -0.9 kcal mol$^{-1}$, which is lower than values found at lower ionic strength. As mentioned, CP-CP interactions are of hydrophobic nature, which mainly gives a favorable entropy rather than enthalpy change. Increasing the ionic strength also enhances hydrophobic effects and should therefore increase the change in entropy. Using the Equations 4.4 and 4.5, we calculated a $T\Delta S$ of -4.8 kcal mol$^{-1}$ and $\sim -2.4$ kcal
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mol for PSS titrations into a CP solution at 0.3 M and 0.6 M NaCl, respectively. This indeed indicates more favorable entropic effects at higher ionic strength.

In this section we compare 6.7 kDa PSS with 70 kDa PSS and show that both lengths of PSS show very similar thermodynamic behavior. Significantly reducing the length of PSS below 6.7 kDa may affect both kinetics and thermodynamics, however, this was not further investigated here. Research involving the interaction between CP and short polyelectrolytes (oligo-ssDNA) is described in Chapter 5 of this thesis. Furthermore, both 6.7 kDa PSS and 70 kDa PSS induce T = 1 particle formation. A T = 1 VLP comprises 60 CPs, thus 600 positive charges, meaning that upon charge compensation approximately 17 chains of 6.7 kDa PSS (~36 negative charges each) and 2 chains of 70 kDa PSS (~380 negative charges each) are encapsulated. It would be interesting to perform similar titrations with even longer PSS, which may lead to formation of particles with a higher T number. Moreover, increasing the length of PSS may lead to a different charge ratio per VLP. This may cause an excess of negative charges, as occurs naturally in the native virus where ~3000 nt (3000 negative charges) ssRNA is encapsulated in a T = 3 (180 CPs = 1800 positive charges). Also, encapsulating high molecular weight PSS as described by Hu et al. yielded negative overcharging, while an excess of positive charges is observed if particles with a higher T number are formed, as was shown by Cadena-Nava et al. when measuring the number of PSS chains per capsid. These variations clearly show that the encapsulation of polyanions is dependent on the assembly conditions used, and does not merely depend on the number of charges. We expect that significantly increasing the length of PSS, or changing the assembly conditions, may affect the thermodynamics of the system. However, according to the data shown in Figure 4.4, the lengths of PSS used here show similar thermodynamic behavior.

4.2.4 Comparison of CP-PSS and CP-ssDNA interactions

To further study the CP-cargo interactions, we applied ITC to compare PSS- or ssDNA-templated VLP assembly. PSS or ssDNA was titrated into a CP solution under identical conditions, while monitoring the heat supplied by the heater coil. Since the CP-cargo interactions are of electrostatic nature, the concentration of CP and the concentration of negative charge added were kept constant during these experiments, to be able to compare the interactions. Furthermore, samples that are compared were prepared from the same batch of CP, to exclude effects
of differences between different batches, e.g. protein purity. Representative integrated ITC data of such measurements are shown in Figure 4.6.

**Figure 4.6:** Integrated data of the heat change per injection, normalized to heat per mol of a fraction containing ten charges, of (a) a 6.7 kDa PSS-solution and (b) an ssDNA-solution into a CP solution (~1 mg mL\(^{-1}\) CP in a pH 7.5 buffer containing 0.3 M NaCl). The fits (solid lines) presented in (a) and (b) are based on a single binding site model.

From Figure 4.6 we observe that, although both systems show exothermic heat effects, upon adding PSS to CP a much larger change in heat is observed than when ssDNA is added under the same conditions. Note that the PSS (~6.7 kDa, ~36 negative charges) and de ssDNA (~500-800 nt, on average 700 negative charges) have a different number of charges per chain. CP-PSS and CP-ssDNA interactions are expected to be primarily of electrostatic nature, therefore, not the number of molecules but the number of charges added to the CP-solution was kept constant. Similar to the work described in Section 4.2.3, where different lengths of PSS are compared, we corrected the integrated ITC data for the number of charges added, giving the heat in kcal mol\(^{-1}\) of segments of 10 charges added.

The ITC data was fitted using the same model described in Section 4.2.3 (Figure 4.6), in order to obtain estimates of the thermodynamic parameters involved in these systems. As before, the 1:1 model we use does not give an exact representation of the assembly system, however the inflection point, although steeper in the case of PSS, in both systems occurs at a charge ratio of ~1 suggesting charge compensation upon binding in both systems. This contrasts observation data published before in which DNA was found to bind with a larger relative number of negative charges (over-compensation).\(^{37}\)
We determined the $K$ of CP-PSS interactions to be almost three times higher than of CP-ssDNA, being $2.8 \times 10^5$ M$^{-1}$ and $1.0 \times 10^5$ M$^{-1}$, respectively. Having $K$ values in the same order of magnitude, no significant differences in affinity are expected between CP and PSS or ssDNA. Slight deviation of the values for CP-PSS interactions described here as compared to section 4.2.3 are likely due to the use of a different batch of CP or experimental and data fitting errors.

Most striking are the differences in $\Delta H$ and $T\Delta S$ observed between these systems. For CP-PSS interactions $\Delta H$ was determined to be $-11.5$ kcal mol$^{-1}$, whereas for CP-ssDNA $\Delta H$ was found to be $-1.8$ kcal mol$^{-1}$. $\Delta H$ for the CP-PSS interactions was six times larger than for CP-ssDNA interactions. In the experiments discussed earlier (section 4.2.3), we noticed that $\Delta H$ is primarily affected by the electrostatic CP-cargo interactions, which suggests that CP has a much stronger electrostatic interaction with PSS than with ssDNA. Possibly, this can be explained by the difference in charge density between PSS and ssDNA, being ~40% higher for PSS.40

Enthalpically the interaction between CP and PSS seems to be favorable, however entropically a completely different picture is drawn. From $K$ and $\Delta H$ we calculated a $T\Delta S$ of $-4.1$ kcal mol$^{-1}$ for CP-PSS interactions and a $T\Delta S$ of 5.0 kcal mol$^{-1}$ for CP-ssDNA interactions. However, to obtain more information on this, further study of the system, for example using PSS and ssDNA of exactly the same length, is required. Yet, the data seems to indicate a much more favorable entropy for the DNA-VLP assembly, in correspondence to hydrophobic interactions, and in line with previously reported data. The discrepancy between the PSS and DNA-induced self-assembly may also lie in part in the different particles that are formed, i.e. $T = 1$ vs $T = 3$ particles. Possibly, the formation of $T = 1$ particles in the case of PSS, with the observed stronger electrostatic interactions, creates more tightly bound cavities with poorer hydrophobic CP-CP interactions at a higher entropic cost. The assembly of VLPs during these ITC experiments was confirmed by studying their reaction products using SEC, DLS, and TEM (Figure 4.7).
Figure 4.7: (a) SEC trace, (b) DLS results, and (c) TEM image of the product obtained after an ITC where PSS is titrated into a CP solution. (d) SEC trace, (e) DLS results, and (f) TEM image of the product obtained after an ITC where ssDNA is titrated into a CP solution (~1 mg mL⁻¹ CP in a pH 7.5 buffer containing 0.3 M NaCl).

Figure 4.7 shows that both with PSS and with ssDNA VLPs are formed in the ITC experiments. For PSS, SEC shows particles eluting at a volume of \( V = 12 \) mL and DLS analysis of these particles (Figure 4.7b) show they have a diameter of \( M_n = 17.7 \pm 3.2 \) nm (\( M_i = 19.5 \pm 3.5 \) nm), which is in line with the particle size observed in TEM of \( 18.8 \pm 2.3 \) nm (Figure 4.7c). For ssDNA, SEC shows more polydispersity in the particle size, resulting in signals at \( V \sim 8 \) mL and \( V \sim 10 \) mL. The reason for this is that, due to volumes required for ITC measurements, we used ssDNA derived from salmon testes, which was fragmented by sonication. Over time, this
ssDNA can reanneal causing the formation of larger templates around which the CP assembles. Isolation of the fraction at ~10 mL shows particles having an Mn diameter of 23.8 ± 4.4 nm (M_\text{i} = 60.5 ± 17.5 nm, indicating the presence of large aggregates) according to DLS measurements, and a diameter of 21.9 ± 2.5 nm according to TEM. A few larger, rod-like structures were observed in TEM, likely involving reannealed DNA, which were ignored during size measurements. Although affecting the assembly into VLPs, the reannealing of the ssDNA is assumed to have little effect on the electrostatic interactions between ssDNA and CP, which dominate the ITC measurements.

We suggest that the large difference in enthalpy gain, primarily caused by electrostatic CP-cargo interactions, may be the main explanation for the results described in section 4.2.1, where we observe that at pH 7.5 – when electrostatic interactions stabilize the capsid – addition of PSS causes CCMV to disassemble and VLPs containing PSS to be formed. Yet, it is important to note that we still lack full understanding of virus assembly and the interactions involved in forming and stabilizing virus particles. The thermodynamic data presented here is based on a model that is far too simple to accurately describe all aspects of particle assembly and only provides an estimate of the binding affinity between the components involved. Using these estimates, and by comparison with non-assembling systems we can get some insight in the assembly parameters. Only by fully understanding assembly pathways, with all its intermediate and final structures, we can accurately determine the strength of all subunit interactions. However, based on our experiments and work described in literature it seems that weak subunit interactions are a general trend in virus assembly.\textsuperscript{24, 41}

These weak interactions can be explained by viruses’ natural infectious pathway. In nature, viruses need to balance between being stable enough to survive various surroundings, while still being able to disassemble in their host’s cells to deliver their viral genome and be able to reproduce. A too strong electrostatic interaction between the CP and the viral genome would hamper disassembly, which in turn would decrease the virus’ potential to reproduce. In other work in our labs, we have noticed that polyelectrolyte-filled VLPs hardly disassemble, even under very high ionic strength. For virus reproduction, such particle stability would be disadvantageous.
4.3 Conclusions

In this chapter, we have studied the interactions between CCMV CP and polyanionic cargos PSS and ssDNA. We found that when we mix CCMV and PSS at neutral pH, CP is transferred from the virus to the PSS to form PSS-filled VLPs. In an attempt to better understand the interactions between CP and a polyanionic cargo, we investigated the structures formed at various PSS to CP charge ratios using dynamic light scattering and microscale thermophoresis. These measurements suggest that at all charge ratios below 1 particles are being formed, with a maximum number of particles at a charge ratio of ~1. Above 1, it seems no structural changes occur, and the excess PSS is free in solution.

We continued the study of PSS-templated VLP assembly using isothermal titration calorimetry, and by separating the electrostatic interactions between CP and cargo involved in the assembly from the complete assembly, we conclude that CP-CP interaction cause an enthalpy change of -3 to -4 kcal mol$^{-1}$. In all our ITC measurements, we found binding stoichiometries of ~1.

We studied the heat effects that are involved when mixing isolated CP and PSS or ssDNA. Significantly higher heat changes were observed when mixing CP with PSS compared to mixing CP with ssDNA. By fitting the ITC data, we estimated that the change in enthalpy caused by the interaction between CP and PSS is approximately six times higher than the change in enthalpy caused by the interaction between CP and ssDNA. Furthermore, a three times higher K value was found for the CP-PSS interaction. These parameters are suggested to cause the observed disassembly of CCMV and the formation of VLPs containing PSS upon mixing CCMV with PSS.

The measurements reported in this Chapter and the tentative interpretation of the data broadens our understanding of virus assembly in general, and specifically adds to our knowledge of the thermodynamics involved. Knowing what the requirements for particle assembly are, may aid in the development of new virus-based materials on one side, and on the other side could help in the development of new anti-viral treatments by understanding what could induce or prevent virus assembly and disassembly.
4.4 Acknowledgements

Prof. Jurriaan Huskens is gratefully acknowledged for the help with fitting and interpreting the data.

4.5 Experimental section

4.5.1 Materials

All chemicals were purchased from Sigma Aldrich and used without further purification unless stated otherwise. 70 kDa PSS and Methacryloxyethyl thiocarbamoyl rhodamine B are purchased from polysciences. The wild-type CCMV virus is obtained according to literature procedures. Solutions were prepared using Milli-Q water (MQ, Millipore, 18.2 mΩ). N-term was purchased from the peptide facility of the Netherlands Cancer Institute.

4.5.2 Wild-type capsid protein isolation

The CP of CCMV was isolated according to procedures described in the literature. Wild-type CCMV is disassembled by dialyzing against protein isolation buffer (50 mM Tris-HCl, 500 mM CaCl₂, 1 mM DTT, pH 7.5) using a 12-14 k MWCO dialysis membrane with 2 times buffer replacement. The high Ca²⁺ concentration causes the viral RNA to precipitate, and it is removed by 2 h centrifugation at 40,000 RPM (179,200 ×g) and 4°C using a Sorvall WX80 ultracentrifuge. The supernatant containing CP dimers was dialyzed against cleaning buffer (50 mM Tris-HCl, 500 mM NaCl, pH 7.5), followed by dialysis, with 3 times buffer replacement, against capsid storage buffer (50 mM NaOAc, 1 M NaCl, 1 mM NaN₃, pH 5) and stored at 4 °C for a maximum of 2 weeks until further use. To ensure the purity of the protein, only CP solutions with a 280/260 nm absorbance ratio of at least 1.5 was used.

4.5.3 Rhodamine-labelled PSS synthesis (R-PSS)

1.0 g of sodium 4-vinylbenzenesulfonate (5 mmol), 34.2 mg of methacryloxyethyl thiocarbamoyl rhodamine B (0.05 mmol), and 14.5 µL of 2-Hydroxyethyl 2-bromoisobutyrate (20.1 mg, 0.1 mmol) are dissolved in 5 mL of MQ. This solution is purged with N₂ gas for 45 min. An excess amount of methanol is purged with N₂ gas for 45 min as well. After, 1.7 mL of purged methanol is added to the aqueous solution using a N₂-purged syringe. The solution is purged with N₂ gas for an additional 5 min before adding 13 mg copper(I) bromide (0.090 mmol) and
30 mg 2,2′-Bipyridyl (0.19 mmol) as a solid while maintaining a N₂ gas purge. After addition of the catalyst, the reaction mixture turns brown, and is stirred under N₂ atmosphere for 24 h at 21 °C. After 24 h, the reaction is terminated by opening the flask, causing the reaction mixture to turn from brown to blue indicating oxidation of the Cu(I) catalyst to Cu(II). The copper is removed by running the mixture over a silica gel column (eluent 1:1 H₂O : MeOH). After, the polymer was precipitated from THF. The precipitated solid was filtered off, redissolved in 1:1 H₂O : MeOH and again precipitated from THF. The purified polymer was dried for 12 h at 60 °C before analysis using NMR (Figure 4.8), FPLC, UV-Vis spectroscopy, and fluorescence spectroscopy ($\lambda_{ex} = 563$ nm and $\lambda_{em} = 583$ nm). Based on the sodium 4-vinylbenzenesulfonate monomer conversion determined by NMR the molecular weight of the R-PSS is ~6.7 kDa.

![Figure 4.8: NMR spectrum of R-PSS after purification.](image)

### 4.5.4 Size-exclusion Chromatography

Analysis and purification by size-exclusion chromatography (SEC) was performed using a Superose 6 10/100 GL column on a fast protein liquid chromatography (FPLC) system (GE), eluting with 2x diluted PSS encapsulation buffer (25 mM Tris; 150 mM NaCl; pH 7.5).
4.5.5 Dynamic light scattering

The particle size distribution of the VLPs in the various buffers was determined using dynamic light scattering (DLS), using a Microtrac Nanotrac Wave W3043. The viscosity and refractive index of water and the refractive index of native CCMV (1.54) were used in data processing.

4.5.6 UV-Vis spectroscopy

UV-Vis spectra were measured in a quartz cuvette using a PerkinElmer Lambda 850 UV–vis spectrometer.

4.5.7 Fluorescence spectroscopy

Fluorescence excitation and emission spectra were measured in a quartz cuvette using a PerkinElmer LS 55 fluorescence spectrometer.

4.5.8 Transmission electron microscopy

For Transmission electron microscopy (TEM), 5 µL of sample is drop casting onto a Formvar carbon coated copper grid. After 1 min of incubation, the remaining liquid is removed using filter paper (Schleicher & Schuell). The samples are stained using 5 µL of a 1% uranyl acetate in MQ water which is removed after 20 s using filter paper. Samples are imaged using a Philips CM300ST-FEG TEM or a Zeiss Merlin (S)TEM. Particle sizes were determined using ImageJ software.

4.5.9 Nuclear magnetic resonance

$^1$H Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker 400MHz NMR.

4.5.10 Mass measurements

Mass spectra were recorded with a Waters electrospray ionization time of flight mass spectrometer operated in positive ion mode (ESI(+)-ToF, Micromass LCT).

4.5.11 Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) ITC measurements were carried out using a Microcal VP-ITC with a cell volume of 1.4115 mL. In general, a solution of polyanion in pH 7.5 buffer (50 mM Tris-HCl, 0.3 M NaCl; unless stated otherwise)
was titrated into a solution containing CP or N-term in the same buffer, while constantly monitoring the heat flux.

## 4.6 References

Chapter 5

Oligonucleotide length dependent formation of virus-like particles

Understanding the assembly pathway of viruses is crucial, both for finding new ways to fight them as well as for the development of virus-based materials. In this study, the cowpea chlorotic mottle virus is used to determine the interactions between the capsid proteins of viruses and their cargo. The assembly of the capsid proteins in the presence of different lengths of short single-stranded DNA is studied at neutral pH, where the protein-protein interactions are weak. Chromatography, electrophoresis, microscopy, and light scattering show that the particle assembly efficiency and speed increase with increasing length of oligonucleotides. The minimal length required for assembly at the conditions used here is shown to be 14 nucleotides. Assembly of particles containing such short strands of ssDNA can take almost a month. This slow assembly process enabled the study of intermediate states, confirming a low cooperative assembly for CCMV and allows for further expansion of current assembly theories.

This chapter has been published as:
5.1 Introduction

Viruses are well-known as infectious and disease-causing particles, but this is not the only way in which they can be viewed. From a material chemist’s point of view, they are wonderful multicomponent systems with highly-defined structures. Their self-assembly into monodisperse nanoparticles is unparalleled by synthetic particles. Viruses and their protein capsids are therefore increasingly studied to produce new functional materials. As such, they have been applied as catalytic particles, to synthesize inorganic nanoparticles, or as medically active agents.1-3 For these and future applications it is relevant to know how a virus assembles and how non-native cargo can be encapsulated in the viral protein cage. Various studies, commonly involving model viruses, have been conducted to gain insight into virus assembly processes. Nevertheless, the physiochemical pathways, and the exact requirements for the protein-protein and protein-cargo interactions, of the assembly of the virus shell and the packaging of the cargo inside are still not yet fully understood.

A well-studied model virus is the Cowpea Chlorotic Mottle Virus (CCMV). CCMV is often studied because of its safety, simplicity, and its controllable disassembly and reassembly behavior.4-6 The native CCMV is a positive sense RNA virus that consists of 90 identical capsid protein (CP) dimers and has a diameter of 28 nm and a T = 3 icosahedral symmetry.7, 8 In vitro studies show that CCMV can easily be disassembled into its RNA and free CP dimers upon raising the pH and increasing the ionic strength – reducing both protein-protein as well as electrostatic protein-cargo interactions. Isolated CP can be reassembled into empty capsids at low pH and high ionic strength. Furthermore, the CP can assemble around non-native polyanionic species, for example polymers9-12, nanoparticles13, 14, or genomic material15-17, forming virus-like particles (VLPs) with T = 1, T = 2, or T = 3 icosahedral symmetry, having a diameter of 18 nm, 23 nm, and 28 nm, respectively, depending on the cargo.18 For these reasons CCMV can be used in a wide array of applications.

In most of the work described in literature, capsid proteins assemble into well-defined, stable capsids that are studied at acidic pH around 5. This is due to stronger CP-CP interactions at this pH, which consist of a combination of hydrophobic attractions and electrostatic repulsions. The latter repulsion is stronger when the acidic residue on the Glu81 (pKa 6.5) position of CCMV is
deprotonated at high pH, thus decreasing the CP-CP strength. This is also evident from the swelling of the native CCMV virus at neutral pH in the absence of divalent salts.1, 19, 20

Next to the CP-CP interactions, also CP-cargo interactions play a role in VLP assembly. This binding results from electrostatic attraction of the negatively charged cargo and the positively charged arginine rich motive (ARM) on the N-terminus of CCMV. In the case of CCMV, this interaction is known to be independent of pH, within the stability range of the virus, but it depends strongly on the ionic strength of the solution.21, 22 The combination of CP-CP and CP-cargo interactions drive the assembly, making the assembly more efficient at acidic pH and less efficient at neutral pH.16 Theoretical studies have been used to investigate the effect of varying these interactions, showing a specific range of conditions required for successful VLP formation.23, 24

Besides VLP formation at pH 5, there is also literature proving that CCMV can form well-defined VLPs at neutral pH, where CP-cargo interactions dominate, in the presence of a sufficiently negative template.9, 14, 25 However, the required strength of this interaction and the exact mechanism of assembly at these conditions are currently not yet fully understood. Therefore, the study described in this chapter seeks to determine the minimal number of charges, i.e. the number of nucleotides (nt), needed to induce the virus assembly around oligonucleotides.

We use short, single-stranded DNA (ssDNA) because of its increased stability over ssRNA while having similar characteristics. The selected ssDNA is synthesized in a sequence that cannot form stable hairpins or self-dimers at room temperature reducing the possibility of (favorable) interactions with the proteins, resulting from their secondary structure or sequence.26-28 Understanding the minimal requirements for assembly may increase our general understanding of virus assembly at neutral pH – i.e. under conditions relevant for many applications, especially in the medical field. Furthermore, the encapsulation of short strands of DNA or RNA may be of great interest for antisense oligo DNA and small interfering RNA (siRNA) delivery. Additionally, it can be used to improve encapsulation of other foreign cargo into viral capsids to develop new materials with applications in, for example, medicine, and nanotechnology.
5.2 Results and discussion

5.2.1 Encapsulation of oligonucleotides at neutral pH

To test whether short, ssDNA can be used for the assembly of CCMV CP into VLPs, we mixed CP with ssDNA of 40 nt. To allow the particles to reach full maturity, the samples were purified using size exclusion chromatography (SEC) after one week (Figure 5.1).

![Figure 5.1: (a) SEC trace of CCMV CP mixed with ssDNA of 40 nt. (b) DLS and (c) TEM analysis of the particles formed by CP and ssDNA of 40 nt.](image)

An incubation time of one week is long, compared with encapsulation times required for longer polynucleotides, however, initial experiments showed that for these short nucleotides assembly is slow and requires a week of incubation. Furthermore, the assembly is also strongly influenced by the ionic strength (I) of the assembly solution. Too low salt concentrations (I < 50 mM) cause the CP to precipitate, while at higher salt concentrations (I > 100 mM) assembly does not occur (Figure 5.2).
The chromatogram (Figure 5.1a) shows two peaks: one at an elution volume (V) around 11 mL and one around V = 17 mL. This peak corresponds to the elution profile of the free ssDNA and possibly some residual capsid protein dimers while the 11 mL peak signifies a significant increase in size and possibly the formation of stable particles. After obtaining the material eluting at V = 11 mL, its size distribution in solution is further analyzed using dynamic light scattering (DLS) indicating the presence of monodisperse particles with a hydrodynamic diameter of 19.2 ± 0.2 nm (Figure 5.1b). Analysis using transmission electron microscopy (TEM) confirmed particle formation with particle sizes ranging from 18 to 24 nm with a number average diameter (Mn) = 20 ± 2 nm (Figure 5.1 b-c). This size range corresponds to similar work on RNA encapsulation in CCMV using 140 nt ssRNA chains at pH 5.15,16 The existence of spherical particles in TEM seems to indicate that these are not pre-capsids but fully assembled VLPs corresponding to T = 1 and pseudo T = 2 triangulation geometry. To further confirm the initial findings, small-angle X-ray scattering (SAXS) experiments were carried out with these samples, as well as with wild-type CCMV (WT-CCMV) at pH 7.5. The data obtained during these measurements show curves indicating highly structured assemblies in solution in all cases (Figure 5.3).
Figure 5.3: SAXS data measured (blue dots), P(r) distribution (red line), and form factor fit (grey line) for (a) 40 nt ssDNA + CP and (c) WT-CCMV pH 7.5. And P(r) distribution curves of (b) 40 nt ssDNA + CP, (d) WT-CCMV pH 7.5.

The Guinier approximation was used to obtain a radius of gyration ($R_g$) for these samples (Table 5.1). Particle radii were determined using a pair-distance distribution function (P(r)-distribution) and by fitting the data with a form factor of a homogeneous sphere.29

Table 5.1: Radius of gyration ($R_g$) and particle radii ($R_{P(r)}$ and $R_{model}$) determined for 40 nt ssDNA filled VLPs and WT-CCMV at pH 7.5 using SAXS data.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$R_g$ (nm)</th>
<th>$R_{P(r)}$ (nm)</th>
<th>$R_{model}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 nt ssDNA VLPs</td>
<td>9.4</td>
<td>12.3</td>
<td>12.0</td>
</tr>
<tr>
<td>WT-CCMV pH 7.5</td>
<td>12.2</td>
<td>15.8</td>
<td>15.5</td>
</tr>
</tbody>
</table>

Table 5.1 shows that particle radii determined using the P(r)-distribution and using the model showed similar results. Figure 5.3 shows that the form factor fits
deviate from the experimental scattering curves at high values of q. We fitted the scattering curves using the form factor of homogeneous spheres, such as gold nanoparticles. At larger length scales (low q), this works very well. At shorter length scales (high q), deviations arise from the fact that VLPs are not homogeneous, since they consist out of a protein shell containing DNA. The particle radius is, therefore, determined using this model for q < 0.027.

Furthermore, the ratios of \(R_g/R_{p(r)}\) and \(R_g/R_{\text{model}}\) are in all cases approximately 0.77 which matches the expected ratio of the \(R_g\) and the hydrodynamic radius (\(R_h\)) for a sphere:\textsuperscript{30}

\[
\frac{R_g}{R_h} = \frac{\sqrt{2}}{5} = 0.775 \quad \text{Eq. 5.1}
\]

The P(r)-distribution plots (Figure 5.3) show symmetrical curves, which indicates that the samples are monodisperse. This again suggests that VLPs are properly formed, since random aggregates are expected to be more polydisperse and the SAXS curves of polydisperse particles would not show the distinct features of the form factor of a homogeneous sphere.

Similar results are observed in assemblies using 30 nt ssDNA. However, these samples were further studied by re-analyzing the isolated VLP fraction to test the stability (Figure 5.4).

![Figure 5.4: SEC elution profile of 30 ssDNA + CP VLPs after isolation, concentration, and reinjection in the SEC setup followed by 280 nm light absorption (red dashed line), and 260 nm light absorption (black line).](image)

Reinjection of the isolated VLP peak into the SEC setup shows partial disassembly of the particles. This could be caused by concentration steps.
required, which were performed by spin filtration before reinjection, as the centrifugation can exert strong forces on the sample. It could also indicate that under the applied conditions the formed particles are dynamic, allowing them to swell and disassemble. This is supported by studies of similar particles in which cryo electron microscopy shows clear swelling at neutral pH, causing large pores to occur in the capsids.\textsuperscript{1, 31}

The VLPs containing ssDNA were treated with DNAses to determine whether the protein shell protects the DNA from digestion. Proper protection from DNAses may indicate proper capsid formation \textsuperscript{20}, and is crucial if these constructs would be used for DNA or RNA delivery purposes. In Figure 5.5a, a sample treated with DNAses is compared with a similar, untreated sample.

Figure 5.5: (a) SEC elution profile following the absorbance at 260 nm of 30 ssDNA + CP VLPs after 1 week incubation (red dashed line), and after 1 week incubation + DNAse treatment (black line). (b) SEC elution profile following the absorbance at 260 nm of 30 nt ssDNA (red dashed line), 30 nt ssDNA that was treated with DNAse (black line), and the disassembled particles obtained using SEC of a DNAse-treated mixture of 30 nt ssDNA and CP (blue broken-dashed line).

In both cases particles are observed, however fewer particles are present in the sample treated with DNAses. We suspect this may be caused by the dynamic nature of the particles at neutral conditions observed above. As suggested by Bancroft \textit{et al.}, this may allow more of the DNAse to reach and digest the DNA, causing fewer particles to be left in solution.\textsuperscript{32} Furthermore, the peak of the free
ssDNA shifts from an elution volume of $V = 18$ mL for the untreated sample to an elution volume of $V = 20$ mL for the digested sample. This clearly indicates that the free ssDNA is indeed digested, causing the smaller DNA fractions to elute at a higher elution volume. The VLPs that were present in the DNase treated sample were isolated and disassembled, to determine whether the ssDNA inside the particles was protected from the DNase. Figure 5.5b shows that the ssDNA from the disassembled particles has the same elution volume as untreated ssDNA in the same buffer. This points to protection of the ssDNA inside the particles in line with proper VLP formation. The results presented above are a clear verification that CCMV CP can assemble around ssDNA into well-formed VLPs at neutral pH.

5.2.2 Minimum ssDNA length required for assembly

After the conformation of assembly at neutral pH, we aimed to use ssDNA to study the cargo charge requirements for assembly. This was done by mixing CCMV CP with various lengths of ssDNA, starting from 10 nt. The used ssDNA sequences are similar to a part of the 40 nt ssDNA sequence, thus having a random sequence while avoiding hairpins and self-dimers. A constant concentration and weight ratio of CP to ssDNA was used in all experiments. The assembly behavior was investigated using SEC, DLS, TEM, and gel electrophoresis.

From SEC, a number of significantly different elution profiles were observed over the range of ssDNA lengths. Figure 5.6a-c show the SEC traces in which a transition in behavior is observed. Short lengths of ssDNA (10-13 nt) have a similar elution profile as 13 nt with CP (Figure 5.6a, black lines). This shows a free CP peak at $V \approx 17$ mL and free ssDNA at $V \approx 18.5$ mL, indicating that the two compounds have little to no stable interaction on the timescale of a SEC measurement. From samples with an ssDNA length longer than 13 nt a transition is observed. These show co-elution of the CP and some of the ssDNA, causing an increase of the 260 nm absorption at the CP elution volume (Figure 5.6a, red lines). Similar trends were observed for all samples containing ssDNA of 14-17 nt, however no VLPs, normally eluding at $V = 8-13$ mL, were observed in these cases (Figure 5.6a and b). Besides an increased 260 nm absorption also a widening of the CP peak to smaller elution volumes, starting before $V = 16$ mL, is observed. This suggests that structures with increased size compared to free CP or ssDNA
alone elute from the column. An explanation for this might be that the CP and ssDNA are associating – e.g. in less defined “pre-capsid structures” – without forming full VLPs.

From 18 nt onwards another transition can be observed: a VLP peak occurs, with an elution volume of $V = 11-12$ mL similar to the peak observed previously using 40 nt ssDNA (Figure 5.6b). Initially particles were formed with very low efficiency, however ssDNA encapsulation was found to become more efficient upon increasing the ssDNA length (Figure 5.6b-c, and Figure 5.7b).

**Figure 5.6:** SEC traces of CCMV CP mixed with ssDNA of (a) 13 and 14 nt, (b) 17 and 18 nt, and (c) 18 and 26 nt. (d) DLS and (e) TEM analysis of the particles formed by CP and ssDNA of 25 nt eluting around $V = 11$ mL from the SEC column.
The VLP fractions were isolated and analyzed using DLS and TEM (Figure 5.6d and e). Both TEM and DLS showed particle sizes between $M_n = 18$ and 24 nm, which matches the size of $T = 1$ and $T = 2$ icosahedral symmetry structures observed for CCMV capsids.$^{18}$

The absorption intensities at 280 nm and 260 nm used to detect the SEC outflow were further analyzed and applied to study assembly behavior (Figure 5.7). The trend in CP-ssDNA interaction was investigated by plotting the $\lambda = 280$ nm absorption divided by the $\lambda = 260$ nm absorption at the CP peak ($V = 17$ mL) for the samples with ssDNA ranging from 10-26 nt.

**Figure 5.7:** (a) Ratio of the 280 nm and 260 nm absorption at the CP peak of the SEC trace ($V = 17$ mL). (b) Encapsulation efficiency at varying number of nt per strand of ssDNA, based on the ratio of $\lambda = 260$ nm absorption of the VLP peak ($V = 11-12$ mL) and the ssDNA peak ($V = 18-20$ mL) in the SEC elution profile.

Overall, two trends are observed in this plot. First, with ssDNA ranging from 10-16 nt, the ratio strongly decreases indicating a higher $\lambda = 260$ nm absorption from the DNA, which suggest more of the ssDNA is co-eluting with the CP. The second trend, with ssDNA ranging from 16-26 nt, the ratio is almost stable. Which is an indication that CP and ssDNA interact in similar ratios. The slight increase in the ratio is due to the fact that as the ssDNA gets longer more of the “pre-capsids” mature into VLPs. Due to overlap of the elution peak of ‘free’ 40 nt ssDNA and ‘free’ CP this sample is not included in Figure 5.7a.

The efficiency of ssDNA encapsulation was investigated by studying the ratio of the maximum $\lambda = 260$ nm absorption of the VLP peak ($V = 11-12$ mL) and the maximum $\lambda = 260$ nm absorption of the free ssDNA peak (Figure 5.7b). The
efficiency is defined as the relative amount of encapsulated DNA versus free DNA. We assume that the CP contribution to the $\lambda = 260$ nm signal has a minimal influence on this analysis. As indicated before, the VLPs were observed starting from an ssDNA length of 18 nt. Initially, for 18-20 nt, the efficiency is low and is almost constant. From a length of 20 nt onwards, the encapsulation efficiency increases linearly with the number of nt (Figure 5.7b).

To confirm the SEC results, agarose gel electrophoresis (AGE) with SYBR safe DNA stain was employed. Figure 5.8 shows the AGE results of CP mixed with various lengths of ssDNA after 1 week incubation without purification.

Figure 5.8: (a) SYBR safe and (b) coomassie stained agarose gel. From left to right, lane 1: CP + ssDNA of 40 nt, lane 2: CP + ssDNA of 26 nt, lane 3: CP + ssDNA of 25 nt, lane 4: CP + ssDNA of 23 nt, lane 5: CP + ssDNA of 22 nt, lane 6: CP + ssDNA of 21 nt, lane 7: CP + ssDNA of 20 nt, lane 8: CP + ssDNA of 19 nt, lane 9: CP + ssDNA of 18 nt, lane 10: CP + ssDNA of 17 nt, lane 11: CP + ssDNA of 16 nt, lane 12: CP + ssDNA of 10 nt, lane 13: 50 bp ladder.

In Figure 5.8, starting from 18 nt two species are observed. One band corresponds to the free ssDNA while the other band has significantly lower gel mobility. This is most likely caused by encapsulation of the ssDNA inside the virus shell, which would yield a larger structure with lower mobility in the gel. To confirm the presence of protein, the same gels were stained with coomassie (Figure 5.8b). This staining also reveals two bands: one at the same position as the slowest ssDNA band and one that runs even slower (if at all). The same position of the protein and the ssDNA confirms that the lower mobility is caused by ssDNA-Protein.
interaction. The slower migration of the other protein band is similar to the CP reference and contains no ssDNA. Migration of the CP most likely originates from the net negative charge of CP at the pH used. Both the SEC and the gel are in accordance, showing that the transition of non-assembly to assembly is at 18 nt ssDNA under the conditions employed.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that most of the CP was intact at the time the assembly was observed, forming a band corresponding to a molecular weight of approximately 20 kDa (data not shown). A faint band at approximately 18 kDa is also observed, corresponding to CP that has lost its ARM on the N-terminus. This indicates that the formation of a capsid stabilizes the protein. To exclude particle formation induced by the small fraction of CP missing its N-terminus a sample containing only CP was analyzed using SEC. No particles are observed in this trace suggesting that the interaction with the ssDNA is indeed required for particle formation (data not shown). It is possible that CP that is missing the ARM co-assembles with intact CP in the formed capsids with DNA, but it is not involved in the initiation of the assembly.

The results shown here were obtained one week after mixing the CP and ssDNA. However, further study of these assemblies, after 3 weeks, pointed to a time dependence of the VLP assembly (Figure 5.9).
Figure 5.9: (a) SYBR gold and (b) coomassie stained agarose gel. Top lanes from left to right, lane 1: CP + ssDNA of 20 nt, lane 2: CP + ssDNA of 19 nt, lane 3: CP + ssDNA of 18 nt, lane 4: CP + ssDNA of 17 nt, lane 5: CP + ssDNA of 16 nt, lane 6: CP + ssDNA of 15 nt, lane 7: CP + ssDNA of 14 nt, lane 8: CP + ssDNA of 13 nt, lane 9: CP + ssDNA of 10 nt, lane 10: CP. Bottom lanes from left to right: lane 1: 2-log DNA ladder, lane 2: ssDNA of 20 nt, lane 3: CP + ssDNA of 40 nt, lane 4: CP + ssDNA of 27 nt, lane 5: CP + ssDNA of 26 nt, lane 6: CP + ssDNA of 25 nt, lane 7: CP + ssDNA of 24 nt, lane 8: CP + ssDNA of 23 nt, lane 9: CP + ssDNA of 22 nt, lane 10: CP + ssDNA of 21 nt.

In contrast to Figure 5.8a, in which a second band becomes apparent starting from 18 nt, in Figure 5.9a this second band appears at 14 nt. This suggests that CP and ssDNA have a sufficiently strong interaction to penetrate the gel as a complex. As observed from the coomassie staining of the gel (Figure 5.9b) ssDNA of 13 or 10 nt show a similar trend as the free CP, suggesting little to no interaction between the protein and the ssDNA. Similar to the SYBR safe staining, the coomassie staining revealed a second band from 14 nt onwards. The fact that this band is observed using both stains shows it contains both protein and ssDNA, which could indicate VLP formation. TEM analysis of the sample containing 14 nt ssDNA proved the presence of spherical particles in this sample (Figure 5.10).
Oligonucleotide length dependent formation of virus-like particles

Figure 5.10: TEM image of particles formed by CP and ssDNA of 14 nt.

For the sample containing 13 nt ssDNA no particles could be detected using TEM, in accordance with the data obtained from AGE. Furthermore, if the presence of particles observed in TEM would be caused by the acidic uranyl acetate staining, as suggested in literature, similar particles should be observed in all samples. The fact that below 14 nt ssDNA no particles are observed suggests the TEM results are not influenced by the staining solution.

Models to explain the assembly of CCMV around RNA have been reported, but these do not include the characteristics of CCMV at neutral pH and in the presence of short nucleic acid strands. To expand the current models, incorporating the data described above, we propose the following mechanism for CCMV CP assembly around oligonucleotides into a VLP (Figure 5.11).

Figure 5.11: Schematic assembly of CP dimers (in green, blue and red) around short ssDNA (orange). Step 1: Electrostatic binding of DNA to CP-dimer. Step 2: Clustering of multiple CPs on one or multiple strands of ssDNA. Step 3: Close proximity and combination of hydrogen, hydrophobic, and divalent salt binding causes interprotein binding and folding into pentamers. Step 4: Highly cooperative assembly into VLPs, which is mainly caused by the CP-CP interactions.
This model is partly based on a low cooperativity model,\textsuperscript{34} which is applicable for this virus\textsuperscript{20, 22}. The first step in the process is the electrostatic interaction between the CP and the ssDNA. This interaction is dependent on the ionic strength of the solution and involves CP-cargo interaction. At very small ssDNA lengths, in our experiments 10-13 nt, this interaction was too weak to be stable during the timescale of SEC or AGE. From 14 nt onwards stable interactions between CP and ssDNA were observed. This interaction could involve only one CP dimer with one or multiple strands of ssDNA, but it could also involve multiple CP dimers clustering around one or multiple strands of ssDNA (Figure 5.11, step 2). We suspect that the initial clustering of multiple CP dimers and multiple ssDNA strands strongly depends on the ssDNA length. At small lengths, a single strand of ssDNA is more likely to interact with the two ARMs of a single CP dimer rather than with two ARMs of two different dimers, causing the slower and less efficient assembly. This may be related to an interaction that exceeds the charge of the ARM of a CCMV CP (+10 charges). Apparently, under the conditions applied here, 4 excess charges are sufficient for the binding of multiple CPs, although with low probability. In the formed clusters the local concentration of CP is much higher than in the bulk solution, increasing the probability of further CP-CP interactions which may lead to a rearrangement towards larger protein structures, for example pentamers (Figure 5.11, step 3) or other protein structures.\textsuperscript{35, 36} Such an initial nucleating event is required for viral assembly, as has been shown previously, and explains the time dependence.\textsuperscript{33} The last stage, step 4, of assembly is the formation of full capsids from the substructures.

In our SEC experiments, we have observed that under the used conditions VLP formation around oligonucleotides is slow, taking up to a month for particles to form. In the case of 14 nt ssDNA, ssDNA-CP interaction was observed within a week after mixing, however no VLPs were formed at this time. Since little material elutes from the SEC column between the VLP peak, eluding around $V = 11 \text{ mL}$, and the CP peak, starting at $V = 16 \text{ mL}$, it is likely that the last step is fast, otherwise intermediately-sized structures should have been observed.

As observed in our experiments, VLP formation can take place as soon as ssDNA-CP interaction was observed, from 14 nt. This suggests that, given enough time, even slow and inefficient clustering leads to particle formation. Calculations were
done to investigate whether the observed results could be caused by a change in ionic strength of the solution, however the ionic strength was found to vary little with increasing ssDNA length (data not shown). Other factors, such as temperature or stirring, are also likely to influence the assembly kinetics, however, this was not investigated during this study.

Based on the law of mass action, the concentration of protein and ssDNA in the samples is another factor that is likely to affect the assembly kinetics. To test the influence of concentration a number of samples are prepared with similar CP : ssDNA ratios, but with varying concentrations. Increasing the concentration of the components in the samples led to an increased encapsulation efficiency (see Figure 5.12).

**Figure 5.12**: SEC elution profiles of (a) 13 nt ssDNA, (b) 14 nt ssDNA, and (c) 28 nt ssDNA mixed with CP at various concentrations. The ratio ssDNA : CP was kept constant.
Moreover, a very small particle peak is observed when mixing 13 nt ssDNA and CP at high concentration, which suggests that the minimum ssDNA length for encapsulation depends on the concentration of the components in the solution. This means that the minimum ssDNA length of 14 nt found in the experiments mentioned above is valid under the applied conditions, however it is likely that different results will be obtained if the conditions are varied.

The increase in efficiency of VLP assembly observed from 20 nt onwards could be explained by the fact that the ARM of CCMV CP contains 10 positive charges. This means that a strand of ssDNA of 20 nt is able to exactly compensate the positive charges of a CP dimer. Longer strands would have an excess of negative charge compared to a single dimer, which may increase the potential for interaction with multiple dimers. Additionally, the radius of gyration of the ssDNA increases with increasing number of nt, making it more likely to interact with multiple species in the solution. The assembly of the virus around longer nucleic acid templates, for example around its native viral RNA, is in line with the presented model since the increased length and charge make it function even more efficiently in bringing multiple CPs in close proximity. This will induce even faster and more efficient assembly. Such effects could also be a driving factor for the preferred encapsulation of native viral genetic material in vivo, required for viral replication. Increasing encapsulation efficiency for longer nucleic acid strands is also verified by recent theoretical work by the group of Van der Schoot.38

The model takes the different cargo-CP and CP-CP interactions into account as proposed by Gelbart et al.16 The pentamer intermediates are included in this model because they are essential for imposing icosahedral symmetry on this type of viruses and because they have been observed during mass spectrometry measurements and simulations.23, 39 Furthermore the proposed model corresponds to recent work on the assembly of TMV virus around non-native cargo; initially there is Langmuir adsorption of the CPs on the cargo followed by Hill-type co-operative reorganization of bound proteins to form the capsid.40 This is similar to the assembly steps described in this paper and therefore the model can also be useful to describe other viruses than just CCMV. Furthermore, the results show that a minimal number of charges on a molecule is required for
encapsulation of foreign material in a virus. This may help in creating virus-based materials, for example gene or siDNA delivery formulations.

5.3 Conclusions

We have investigated the assembly of CCMV CP around oligonucleotides. Under the conditions used here, VLP formation starts to occur from an ssDNA length of 14 nt, relating to a minimum of 14 negative charges per strand. From a length of 20 nt onwards we observed a linear increase in the encapsulation efficiency. To explain the observed behavior, we have proposed a pathway for the assembly of CCMV CP around ssDNA. Further study of the assembly is required to confirm the suggested pathway, to study the influence of the law of mass action on the system, and to determine the kinetics of the various equilibria. The minimal charge requirement for the encapsulation of charged species inside a protein cage gives us information about the interactions required for assembly, which in turn could lead to better understanding of virus and virus-like particle assembly in general.

5.4 Acknowledgements

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5.5 Experimental Section

5.5.1 Materials

Chemicals were purchased from Sigma Aldrich unless stated otherwise. The ssDNA '5'-GTAGATGGAACCCGAGAAGGCTGTGGAGTCAATGGA-3' with decreasing length (from '3 to 5' direction) and polyadenosine oligonucleotide sequences were synthesized by Eurofins genomics. Milli-Q water has been obtained by ultrafiltration (MQ, Millipore, 18.2 MΩ). The native wild type CCMV virus is obtained according to literature procedures.41, 42
5.5.2 CCMV coat protein isolation

The CP of CCMV was isolated according to procedures described in the literature.\textsuperscript{42, 43} Wild-type CCMV is disassembled by dialyzing against protein isolation buffer (50 mM Tris-HCl, 500 mM CaCl$_2$, 1 mM DTT, pH 7.5) using a 12-14 k MWCO dialysis membrane with 2 times buffer replacement. The high Ca$^{2+}$ concentration causes the viral RNA to precipitate, and it is removed by 2 h centrifugation at 40,000 RPM (179,200 × g) and 4°C using a Sorvall WX80 ultracentrifuge. The supernatant containing CP dimers was dialyzed against cleaning buffer (50 mM Tris-HCl, 500 mM NaCl, pH 7.5), followed by dialysis, with 3 times buffer replacement, against 5x assembly buffer (250 mM Tris-HCl, 250 mM NaCl, 50 mM KCl, 25 mM MgCl$_2$, pH 7.2). CP was used within 1 day after isolation, and only CP solutions with a 280/260 nm absorbance ratio of at least 1.5 was used.

5.5.3 Encapsulation of ssDNA in CCMV-based virus-like particles

CCMV CP in 5x assembly buffer was diluted to a concentration of 7.5 mg/ml. The ssDNA was dissolved in MQ to a concentration of 1 mg/ml. For the encapsulation, CCMV-CP and ssDNA were mixed in a 5:1 mass ratio of CP to ssDNA and a 4:1 (v/v) ratio of MQ to buffer by adding more MQ, yielding a 1x assembly buffer solution containing 50 mM Tris-HCl, 50 mM NaCl, 10 mM KCl, and 5 mM MgCl$_2$ with a pH of 7.2. This was subsequently incubated for 1 to 3 weeks at 4°C before analysis.

5.5.4 Digestion study

The DNA digestion study was performed by adding 1 µL of benzoase nuclease and 1 µL of DNase I to 50 µL of mixture of 30 nt ssDNA and CP that was incubated for 1 month. After addition of the nucleases, the sample was digested for 1 hour at room temperature before analysis using size-exclusion chromatography (SEC). The SEC-fractions containing particles were isolated and concentrated using spin-filtration. Next, the particles were disassembled by dialysis to a buffer solution containing 50 mM NaCl and 1 M NaCl at pH 7.5, and again analyzed using SEC.
5.5.5 Size-exclusion chromatography

Analysis and purification by SEC was performed using a Superose 6 10/100 GL column on a FPLC-system (GE), eluting at 0.5 mL/min with 1x assembly buffer. Absorption was monitored at 260 nm and 280 nm.

5.5.6 Agarose gel electrophoresis

A 1% agarose gel in TAE buffer with either 1x SYBR safe or SYBR Gold (Thermo Fisher) was loaded with 5 µL unpurified sample mixed with 2 µL gel loading dye and run for 1 hour at 100 V. The gel was imaged using a Gel Doc™ EZ on a UV tray (Bio-Rad). The gel was then destained in MQ followed by 1 hour incubation with bio-Safe Coomassie G-250 stain (Bio-Rad) followed by destaining. It was again imaged on a Gel Doc™ EZ for determination of protein content.

5.5.7 Dynamic light scattering

The size distribution of VLPs in assembly buffer pH 7.2 was measured with dynamic light scattering (DLS) using a Microtrac Nanotrac Wave W3043. Viscosity and refractive index of water and the refractive index of native CCMV (1.54) were used.

5.5.8 Transmission electron microscopy

Samples (5 µL) were applied onto Formvar-carbon coated grids. After 1 min, the excess of liquid was drained using filter paper. Uranyl acetate (5 µL, 1% w/v) was added and the excess of liquid was drained after 15 sec and dried for 30 min at room temperature. The samples were examined on a FEG-TEM (Phillips CM 30) operated at 300 kV acceleration voltages. The size distribution was measured with ImageJ.

5.5.9 SDS-PAGE and densitometry analysis

SDS-PAGE samples are prepared by mixing 10 µL of sample with 9 µL of sample buffer (125 mM Tris-HCl, 20% (v/v) glycerol, 5% (w/v) sodium dodecyl sulfate, 0.02% (w/v) bromophenol blue, pH 6.8) and 1 µL 2-mercaptoethanol. The mixture was heated at 99 °C for 5 minutes to denature the protein, after which the mixture was used to fill the wells of 4-15% stain free pre-cast poly acryl amide gels (Bio-Rad). Precision Plus Protein™ Unstained Protein Standard was added in a separate well. Electrophoreses was conducted at 100 V for 5 min followed by 200
V for approximately 20 minutes. Gels where activated with UV for 5 min on a stain-free enabled UV transilluminator and imaged with a Gel Doc™ EZ system with Image Lab software (Bio-Rad). Using 5 different concentrations of CP as reference the CP content of the purified samples was determined.

### 5.5.10 UV-Vis analysis

All UV-visible absorbance measurements were performed on a Perkin Elmer Lambda 850 spectrometer. Standard Quartz cuvettes with a 1 cm path length were used.

### 5.5.11 Small-angle x-ray scattering

Small-angle x-ray scattering experiments were performed at the B21 beam line of the Diamond Light Source Synchrotron (Harwell, UK). Samples were loaded into a 96-well plate which was placed in an EMBL BioSAXS robot. An automatic sample changer injected 25 µL aliquots of solution into a quartz capillary with a radius of 1.6 mm. This capillary was enclosed in a vacuum chamber, reducing the amount of parasitic scattering. When the sample was present at the right position in the capillary 60 frames of 1 second were collected. Bm 21 has a fixed energy of 12.4 KeV and a sample to detector distance was 4.01 m, resulting in a q-range of 0.0039 < q < 0.40 Å⁻¹. The collected SAXS data were processed (radial averaged, background subtracted and converted to absolute units), using the program SCÅTTER. The latter program was also used to perform a Guinier analysis and to obtain the pair distance distribution function, yielding the radius of gyration and the P(r), respectively. We used Excel for fitting the form factor of a homogeneous sphere to the scattering curves.

### 5.6 References


In biology, a variety of nanometer-size protein cages is found with different functions. Understanding the physio-chemical properties, in particular inside the confinement of a protein cage, helps to predict the behavior and the properties of new materials based on such particles. Here, we investigate the relation between the bulk solution pH and the local pH in the cavity of a model protein cage, based on virus-like particles (VLPs) built from the capsid proteins (CPs) of the cowpea chlorotic mottle virus (CCMV). The pH is a crucial parameter in a variety of processes, e.g., protein-cargo interactions, enzymatic, catalytic, or assembly processes and it is potentially significantly influenced by the high concentration of amino acid residues residing on the interior of the VLPs. The internal pH conditions are studied by the inclusion of a negatively charged, pH-responsive polymer and the results show a systematic more acidic pH of 0.5 unit inside the VLP compared to that of the bulk solution for pH values above pH 6. We explain this shift by invoking a theoretical model based on a Donnan equilibrium, caused by an imbalance between the number of charges on the polyanions and those on the cationic RNA binding domain of the CPs. The model agrees with the experimental data over almost 2 orders of magnitude, while below pH 6 the experimental data points to a buffering capacity of the VLP. These results are a first step in a better understanding of the physio-chemical conditions inside a protein cage and more specifically inside a virus-like particle.
6.1 Introduction

Over the years a variety of protein cages, in particular viruses, have been studied for their application in nanotechnology, which has yielded knowledge about their biological, chemical and physical properties. Due to their diversity, homogeneity, and well-defined dimensional properties, there is increasing interest in the use of viruses or virus-like particles (VLPs); for example, in the fields of biomedicine, materials science, and nanotechnology. Some examples of this work involve encapsulating non-native cargo such as enzymes, polymers, or metal nanoparticles. Virus particles are also used for the formation of larger-scale structures. Improving our understanding of viruses and VLPs is crucial to further advance this field and to find new applications for these particles.

A significant amount of data on the structure of many viruses is already available with sub-nanometer resolution. This data is commonly obtained by crystallography, (cryo) electron microscopy, and atomic force microscopy. Furthermore, the physical properties of viruses, such as subunit interactions, assembly and disassembly behavior, and their response to environmental conditions have been extensively studied, both theoretically and experimentally.

Still, many aspects concerning their physio-chemical properties remain poorly understood. Even for the simplest viruses, that is, those consisting of a single type of protein forming a capsid around the viral genetic material, the exact pathway for subunit assembly that leads to the formation of a virus particle is still under debate. Furthermore, the effects of the protein cage on its cargo, due to crowding of the cargo, the presence of high concentrations of charged species, or possible diffusion limitations across the capsid shell, are not fully understood.

We aim to gain insight in the physio-chemical conditions inside a protein cage. Confinement effects may cause large differences in the physical conditions inside a capsid compared to those in bulk. For example, much work is done towards optimizing catalytic reactions inside protein cage structures. Such processes are often strongly affected by the conditions, such as temperature, pH, viscosity – under which they take place.
Experimental and theoretical determination of the pH inside a virus-like particle

In order to obtain more insight in the interior physio-chemical conditions of VLPs, we determine the pH on the inside of a VLP and relate this to the pH of the bulk solution. Many viruses rely on electrostatic interactions between a positively charged section of the CP and the negatively charged viral genetic material for capsid assembly and stability.\textsuperscript{41, 42} This means that after assembly a large number of charged species are concentrated on the inside of the capsid, which might influence the local proton concentration, \textit{i.e.}, the pH. Previous studies already showed that the isoelectric point inside the PP7 virus capsid (pH \(\approx 11.7\)) differs significantly from the isoelectric point on the outside of the capsid (pH \(\approx 3.8\)), yielding a difference in charge density between the inside and the outside.\textsuperscript{43}

To investigate the pH conditions inside a virus capsid, we encapsulated a negatively charged pH-responsive polymer probe in a capsid formed by cowpea chlorotic mottle virus (CCMV) capsid proteins (CPs). The pH probe is synthesized by including fluorescein methacrylate (FMA) subunits in a polystyrene sulfonate (PSS) polymer chain, forming fluorescein-containing PSS (FMA-PSS). Fluorescein and its derivatives are commonly used for pH sensing purposes in biological systems due to their strong response in the near-neutral pH range.\textsuperscript{44-46} The absorption spectrum and fluorescence emission of the fluorescein subunits are strongly pH-responsive, due to the different protonation states that the molecule can adopt.\textsuperscript{47, 48} At high pH, the fluorescence properties are mainly determined by the dianionic state, while upon lowering the pH the monoanionic state becomes more pronounced.\textsuperscript{48} This allows for a ratiometric pH determination, using the ratio of the fluorescence emission intensity at an excitation wavelength corresponding to the monoanionic maximum excitation wavelength (\(\lambda \approx 490\) nm) divided by the emission intensity while exciting at the dianionic excitation wavelength (\(\lambda \approx 450\) nm). Comparing the fluorescence response of the encapsulated probe with the response of the unencapsulated probe at various pH values gives an indication of the pH conditions inside the capsid. In order to explain the observed differences in pH inside the CCMV-based protein cage, we assumed a Donnan equilibrium of the charged species, which resulted in a model that correlates well in the relevant pH range.
6.2 Results and discussion

6.2.1 FMA-PSS pH sensitivity

To verify the FMA-PSS its’ sensitivity to pH changes, fluorescence spectra were obtained of the polymer solutions at a concentration of 0.5 mg mL\(^{-1}\) (~25 µM) with pH varying from 5.0 to 7.5 (Figure 6.1).

![Excitation spectra at \(\lambda_{em} = 523\) nm and emission spectra at \(\lambda_{ex} = 499\) nm of FMA-PSS at various pH conditions. All spectra are normalized to the maximum intensity at pH 7.5 and point to an increased intensity at higher pH.]

6.2.2 FMA-PSS encapsulation

To test the pH response of FMA-PSS inside a VLP, the polymer was encapsulated inside CCMV based VLPs by mixing the polymer with free CP dimers in solution at neutral pH.\(^{39}\) The formation of capsids was evident from SEC with a UV/Vis detector, which was also used for the purification of the VLPs (Figure 6.2a). The absorbance data is normalized to show an overlapping signal at the different detection wavelengths at an elution volume of approximately \(V = 11\) mL, which is the characteristic elution volume for VLPs. The peak was isolated and analyzed by DLS and TEM (Figure 6.2b and 6.2c). DLS showed that the isolated peak contains particles of \(19.0 \pm 3.8\) nm, which is confirmed by TEM analysis showing spherical particles with an average diameter of \(19.9 \pm 1.1\) nm. These data are in line with the previously confirmed \(T = 1\) icosahedral symmetry for CCMV VLPs formed at neutral pH on a polyanionic template.\(^{39,49}\)
Experimental and theoretical determination of the pH inside a virus-like particle

Figure 6.2: a) Normalized SEC traces by UV/Vis detection of the product formed after mixing FMA-PSS with CCMV CP at neutral pH. b) DLS and c) TEM analysis of the isolated particles after SEC.

The pH response of the FMA-PSS containing particles was measured using fluorescence spectroscopy over a range of pH values in which the VLPs are stable (pH 4-8), as was confirmed by DLS (see Figure 6.3).

Figure 6.3: Diameter of the FMA-PSS VLPs at various pH measured by DLS. Swelling of the particle is observed at increasing pH.
Comparable to the polymer free in solution, the encapsulated polymer responded strongly to the changes in pH, becoming more fluorescent at higher pH (Figure 6.4).

**Figure 6.4:** Excitation spectra at $\lambda_{em} = 523$ nm and emission spectra at $\lambda_{ex} = 499$ nm of VLPs containing FMA-PSS at various pH conditions. All spectra are normalized to the maximum intensity at pH 7.5.

Overlaying the excitation and emission spectra of FMA-PSS in solution and the encapsulated FMA-PSS at the same bulk solution pH clearly shows that the polymer responds different in the two situations (Figure 6.5).

**Figure 6.5:** Comparison of the excitation spectra at $\lambda_{em} = 523$ nm and emission spectra at $\lambda_{ex} = 499$ nm of FMA-PSS in solution (red, dashed line) and encapsulated (black, solid line) FMA-PSS at a) pH 7.0 and b) pH 6.5.

To further study the pH-response of FMA-PSS in bulk solution and inside the VLPs, the ratio between the maximum emission at $\lambda_{ex} = 458$ nm and the maximum emission at $\lambda_{ex} = 499$ nm is compared. Since the protonation state of fluorescein is
strongly dependent on the pH of the solution, the ratio of the emission at both excitation wavelengths is used as a measure for the pH of the solution.\textsuperscript{47, 48} In Figure 6.6 this ratio is plotted against the pH of the solution for both samples.

![Graph showing the ratio of maximum emission at $\lambda_{\text{ex}} = 458$ nm and at $\lambda_{\text{ex}} = 499$ nm at varying pH for FMA-PSS free in solution (red, dashed line) and encapsulated FMA-PSS (black, solid line).]

**Figure 6.6:** Ratio of maximum emission at $\lambda_{\text{ex}} = 458$ nm and at $\lambda_{\text{ex}} = 499$ nm at varying pH for FMA-PSS free in solution (red, dashed line) and encapsulated FMA-PSS (black, solid line).

The pH response of FMA-PSS inside a VLP is different from the pH response of FMA-PSS free in solution, as shown in Figure 6.6. Between pH 6.0 and 8.0 a pronounced shift is observed, corresponding to a more acidic environment for encapsulated FMA-PSS compared to free FMA-PSS. For example, the response of the polymer inside the capsid in a pH 6.5 buffer is similar to the free polymer in a pH 6.0 buffer. This suggests that within this range the polymer senses a more acidic environment inside the capsid than outside.

Between pH 5 and 6, the pH response inside the capsid is almost stable, whereas the free polymer still has a linear response in this range. The lack of change in response suggests that the degree protonation of the fluorescein inside the capsid does not change in this range, implying a much smaller pH variation is sensed by the polymer inside the capsid than outside. This might be due to a buffering effect caused by the carboxylic acid pairs that increase the CP-CP interaction and stabilize the capsid.\textsuperscript{50-52}

To investigate whether the observed response is caused by the encapsulation of the polymer rather than the presence of charged species, several control experiments were performed (Figure 6.7).
Figure 6.7: Ratio of maximum emission at $\lambda_{ex} = 458$ nm and at $\lambda_{ex} = 499$ nm at varying pH for FMA-PSS free in solution (red, dashed line), FMA-PSS with CPΔN26 in solution (black, solid line), FMA-PSS with the N-terminal peptide fragment (N-term) in solution (blue, dashed-dotted line), and FMA-PSS with a high concentration of salt (1.8 M NaCl) in the solution (orange, dotted line).

First of all, the response of the FMA-PSS in combination with CCMV CP lacking 26 amino acids on its N-terminus (CPΔN26) was measured. The N-terminus can be cleaved from the unassembled CP upon prolonged standing. The absence of the N-terminus was confirmed by SDS, showing a reduction of protein mass by approximately 2 kDa, matching the weight of the 26 amino acids (Figure 6.8).

Figure 6.8: SDS-page gel containing: Lane 1: Precision Plus Protein ladder, Lane 2: CPΔN batch 1, Lane 3: CCMV batch 1, Lane 4: CCMV batch 2, Lane 5: CCMV batch 3, Lane 6: CCMV batch 4, Lane 7: CCMV batch 5, Lane 8: CPΔN batch 2, Lane 9: Precision Plus Protein ladder.
These 26 amino acids, known as the arginine rich motif (ARM), form the positively charged tail that points towards the inside of the CCMV capsid. The ARM causes the interaction with negatively charged cargo; without that section, the protein-cargo interaction is reduced and cargo-templated assembly is no longer possible. However, assembly of CPΔN26 can still occur at acidic pH. As can be seen from Figure 6.9, in the presence of CPΔN26 the FMA-PSS responds similarly to the polymer free in solution between pH 5.5 and 8. Below pH 5.5, a deviation from the response of the free probe is observed. Comparable to the encapsulated probe, the probe mixed with CPΔN26 shows a stable response at low pH, presumably caused by a buffering effect of the protein. Competition between the protonation of the protein and the protonation of the fluorescein causes a decreased pH-response below pH 5.5 in comparison to the free polymer.

To specifically test the effect of the presence of the ARM, the pH response of FMA-PSS in the presence of a 26 amino acid peptide matching the ARM of CCMV CP (N-term) was tested. The concentration of N-term was chosen such that it matches the local concentration inside a CCMV capsid. Similarly, to the case of CPΔN26, the response of the polymer in the presence of N-term varied little from the polymer free in solution from pH 5.5 to 8. Below pH 5.5, a slight variation is again observed; however, it is less pronounced compared to CPΔN26.

Finally, the effect of a high concentration of sodium chloride was studied, to test whether a high concentration of charged species affects the protonation of fluorescein at a pH < 5.5 (FMA-PSS HS). Under these conditions, the pH response of FMA-PSS was comparable to that of the polymer in the presence of N-term. This suggests that the observed variations in the presence of N-term are indeed an effect of the high concentration of charged species.

In order to get an understanding of the molecular origin of the pH shift over the protein cage barrier, we developed a model to describe the acidity of the capsid cavity that is based on a Donnan equilibrium across the protein shell. To do this, we consider a virus capsid with a polyanionic cargo that interacts with the polycationic RNA-binding domains on the CPs lining the cavity of the protein shell. Complexation of the polycationic and polyanionic species does not necessarily lead to charge neutralisation. There are strong indications for overcharging, implying the total number of charges on the negatively charged species is larger than that on the positively charged species. If indeed the case, this creates a Donnan potential across the protein shell that draws in mobile ionic...
species, in particular positively charged ones. This implies that the acidity inside of the capsid must be lower than the acidity outside of it.

This is confirmed by the next model: presume there are positively charged sodium ions, negatively charged chloride ions and positively charged hydronium ions in the solution that acts as a reservoir for these species. Sodium and chloride ions are associated with added salt and in the model in addition act as counter ions of the polyionic species present in the capsid’s cavity as well as that for any acid present in the solution. Whether the actual ions present in the solution are sodium and chloride is irrelevant to the model, we merely take them for the sake of assigning names to them.

Let the mole fraction of these ions be denoted $X_\alpha$ with $\alpha = \text{Na, Cl, H}$. The ions can freely move between the bulk solution and the cavity of the capsid through the holes in the shell that are known to be large enough to let ions pass.\textsuperscript{52, 56} Chemical equilibrium between the mobile ionic species across the shell presumes equal chemical potentials. This implies $\ln X_\alpha^\mathcal{C} \pm \phi = \ln X_\alpha^\mathcal{S}$, where the superscripts $\mathcal{C}$ and $\mathcal{S}$ indicate whether they refer to the capsid and solution regions, respectively. Here, $\phi$ denotes the (dimensionless) Donnan potential, entering the equations with a positive sign for the positively charged species and with a negative sign for the negatively charged species. Note that any differences in reference chemical potential is tacitly absorbed in the Donnan potential.

This set of equations needs to be closed by insisting on charge neutrality inside the capsid and charge neutrality in the bulk solution. This gives additional equations 6.1 and 6.2.

\begin{align*}
X_{\text{Na}}^\mathcal{C} &- X_{\text{Cl}}^\mathcal{C} + X_{\text{H}}^\mathcal{C} - Q = 0 & \text{Eq. 6.1} \\
X_{\text{Na}}^\mathcal{S} &- X_{\text{Cl}}^\mathcal{S} + X_{\text{H}}^\mathcal{S} = 0. & \text{Eq. 6.2}
\end{align*}

Here, $Q$ is the net mole fraction of negative charges on the polyionic cargo. If $Q < 0$ then the cavity is undercharged, if $Q > 0$ it is overcharged. The mole fractions of the chlorine species and that of the hydronium species in the bulk solution are known quantities. The quantity $Q$ we fix by fitting the measured acidities in the capsids and the bulk soluiton, $pH^\mathcal{C}$ and $pH^\mathcal{S}$, to the theory. Charge neutrality sets $X_{\text{Na}}^\mathcal{C}$ and $X_{\text{Na}}^\mathcal{S}$.

All other quantities can be expressed in terms of $X_{\text{H}}^\mathcal{S}$ and $X_{\text{Cl}}^\mathcal{C}$. By solving the coupled set of equations, we find that the ratios $X_{\text{Cl}}^\mathcal{C}/X_{\text{Cl}}^\mathcal{S}$ and $X_{\text{H}}^\mathcal{C}/X_{\text{H}}^\mathcal{S}$ are inversely
proportional, so \( \frac{X^C_H}{X^S_H} = \frac{X^S_{Cl}}{X^C_{Cl}} \equiv \alpha \). Notice that \( \log \alpha = \text{pH}^S - \text{pH}^C \).
Furthermore, we find that \( \alpha = \exp \phi \) and that \( \alpha \) obeys a simple quadratic equation:

\[
\alpha^2 = 1 + (Q/X^S_{Cl})\alpha \\
\text{Eq. 6.3}
\]

This already tells us that if the concentration of salt is sufficiently high and \( Q/X^S_{Cl} \ll 1 \), we have \( \alpha = 1 \) and \( \text{pH}^S = \text{pH}^C \). The quadratic equation can be solved exactly, to give equation 6.3.

\[
\alpha = \frac{1}{2} \left( Q/X^S_{Cl} \right) + \frac{1}{2} \sqrt{\left( Q/X^S_{Cl} \right)^2 + 4} \\
\text{Eq. 6.4}
\]

Notice that this does not depend on the pH of the solution. This means the same shift of the pH in the capsid compared to that in the solution for all pH values.

The experimental shift of the pH found in experiments on CCMV CPs, encapsulating FMA-PSS in 150 mM salt in \( T = 1 \) particles, equals 0.37. This corresponds to \( \alpha = 2.3 \), and implies that \( (Q/X^S_{Cl}) = 1.9 \). To verify if this makes sense, we make use of the fact that a \( T = 1 \) virus consists of 60 proteins, where the RNA-binding domains of each protein bear 10 positive charges. In that case, we can write:

\[
Q/X^S_{Cl} = \gamma \times (60 \times 10)/(N_A \times V_C \times [\text{NaCl}] \times 10^3) \\
\text{Eq. 6.5}
\]

Where \( V_C = 4\pi R^3/3 \) the volume of the cavity in m³, with \( R \) its radius in m, \( N_A = 6 \cdot 10^{23} \text{ mol}^{-1} \) Avogadro’s number and [NaCl] the ionic strength in M. Here, \( \gamma \) is a number measuring the degree of overcharging. Estimating \( R = 4 \cdot 10^{-9} \) m, we get \( \gamma = 1.9/25 = 0.076 \) implying an overcharging of a mere 8%. Figure 6.9 shows the validity of the model between pH 6 and 8. At pH < 6 effects of the protein buffering come into play, which has not been accounted for in the model.
Figure 6.9: a) Ratio of maximum emission at $\lambda_{ex} = 458$ nm and at $\lambda_{ex} = 499$ nm excitation at varying pH for FMA-PSS free in solution (blue, solid line), encapsulated FMA-PSS (red, broken line), and encapsulated FMA-PSS shifted over the average difference between a) and b) between pH 6 and 7 (black, broken line). b) pH measured inside the capsid plotted against the bulk pH (blue, solid line), and the line pH inside = pH outside + the average shift between pH 6 and 7 (red, broken line).

Figure 6.9 shows that at pH > 6 the shift caused by the encapsulation is independent of the pH of the bulk solution. When shifting the response of the encapsulated probe (Figure 6.9a; black, solid line) over the average pH difference between the free and the encapsulated probe between pH 6 and 7, it becomes clear that the trend in the response is similar to the free probe at pH above 6; the shifted encapsulated probe’s response (Figure 6.9a; blue, dashed-dotted line) and the free probe’s response (Figure 6.9a; red, dashed line) overlap in this range. Figure 6.9b shows the line of the pH measured inside the capsids plotted against the bulk pH (Figure 6.9b; red, dashed line). Above pH 6 this line shows a linear trend that matches the line pH inside = pH outside + the average shift between pH 6 and 8, which further emphasizes that above pH 6 the encapsulation causes a pH shift that is independent of the bulk pH. This is in line with the outcome of the described model based on a Donnan equilibrium.

6.3 Conclusions

In order to better understand the physio-chemical conditions inside a model protein cage, we measured the pH inside a virus-like particle and compared it to the bulk solution conditions. The obtained data shows that the pH inside such a
Experimental and theoretical determination of the pH inside a virus-like particle

capsid is approximately 0.5 units more acidic between pH 6 and 8, while at pH < 6 the measured response inside the capsid remains constant. The observed shift can be explained by a simple model based on a Donnan equilibrium over the protein shell. This model suggests a slight negative overcharging of the capsid of approximately 8% and explains the experimental pH shift inside the capsid compared to the bulk pH.

Insight in the physio-chemical conditions inside nanometer-size protein cages is crucial for a better understanding of the assembly and dis-assembly processes of these containers, but also their biological relevance. Furthermore, it will be of aid in the design and synthesis of artificial, e.g., virus-based, protein cages that find application in nanoreactors, materials and medicine. The presented combination of experimental and theoretical analyses yield insight that can potentially be generalized to other protein cages, beyond the CCMV VLP discussed in this contribution. While the encased polymer probe gave us insight in the increase acidity inside the CCMV VLP as a function of the bulk pH, only with the help of the theoretical model we were able to present a chemical mechanistic explanation in terms of a Donnan potential caused by the charge imbalance between the cationic RNA binding domains of the CCMV CPs and the encapsulated polyanions.

6.4 Acknowledgements

Prof. Paul van der Schoot is gratefully acknowledged for the theoretical analysis of the data.

6.5 Experimental section

6.5.1 Materials

All chemicals were purchased from Sigma Aldrich and used without further purification. The wild-type CCMV virus is obtained according to literature procedures. Solutions were prepared using Milli-Q water (MQ, Millipore, 18.2 mΩ).

6.5.2 Size-exclusion Chromatography

Analysis and purification by size-exclusion chromatography (SEC) was performed using a Superose 6 10/100 GL column on a fast protein liquid
chromatography (FPLC) system (GE), eluting with 2x diluted PSS encapsulation buffer (25 mM Tris-HCl, 150 mM NaCl, pH 7.5).

6.5.3 Dynamic light scattering

The particle size distribution of the VLPs in the various buffers was determined using dynamic light scattering (DLS), using a Microtrac Nanotrac Wave W3043. The viscosity and refractive index of water and the refractive index of native CCMV (1.54) were used in data processing.

6.5.4 UV-Vis spectroscopy

UV-Vis spectra were measured in a quartz cuvette using a PerkinElmer Lambda 850 UV–vis spectrometer.

6.5.5 Fluorescence spectroscopy

Fluorescence excitation and emission spectra were measured in a quartz cuvette using a PerkinElmer LS 55 fluorescence spectrometer.

6.5.6 High-pressure liquid chromatography

Analysis using high-pressure liquid chromatography (HPLC) was performed using a Waters 2535 Quaternary Gradient Module equipped with an X-Bridge C18 reverse phase column. Analytical HPLC was operated at 0.5 mL/min, always in the presence of 0.1% trifluoroacetic acid (TFA). Elution was performed for 10 min at 90:10 water:acetonitrile, followed by a linear gradient to 100% acetonitrile in 65 min, which was maintained for another 25 min.

6.5.7 Transmission electron microscopy

For Transmission electron microscopy (TEM), 5 µL of sample was drop-cast onto a Formvar carbon-coated copper grid. After 1 min of incubation, the remaining liquid was removed using filter paper (Schleicher & Schuell). The samples were stained using 5 µL of a 1% uranyl acetate in MQ water which was removed after 20 s using filter paper. Samples were imaged using a Philips CM300ST-FEG TEM or a Zeiss Merlin (S)TEM.

6.5.8 Nuclear magnetic resonance

1H Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker 400MHz NMR.
6.5.9 FMA-PSS synthesis

878.4 mg of NaSS (4.26 mmol), 85 mg of FMA (0.21 mmol), and 14 µL of (BIBOE)2S2 (20.7 mg, 0.0457 mmol) were dissolved in 4.2 mL of MQ water. This solution was purged with N2 gas for 45 min. An excess amount of MeOH was also purged with N2 gas for 45 min. Next, 1.4 mL of purged MeOH was added to the aqueous solution using a N2-purged syringe. The solution was purged with N2 gas for an additional 5 min before adding 13.6 mg Cu(I)Br (0.095 mmol) and 29.6 mg BPY (0.19 mmol) as a solid while maintaining a N2 gas purge. After addition of the catalyst, the reaction mixture turned brown, and was stirred under N2 atmosphere for 48 h at RT. After 48 h, the reaction was terminated by opening the flask, causing the reaction mixture to turn from brown to blue indicating oxidation of the Cu(I) catalyst to Cu(II). The copper was removed by running the mixture over a silica gel column (eluent 1:1 H2O:MeOH). After that, the polymer was precipitated from THF. The precipitated solid was filtered off, redissolved in 1:1 H2O:MeOH and again precipitated from THF. The purified polymer was dried for 12 h at 60 °C before analysis using NMR, HPLC, UV-Vis spectroscopy, and Fluorescence spectroscopy.

6.5.10 CCMV Capsid Protein isolation

The CP of CCMV was isolated according to procedures described in the literature.58, 59 Wild-type CCMV is disassembled by dialyzing against protein isolation buffer (50 mM Tris-HCl, 500 mM CaCl2, 1 mM DTT, pH 7.5) using a 12-14 k MWCO dialysis membrane with 2 times buffer replacement. The high Ca2+ concentration causes the viral RNA to precipitate, and it is removed 2 hour centrifugation at 40,000 RPM (179,200 ×g) and 4°C using a Sorvall WX80 ultracentrifuge. The supernatant containing CP dimers was dialyzed against cleaning buffer (50 mM Tris-HCl, 500 mM NaCl, pH 7.5), followed by dialysis, with 3 times buffer replacement, against capsid storage buffer (50 mM NaOAc, 1 M NaCl, 1 mM NaN3, pH 5) and stored at 4 °C for a maximum of 2 weeks until further use. To ensure the purity of the protein, only CP solutions with a 280/260 nm absorbance ratio of at least 1.5 was used.

6.5.11 Removing the N-terminus from capsid protein

For experiments involving CP missing a part of its N-terminus (CPΔN26), the N-terminus was removed as described before.60 Here, CPΔN26 is prepared by
dialyzing isolated CP against PSS encapsulation buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5) for at least two weeks. After, the CP is purified using SEC, and analyzed by SDS-PAGE.

6.5.12 FMA-PSS encapsulation in CCMV VLPs

CP (5-10 mg mL\(^{-1}\)) in PSS encapsulation buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5) was mixed 1:1 into a solution of FMA-PSS in MQ water (weight ratio CP:FMA-PSS = 2.4:1). The mixture was stirred at 4 °C for 1 h. The VLPs formed were purified using size-exclusion chromatography using a 2x diluted PSS encapsulation buffer (25 mM Tris-HCl, 150 mM NaCl, pH 7.5) as eluent. After SEC, the VLPs were concentrated using spin filtration and analyzed using DLS, TEM, UV-Vis spectroscopy, and fluorescence spectroscopy.

6.5.13 pH measurements inside VLPs

For pH measurements inside VLPs, the purified FMA-PSS containing VLPs were dialyzed to a phosphate buffer (25 mM sodium phosphate, 150 mM NaCl, pH 6.0-8.0) or acetate buffer (25 mM NaOAc, 150 mM NaCl, pH 4.0-6.0) of the desired pH, ranging from 4 to 8. The VLPs were concentrated to a concentration of approximately 1.5 mg mL\(^{-1}\), giving a sufficiently high concentration to be able to measure fluorescence at acidic pH. DLS and UV-Vis spectroscopy measurements were performed to check the particle size and concentration. Fluorescence spectroscopy was used to obtain excitation and emission spectra over a range of pH. Excitation spectra were measured using an emission wavelength of \(\lambda_{em} = 523\) nm and emission spectra were measured using both \(\lambda_{ex} = 458\) nm and 499 nm excitation wavelengths.

Control experiments involving CP missing a part of its N-terminus (CP\(\Delta N26\)) were performed using a similar procedure except that CP was replaced by CP\(\Delta N26\).

6.5.14 pH measurements in buffer

For pH measurements in buffer 1 mg mL\(^{-1}\) FMA-PSS in MQ water was mixed 1:1 with a phosphate buffer (50 mM sodium phosphate, 300 mM NaCl, pH 6.0-8.0) or acetate buffer (50 mM NaOAc, 300 mM NaCl, pH 4.0-6.0) of the desired pH. UV-Vis spectroscopy was performed to compare the FMA concentrations in the solutions and fluorescence spectroscopy was used to obtain excitation and
emission spectra over a range of pH. Excitation spectra were measured using an emission wavelength of 523 nm and emission spectra were measured using both $\lambda = 458$ nm and 499 nm excitation wavelengths.

Control experiments involving only the last 26 amino acids of the N-terminus of the CP (N-term) or involving high Ionic strength were performed following the same procedure as used for the pH measurements in buffer, except for the addition of N-term (0.32 mM) or the addition of NaCl (final NaCl concentration in experiment = 1.8 M) respectively to the solutions in these control experiments.

6.6 References

Experimental and theoretical determination of the pH inside a virus-like particle


Chapter 7

New methods for assembly and cargo loading

Various approaches have been developed to use viruses or their components in the formation of new nano-sized materials. A well-studied virus for such purposes is the cowpea chlorotic mottle virus (CCMV), because of its controllable assembly and disassembly behavior. Templated assembly of CCMV based particles generally relies on electrostatic interactions between the capsid protein (CP) and the cargo. Functionalization of the CP often involves protein engineering. Here, we extend the toolbox for CCMV based particle assembly and loading of cargo in such structures. We use nickel-histidine interactions for assembly templated by nickel-polyethylenimine complexes and for the introduction of nitrilotriacetic acid-functionalized ligands. Furthermore, we introduce a single-step kinetic labelling approach to functionalize CCMV CP.
7.1 Introduction

Viruses are emerging as new components in nanotechnology and are used in various fields of research. Application vary from using the full viruses, for example to develop structured materials such as super lattices,\(^1\) to using their components, e.g. their coat proteins, to form particles containing non-natural cargos.\(^6\)-\(^12\) The cowpea chlorotic mottle virus (CCMV) and its capsid protein (CP) are commonly studied for such applications. The assembly of CCMV-based virus-like particles (VLPs), either by CP self-assembly or through templated assembly by polyanionic species, has been studied extensively, and is discussed in chapters 3, 4, and 5 of this thesis.

Besides using electrostatics or protein-protein interactions to form VLPs, other approaches for capsid assembly and loading cargo into CCMV are described in literature. These often involve modification of the capsid protein’s N-terminus, to introduce a functionality that can be utilized to induce capsid assembly or for the coupling of cargo. For example, to introduce a new assembly pathway, elastin-like polypeptides (ELPs) were fused to the CCMV CP (ELP-CP).\(^13\), \(^14\) This fusion product retained the pH responsive assembly behavior of the CCMV coat protein, but capsid formation can also be triggered by a salt- and temperature-response of the ELP part. Well-defined spherical particles of different sizes were observed for the two assembly pathways. It was furthermore shown that the addition of metal ions enhanced the stability of these particles, presumably due to the interaction of the hexahistidine sections in the ELP segment, which are used for protein purification. This allows the use of these ELP-CP VLPs under conditions at which an enzymatic cargo was active,\(^15\) opening up a new approach for the use of non-charged (bio) polymers as templates for capsid formation and for the formation of new responsive materials.

To load cargo into VLPs, without relying on electrostatic or random encapsulation, cargo has been attached non-covalently by means of coiled-coil peptides which were attached to the CPs N-terminus and to the cargo.\(^16\), \(^17\) Another non-covalent approach involves the interaction between Ni\(^{2+}\) and a hexahistidine group (His-tag) that was attached to both the CP’s N-terminus and the cargo.\(^18\) Covalent attachment of cargo to CCMV CP’s N-terminus has been used for capsid loading as well, either by means of protein engineering,\(^17\) or through a sortase A-mediated enzymatic coupling of a cargo to engineered
CCMV CP. A disadvantage of these approaches is that they require modification of the CP through protein engineering, to introduce the required functionalities at the N-terminus. Recently, a two-step approach is reported to covalently functionalize ELP-CP using a 2-pyridinecarboxaldehyde to site-specifically modify the N-terminus, followed by the coupling of a functional ligand using a tetrazine ligation. Since this system is independent of the protein’s N-terminus, it is likely that the same approach can be applied to functionalize wild-type CP instead of ELP-CP.

Here, we describe some of the work we have done towards extending the range of methods to assemble and functionalize CCMV-based VLPs. First, we use the interaction between Ni$^{2+}$ and CP that is N-terminally modified with a His-tag (His-CP) to induce virus assembly around a metal chelating polymer. Second, we use the same interaction to attach metal chelating ligands to His-CP to reversibly introduce functional groups into the VLPs. And finally, we attach functional ligands to wild-type CP, thus avoiding the need for protein engineering of the CP. This is done using a one-step labelling approach, where we specifically target the CP’s N-terminal amine by controlling the pH and the reactants’ concentrations during modification, employing the lower pKa of N-terminal amines (pKa $\approx 6.8$) compared to the pKa of the other amines in the protein (pKa of lysines $\approx 10.5$).

### 7.2 Results and discussion

#### 7.2.1 Assembly of virus-like particles using metal chelating polymers

In an attempt to develop a new method to assemble VLPs at neutral pH, that is not based on electrostatic interactions, we studied the assembly of CCMV CPs around metal chelating polymers. The polymer selected for this purpose is polyethylenimine (PEI), due to its water solubility and metal chelating properties without having negatively charged groups. The interaction between the CCMV CP and the metal-polymer complex is targeted by the N-terminal modification of the CP with a His-tag (His-CP) by means of protein engineering. This system has been shown to allow protein-metal interactions.

PEI was incubated with NiCl$_2$ and dialyzed against pH 7.5 buffer to remove unbound Ni$^{2+}$ prior to mixing with His-CP. The product of mixing was analyzed.
using size-exclusion chromatography (SEC), dynamic light scattering (DLS), and transmission electron microscopy (TEM) (Figure 7.1).

![SEC trace by UV/Vis absorbance of His-CP mixed with Ni-PEI complex. b) DLS measurement and c) TEM image of the fraction eluting from SEC at ~11 mL. For details see Experimental section.](image)

**Figure 7.1:** a) SEC trace by UV/Vis absorbance of His-CP mixed with Ni-PEI complex. b) DLS measurement and c) TEM image of the fraction eluting from SEC at ~11 mL. For details see Experimental section.

The SEC results in Figure 7.1a show a peak at an elution volume of ~11 mL and ~17.5 mL, which correspond to VLPs and free His-CP, respectively. The VLP fraction is isolated for further analysis using DLS and TEM (Figure 7.1b and c). Both techniques show that the fraction contains well-defined particles with a number average particle size of $M_n = 19.3 \pm 3.7$ nm ($M_i = 21.8 \pm 4.5$ nm) according to DLS, and an average diameter of $19.8 \pm 1.7$ nm according to TEM. The second peak, at ~17.5 mL belonging to unassembled His-CP, suggests that the assembly is not complete, and may require more of the PEI-Ni template. Interestingly, when we added a two-fold molar excess of the Ni-PEI complex and increase both the protein as well as the complex concentrations no increase in the assembly efficiency was observed (Figure 7.2). In this case also free Ni-PEI complex, eluting at ~19 mL, is observed.
To be able to compare the assembly efficiencies under the different conditions, we compared the maximum $\lambda = 280$ nm absorption of the VLP peak divided by the maximum $\lambda = 280$ nm absorption of the CP peak, both corrected for the coeluting polymer. In the mixture containing stoichiometric amounts of His-CP and Ni-PEI complex, an encapsulation efficiency of 48% is found. Using an excess of Ni-PEI complex over His-CP, an encapsulation efficiency of 47% is found. These results suggest a limit to the amount of His-CP that assembles that is not related to the protein-complex ratio or the components’ concentrations. An explanation for this may be that CP is known to degrade over time, losing part of its N-terminus. Possibly some of the His-CP lost its N-terminus, and consequently the metal ion-binding His-tag, which prevents it from interacting with Ni-PEI complex. Indeed, upon analyzing the stock solution of His-CP using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), we observe several smaller molecular weight bands below the band of intact His-CP (Mw ~22.4 kDa), which indicate degraded protein. Based on the intensities, we determined that approximately 50% of the protein is still intact, which explains why only ~50% of the His-CP was found to assemble. At this point, no attempts to purify the materials, e.g. by affinity chromatography, have been made.

To prove that indeed interactions of the protein with the metal-chelating PEI are responsible for the assembly of CP into VLPs, we performed several control experiments, which are presented in Figure 7.3.
Figure 7.3: (a), (c), (e), and (g) show SEC traces and (b), (d), (f), and (h) show DLS measurements of a mixture of (a)-(b) His-CP and Ni, (c)-(d) His-CP and PEI, (e)-(f) WT-CP, PEI, and Ni, and (g)-(h) VLPs formed by mixing His-CP with PEI and Ni\textsuperscript{2+}, which were isolated and incubated with EDTA.

We tested the assembly while systematically excluding either the Ni\textsuperscript{2+}, the PEI, or the His-tag on the CP. Furthermore, we tested the stability of the VLPs formed when all components are present upon addition of an excess of a strong metal ion-chelater, ethylenediaminetetraacetic acid (EDTA). Note that none of the SEC traces in Figure 4.3 show elution of VLPs, which generally appear at an elution volume of V \textasciitilde 11 mL. The main signals in Figure 7.3a, -c, and –d with an elution
volume of V ~17.5 mL contain CP. In Figure 7.3g, the main peak (V ~21 mL) likely contains excess EDTA and EDTA-Ni complex, while a small CP peak is observed at V ~17.5 mL. In line with SEC data, DLS measurements of these samples show particle sizes smaller than 6 nm, which is significantly smaller than the size of VLPs (~19 nm) and is in fact near the lower limits of detection for our DLS setup.

The experiments shown in Figure 7.3 prove that all components – Ni\textsuperscript{2+}, PEI, and the His-tag on the CP – are required for VLP formation under the applied conditions. Moreover, removal of the Ni\textsuperscript{2+} from isolated VLPs causes disassembly of the particles. These results show that templated assembly of CCMV CP into VLPs can be induced without relying on electrostatic interactions with a negatively charged template, thus widening the range of cargos that can be introduced. Further research towards using other interactions, such as host-guest systems, is ongoing in our labs. By using stimulus-responsive components, these systems could be triggered, for example by light or an electric potential, to assemble or disassemble.

### 7.2.2 Using trivalent Ni-NTA ligands for VLP functionalization

Continuing on the successful assembly of VLPs templated by Ni-PEI complexes as described in section 7.2.1, here we use nitrilotriacetic acid (NTA) as a Nickelator to couple functional groups to the His-tag on the N-terminus of His-CP. We started by using a commercially available, fluorescently labeled Ni-NTA (F-NTA) to functionalize His-CP’s N-terminus (Figure 7.4).
In the SEC trace of the mixture of His-CP and F-NTA at pH 5, shown in Figure 7.4a, we see a peak at V ~9 mL, corresponding to His-CP VLPs, which form spontaneously at pH 5. Second, we see a small peak at V ~17.5 mL corresponding to unassembled CP, and a peak at V ~21.5 mL having a strong absorption at λ = 500 nm which corresponds to the F-NTA. No absorption at λ = 500 nm is observed in either of the other two peaks. In Figure 7.4b, which shows the SEC trace of the mixture of His-CP and F-NTA at pH 7.5, we see two peaks: first of all, at V ~17.5 mL, the unassembled His-CP elutes, and second, at V ~21.5 mL, the F-NTA elutes. As at pH 5, no coelution of His-CP and F-NTA is observed. From these measurements we conclude that complexation of F-NTA to the His-tag on the CP was unsuccessful. An explanation for this could be the low binding affinity of a Ni-NTA complex with a His-tag. According to literature, changing from a single NTA group to a three NTA groups on a ligand improves the binding affinity three orders of magnitude, with K_D going from µM to nM.\textsuperscript{25} In an attempt to improve the affinity between His-CP and our ligand, we switched from F-NTA to a fluorescently labeled tris-NTA (F-3NTA) (Figure 7.5).
When we mix His-CP with F-3NTA at neutral pH in the presence or absence of Ni$^{2+}$ (Figure 7.5a and b), we see very different structures being formed when we analyze with SEC. When Ni$^{2+}$-ions are present, they can form a complex together with F-3NTA and histidine groups on the His-CP. Since we are working at neutral pH, where no CP self-assembly into capsid occurs, we expected this complexation to show in coelution of His-CP and F-3NTA, giving an absorbance at $\lambda = 490$ nm at the elution volume of His-CP ($V \sim 17.5$ mL). However, the SEC trace in Figure 7.5a shows a peak at $V \sim 11$ mL, corresponding to VLPs. This peak shows absorbance at $\lambda = 490$ nm, indicating the presence of F-3NTA. We isolated this peak and analyzed the particles using DLS and TEM (Figures 7.5c and d) and
found well defined particles with $M_n = 20.1 \pm 4.7 \text{ nm} \ (M_i = 23.3 \pm 3.0 \text{ nm})$ according to DLS and a diameter of $21 \pm 2 \text{ nm}$ in TEM. This indicates that we are indeed forming well defined VLPs. Since we are using an excess of NTA groups (one F-3NTA comprises three NTA groups) compared to Ni$^{2+}$ to prevent unbound Ni$^{2+}$ ions from inducing assembly of the His-CP, we suspect that it is not free Ni$^{2+}$ ions but the multivalent nature of the F-3NTA that induces VLP assembly, even at neutral pH, probably by clustering several His-tags. As observed in Figure 7.5b, in the absence of Ni$^{2+}$ VLPs are not formed. This is also confirmed by DLS measurements of mixtures of His-CP and F-3NTA without Ni$^{2+}$, where inconsistent particle sizes below 10 nm were measured, indicating that no defined particles are formed.

In Figure 7.5a, the presence of the peak at $V \sim 17.5 \text{ mL}$ – the elution volume corresponding to His-CP dimers – indicates that not all protein participates in the assembly. Based on the absorption at $\lambda = 280 \text{ nm}$ in SEC, we calculated that $\sim 50\%$ of the His-CP participates in assembly. Moreover, a peak at $\sim 21 \text{ mL}$ with a high absorbance at $\lambda = 490 \text{ nm}$ suggests that unbound F-3NTA is also present in our sample. This can indicate a shortage of Ni$^{2+}$ ions in our mixture. However, we have used 0.9 Ni$^{2+}$ ions per NTA group, which would leave a maximum of 10% of the F-3NTA to be lacking Ni$^{2+}$ if all Ni$^{2+}$ is chelated by NTA. Comparing the $\lambda = 490 \text{ nm}$ absorbance at 11 mL and 21 mL, we see that significantly more than 10% of our F-3NTA is unbound. This means that either not all Ni$^{2+}$ is being chelated by NTA upon incubation, or a large fraction of our F-3NTA-Ni complexes is unable to bind to the His-CP.

We observed similar behavior in the assembly of His-CP with Ni-PEI complexes, where not all His-CP would participate in assembly. We found that part of the His-CP in the stock solution has lost part of its N-terminus, thus lacking the His-tag that interact with the Ni-PEI complexes (section 7.2.1). Since the same batch of His-CP was used in these experiments, we deem it more likely that partial loss of N-terminus explains why not all His-CP shows an interaction with the F-3NTA-Ni complexes, rather than a shortage of Ni$^{2+}$.

The data shown in this section indicate that tris-NTA ligands can be used to functionalize capsids formed by His-CP. Interestingly, the tris-NTA ligands do not just bind to the hexahistidine sequences on the His-CP’s N-terminus, which would cause the formation of functionalized His-CP dimers, but can induce assembly of His-CP dimers into functionalized capsids even at neutral pH. In
further studies, this system can be used as a new way to reversibly introduce functional groups at the interior of CCMV based protein cages.

7.2.3 Covalent N-terminal functionalization of CCMV CP

The approaches to functionalize CCMV CP and their capsids discussed in sections 7.2.1 and 7.2.2 are both based on the interaction between a hexahistidine sequence on the CP’s N-terminus and Ni²⁺ ions. CCMV CP does not naturally have this hexahistidine sequence at its N-terminus, thus this approach requires protein engineering in order to add this fragment to the CP. Here, we discuss initial experiments to functionalize the CP covalently, without having to modify CP first. Furthermore, we specifically want to target the CP’s N-terminus, to ensure that the functional group that is introduced will end up on the inside of a fully formed capsid. To do this, we make use of the much lower pKₐ of the N-terminal amine (pKₐ ~6.8) compared to the other amines in the protein (pKₐ of lysines ~10.5). Amines are a common target in protein modification, and can, for example, form an amide bond when reacting with N-hydroxysuccinimide (NHS) esters prepared from ligands containing a carboxylate group. By performing such modification reactions at neutral pH, we can specifically target the CP’s N-terminal amine, since they will be deprotonated and available for coupling reactions for a large degree, while other amines remain protonated at this pH.

To covalently modify the N-terminus, we isolated CCMV CP and mixed it with an Alexa Fluor 488 (A488)-labeled NHS ester at pH 7.5. Over a period of one hour, 1.1 equivalent A488 was added to the CP. After coupling, the mixture was analyzed using SEC to see whether the CP was labeled.
SEC analysis of the CP after incubation with A488-labeled NHS ester shows a main peak at $V \approx 17.5$ mL, where CP generally elutes (Figure 7.6a). This fraction also shows absorption of $\lambda = 499$ nm, indicating A488 is attached to the CP (CP-A488). This is also confirmed by UV/Vis spectroscopy, where, after isolation of the fraction eluting at $V \approx 17.5$ mL in SEC, we see an absorption peak at 280 nm related to the protein and a peak at $\lambda = 500$ nm related to the dye (Figure 7.6b).

During preliminary experiments using a more hydrophobic dye, we observed that the modification impaired VLP assembly (data not shown). To test if the CP-A488 can still assemble after modification, we dialyzed it to pH 5 to induce self-assembly into empty capsids and analyzed this sample using DLS (Figure 7.6c). DLS measurement shows that at pH 5 well-defined particles with $M_n = 22 \pm 4.4$ nm ($M_i = 33 \pm 5.6$ nm) are present in the solution, which indicates that the modification does not impair CP assembly.

To identify the location of modification – i.e. whether we succeeded at targeting the N-terminus – we performed a digestion study. This involves digestion of the modified protein using trypsin, which cleaves the protein at the carboxylic side
of lysine and arginine residues. The resulting peptide fractions are subsequently analyzed using mass spectrometry. Due to the specificity of the trypsin, we can \textit{in silico} calculate the mass fractions that are expected based on the amino acid sequence of CP. By comparing the mass spectrum after digestion of the modified CP to the unmodified CP we can determine what mass fractions are added to the spectrum due to the modification, and to what peptide fraction the modification is attached. We were able to identify a few peaks in the spectrum of the modified CP that could be related to the modification using A488 (Figure 7.7).

\textbf{Figure 7.7}: Mass spectra of A488-labeled CP after digestion, zooming on the peak at an m/z (a) 1333 Da (residues 1-8 + A488 + K\textsuperscript{+}), (b) 1665 Da (residues 1-11 + A488 + H\textsuperscript{+}), and (c) 1688 Da (residues 1-11 + A488 + Na\textsuperscript{+}).

The spectrum in Figure 7.7a shows a peak at 1333 Da, which can be correlated to the M+K peak of the first eight amino acid residues of CP (m/z = 780 Da) modified with A488 (Mw = 532 Da, adding 514 Da to the peptide fraction due to loss of H\textsubscript{2}O upon binding). The peaks shown in Figure 7.7b and c, with a m/z of 1665 Da
and 1688 Da, respectively, are correlated to the M+H and M+Na peak of the first eleven amino acid residues of CP, having one missed cleavage site in digestion, (m/z = 1151 Da) modified with A488. Unfortunately, other peaks that are expected based on these results, such as the M+H and M+Na peak of the first eight amino acid residues of CP modified with A488 and the M+K peak of the first eleven amino acid residues of CP modified with A488, could not be identified due to overlap with other peptide fractions formed during digestion of CP. Besides the peaks shown in Figure 7.7, no other peaks related to A488 modified peptide sections could be identified, which is an indication that the modification has indeed only occurred at the CP’s N-terminus.

To obtain more insight in the location of the modification, we attempted to use Förster resonance energy transfer (FRET) to determine the distance between the A488 on our protein acting as a FRET donor, and rhodamine dye on a polystyrene sulfonate (PSS)-based cargo as a FRET acceptor. Upon encapsulation of the rhodamine-labeled PSS (R-PSS) by the CP-A488, the two dyes should be in close enough proximity for FRET to occur, and the inter-dye distance can be calculated according to equations 7.1 and 7.2.

\[
E = \frac{I_{AD}A_{AA} - I_{AA}A_{AD}}{I_{AD}A_{DD}} \quad \text{Eq. 7.1}
\]

\[
E = \frac{R_0^6}{R_0^6 + R^6} \quad \text{Eq. 7.2}
\]

Where \(E\) is the FRET efficiency, \(I_{AD}\) is the acceptor intensity at donor excitation, \(I_{AA}\) is the acceptor intensity at acceptor excitation, \(A_{AA}\) is the acceptor absorbance at the acceptor excitation wavelength, \(A_{AD}\) is the acceptor absorbance at the donor excitation wavelength, \(A_{DD}\) is the donor absorbance at the donor wavelength, \(R_0\) is the distance at which the FRET efficiency is 50%, and \(R\) is the inter-dye distance.

For FRET experiments, we prepared VLPs having both A488-labeled CP and rhodamine-labeled PSS. As controls, also VLPs lacking one of the two dyes were prepared. After mixing, the VLPs were purified using SEC and analyzed using DLS (Figure 7.8).
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Figure 7.8: (a), (c), and (e) show SEC traces and (b), (d), and (f) show DLS measurements of a mixture of (a)-(b) CP-A488 and R-PSS, (c)-(d) CP-A488 and PSS, and (e)-(f) CP and R-PSS.

In the SEC measurements shown in Figure 7.8, we observe a VLP peak with an elution volume of ~12 mL in all samples. Moreover, the absorbance at \( \lambda = 499 \) nm and \( \lambda = 563 \) nm show the presence of CP-A488 and R-PSS, respectively. Indeed, absorbance for both dyes is observed in the VLP peak of the mixture of CP-A488 with R-PSS (Figure 7.8a), the VLP peak of the mixture of CP-A488 and unlabeled PSS only shows absorbance at \( \lambda = 499 \) nm (Figure 7.8b), and the VLP peak of the mixture of CP and R-PSS only shows absorbance at \( \lambda = 564 \) nm (Figure 7.8e). DLS measurements show that all the isolated VLPs indeed have similar diameters, having a \( M_n = 16-17 \) nm (Figures 7.8b, d, and e).
Figure 7.9: (a) Excitation spectra at $\lambda_{em} = 519$ nm and emission spectra at $\lambda_{ex} = 499$ nm of mixtures of CP-A488 and R-PSS (red, dashed line), and CP-A488 and PSS (black, solid line). (b) Excitation spectra at $\lambda_{em} = 583$ nm and emission spectra at $\lambda_{ex} = 563$ nm of mixtures of CP-A488 and R-PSS (red, dashed line), CP-A488 and PSS (black, solid line), and CP-A488 and R-PSS at a higher A488 to R-PSS ratio (blue, dash-dotted line).

Figure 7.9 shows that in VLPs labeled with A488 and containing R-PSS, FRET between the two dyes occurs. This is concluded from the presence of a peak at $\lambda_{em} = 583$ nm when exciting at $\lambda_{ex} = 499$ nm (Figure 7.9a, red, dashed line, FRET signal indicated by the black arrow) and from the increase in the peak at $\lambda_{ex} = 499$ nm while following the emission at $\lambda_{em} = 583$ nm (Figure 7.9b, red, dashed line) compared to VLPs containing unlabeled PSS. Furthermore, at the same CP and A488 concentrations, A488-labeled VLPs containing R-PSS show lower fluorescence intensity originating from the A488 dye compared to those containing unlabeled PSS, due to energy transfer from A488 to R-PSS. Interestingly, when increasing the ratio of A488 to rhodamine dyes, we observe a higher $\lambda_{em} = 583$ nm (R-PSS emission wavelength) fluorescent emission when exciting at $\lambda_{ex} = 499$ nm (A488 excitation wavelength) than at $\lambda_{ex} = 563$ nm (R-PSS excitation wavelength). Even when subtracting the fluorescence emission of A488 at $\lambda_{em} = 583$ nm, we calculate a two times higher fluorescence emission due
to FRET from A488 to R-PSS compared to direct excitation of R-PSS. In this case, the excess of A488 dyes act as antennas and can transfer more energy to the rhodamine dye than the excitation source does, which indicates that the dyes most be in close proximity.

Based on equations 7.1 and 7.2, we calculated an inter-dye distance of 5 to 6 nm. Unfortunately, based on this result we cannot conclude whether the A488 is located on the inside or the outside of the capsid. The protein capsid is ~5 nm thick, which means that when the A488 is on the outer surface of the capsid and the rhodamine on the PSS is very close to the inner surface, the present FRET results are not conclusive.

The measurements that we performed to determine the location of the modification of CP seem to point to modification of the N-terminal amine, especially based on mass spectrometry, however, the results remain inconclusive. To obtain conclusive data, a combination of liquid chromatography and mass spectrometry (LC-MS) could be used, in which the peptide fragments are first separated by chromatography, e.g. high-pressure liquid chromatography (HPLC), followed by mass spectrometry of the separated fractions. By analyzing the flow after chromatography using a UV/Vis detector before mass spectrometry, the fraction showing an absorption of A488 (λ = 499 nm) can be directly related to a certain mass, giving a clear indication of what mass peaks are a result of the modification using A488.

### 7.3 Conclusions

In this chapter, we discuss our initial work to develop new methods to assemble CCMV CP into VLPs, and to functionalize the interior of CCMV CP capsids, based on non-electrostatic CP-cargo interactions. It is shown that assembly of His-CP into VLPs can be induced using metal-to-ligand polymer (i.e. Ni-PEI) complexes. Furthermore, when using the same Ni$^{2+}$-histidine interactions to functionalize His-CP, we show that Tris-NTA ligands chelating Ni$^{2+}$ also induce, unprecedented, VLP assembly of His-CP. In this approach, the functional group – here a fluorescent dye – is introduced into the capsids, which makes this an interesting pathway to reversibly functionalize CCMV CP capsids. We think that the work shown here can be extended to other interactions than metal-to-ligand interactions, for example those relying on host-guest chemistry.
Finally, we studied the covalent functionalization of wild-type CCMV CP. Using a kinetic labeling approach we attempted to target the CP’s N-terminal amine. Mass spectrometry and fluorescence spectroscopy give an indication that this approach may be successful, however the data are remain inconclusive and more experiments are required to prove the location of the modification. If successful, this approach allows for simple modification of wild-type CCMV CP using carboxylate group-bearing ligands, thus allowing for introduction of a cargo without relying on electrostatic interactions or requiring protein engineering of the CP. The work described in this chapter gives us new handles for VLP assembly and functionalization, which broadens the possibilities we have to create new, virus-based materials.

7.4 Experimental section

7.4.1 Materials

All chemicals were purchased from Sigma Aldrich and used without further purification unless stated otherwise. Alexa Fluor 488 succinimidyl ester is purchased from Thermo Fisher. PEI (branched, Mw ~10,000 Da) and methacryloxyethyl thiocarbamoyl rhodamine B are purched from polysciences. The wild-type CCMV virus is obtained according to literature procedures.26, 27 Solutions were prepared using Milli-Q water (MQ, Millipore, 18.2 mΩ).

7.4.2 Size-exclusion Chromatography

Analysis and purification by size-exclusion chromatography (SEC) was performed using a Superose 6 10/100 GL column on a fast protein liquid chromatography (FPLC) system (GE), eluting with 2x diluted PSS encapsulation buffer (25 mM Tris; 150 mM NaCl; pH 7.5).

7.4.3 Dynamic light scattering

The particle size distribution of the VLPs in the various buffers was determined using dynamic light scattering (DLS), using a Microtrac Nanotrac Wave W3043. The viscosity and refractive index of water and the refractive index of native CCMV (1.54) were used in data processing.
7.4.4 UV-Vis spectroscopy

UV-Vis spectra were measured in a quartz cuvette using a PerkinElmer Lambda 850 UV−vis spectrometer.

7.4.5 Fluorescence spectroscopy

Fluorescence excitation and emission spectra were measured in a quartz cuvette using a PerkinElmer LS 55 fluorescence spectrometer.

7.4.6 Transmission electron microscopy

For Transmission electron microscopy (TEM), 5 µL of sample is drop casting onto a Formvar carbon coated copper grid. After 1 min of incubation, the remaining liquid is removed using filter paper (Schleicher & Schuell). The samples are stained using 5 µL of a 1% uranyl acetate in MQ water which is removed after 20 s using filter paper. Samples are imaged using a Philips CM300ST-FEG TEM or a Zeiss Merlin (S)TEM. Particle sizes were determined using ImageJ software.

7.4.7 Nuclear magnetic resonance

$^1$H Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker 400MHz NMR.

7.4.8 Mass measurements

Mass spectra were recorded with a Waters electrospray ionization time of flight mass spectrometer operated in positive ion mode (ESI(+)-ToF, Micromass LCT).

7.4.9 Wild-type capsid protein isolation

The CP of CCMV was isolated according to procedures described in the literature. Wild-type CCMV is disassembled by dialyzing against protein isolation buffer (50 mM Tris-HCl, 500 mM CaCl$_2$, 1 mM DTT, pH 7.5) using a 12-14 k MWCO dialysis membrane with 2 times buffer replacement. The high Ca$^{2+}$ concentration causes the viral RNA to precipitate, and it is removed 2 hour centrifugation at 40,000 RPM (179,200 ×g) and 4°C using a Sorvall WX80 ultracentrifuge. The supernatant containing CP dimers was dialyzed against cleaning buffer (50 mM Tris-HCl, 500 mM NaCl, pH 7.5), followed by dialysis, with 3 times buffer replacement, against capsid storage buffer (50 mM NaOAc,
M NaCl, 1 mM NaN₃, pH 5) and stored at 4 °C for a maximum of 2 weeks until further use. To ensure the purity of the protein, only CP solutions with a 280/260 nm absorbance ratio of at least 1.5 was used.

7.4.10 His-tag modified capsid protein expression and purification

His-CP is expressed according to the procedure described by Minten. Briefly, His-CP is expressed in a colony of BL21(DE3)pLysS (E. coli) cells. One colony is used to inoculate 10 mL of Lysogeny Broth (LB) with ampicillin (0.050 g·L⁻¹) and chloramphenicol (0.025 g·L⁻¹). After 16 h of growth at 37 °C, the culture is transferred to 800 mL of LB and grown until an optical density of 0.4-0.6 is reached. Protein expression is induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 5 hours expression at 32 °C the bacteria are pelleted by centrifugation at 10,000 rpm and 4 °C for 15 minutes. The supernatant is discarded and the pelleted cells are stored at -20 °C.

The cells is lysed using BugBuster® Protein Extraction Reagent according to the manufacturers protocol (Novagen). His-CP is purified by incubating the BugBuster solution with 2 mL of Ni-NTA agarose beads for 1 h at 4 °C. After settling in a column, the flow-through is collected and the column is washed with 40 mL of wash buffer (50 mM Tris-HCl, 25 mM imidazole, and 1.5 M sodium chloride, pH 8.0). The capsid protein is then eluted from the column using approximately 10 mL of elution buffer (50 mM Tris-HCl, 250 mM imidazole, and 1.5 M sodium chloride, pH 8.0). The outflow was collected in small fractions and the presence of protein in these fractions was checked using UV/Vis spectroscopy. The fractions containing His-CP were dialyzed to cleaning buffer (50 mM Tris-HCl, 0.5 M sodium chloride, pH 7.5) for 16 hours, to remove the excess of imidazole. Further purification was performed using SEC, after which the purified His-CP was concentrated using spin filtration, and dialyzed to capsid storage buffer (50 mM sodium acetate, 1 M sodium chloride, 1 mM sodium azide, pH 5) and stored at 4 °C until further use.

7.4.11 Ni-PEI complex templated VLP assembly

A solution of PEI (3 mg mL⁻¹ in MQ) was mixed with a solution of NiCl₂ (10 mg mL⁻¹ in MQ) at a volume ratio of 4:1. After 1 h incubation, the Ni-PEI complex was dialyzed to pH 7.5 Tris buffer (50 mM Tris-HCl, 1 M sodium chloride) both
to change the solvent and to remove unbound Ni\(^{2+}\). His-CP (2 mg mL\(^{-1}\) in pH 7.5 Tris buffer) is mixed with the Ni-PEI complex after dialysis in a volume ratio of 3:1. Samples were incubated at 4 °C for 16 h on a rotator before analysis.

### 7.4.12 Tris-NTA ligand synthesis

Tris-NTA ligands were synthesized according to the reaction steps described in Scheme 7.1. In this scheme, functional groups can be introduced by modifying the amine of product 5 (before NTA deprotection) or product 6 (after deprotection) depending on the properties – e.g. solubility and stability under acidic conditions – of the functional group.

**Scheme 7.1:** Reaction scheme for tris-NTA ligand synthesis
For the synthesis of the various compound we followed procedures described in literature\textsuperscript{29} as described below. Several of the reactions were performed multiple times to increase the yield.

**Compound 1**

H-Lysine(Z)-OtBu.HCl (0.5 g, 1.3 mmol) is suspended in DMF (50 mL), and tert-butyl bromoacetate (1.96 mL, 13.3 mmol) and N,N-diisopropylethylamine (DIPEA) (1.2 mL, 6.6 mmol) are added under nitrogen. The reaction mixture is stirred for 16 h at 55 °C, after which the volatiles are evaporated at 65 °C under reduced pressure. The residue is extracted with cyclohexane : ethyl acetate (3:1) three times. The combined extracts are then concentrated and purified using silica gel column chromatography using hexane/ethyl acetate (4:1) as eluent. Yield: 0.5 g (66 \%) with respect to (wrt) H-Lysine(Z)-OtBu.HCl. Theoretical mass: 564.7, found m/z 565.5 (Mass+H\textsuperscript{+}). 1H NMR (400 MHz, CDCl\textsubscript{3}), δ (TMS, ppm): 1.42 (s, 18H), 1.45 (s, 9H), 1.53 (m, 4H), 1.62 (m, 2H), 3.20 (m, 2H), 3.31 (t, J = 7.2, 1H), 3.44 (dd, J = 16, 8.4, 4H), 5.08 (s, 2H), 7.34 (m, 5H).

**Compound 2**

Compound 1 (0.5 g, 0.88 mmol) is dissolved in 10 mL chloroform, and 1.4 mL trifluoroacetic acid (TFA) and 0.075 mL triisopropylsilane (TIS) are added. The reaction mixture is stirred at 21 °C. After 3 h, methanol (7 mL) and water (4 mL) are added to the reaction mixture. The volatiles are evaporated under reduced pressure. The residue is dried azeotropically with toluene and precipitated in anhydrous ethyl ether. The white precipitate is collected and dried under high vacuum. Yield: 0.1 g (28.5 \%) wrt 1. Theoretical mass: 396.4, found m/z 397.3 (Mass+H\textsuperscript{+}) 1H NMR (400 MHz, CDCl\textsubscript{3}), δ (TMS, ppm): 1.42-1.53 (m, 4H), 1.66 (m, 1H), 1.78 (m, 1H), 3.15 (m, 2H), 3.45 (t, J = 12.8, 1H), 3.63 (dd, J = 18, 9.6, 4H), 5.08 (s, 2H), 7.36 (m, 5H).

**Compound 3**

Compound 1 (0.34 g, 0.6 mmol) is dissolved in 10 mL methanol and 10 \% Pd/C (30 mg) is added under nitrogen atmosphere. The mixture is stirred vigorously for 16 h under H\textsubscript{2} atmosphere at 21 °C. Pd/C is filtered off and solvent of the filtrate is evaporated under reduced pressure. Yield: 0.25 g (97 \%) wrt compound 1. Theoretical mass: 430.6, found m/z 431.6 (Mass+H\textsuperscript{+}). 1H NMR (400 MHz,
New methods for assembly and cargo loading

CDCl₃), δ (TMS, ppm): 1.44 (s, 18H), 1.45 (s, 9H), 1.63 (m, 6H), 2.87 (t, J = 7.2, 2H), 3.29 (t, J = 7.6, 1H), 3.44 (dd, J = 17.2, 9.2, 4H).

**Compound 4**

Compound 2 (83 mg, 0.21 mmol) is dissolved in 4.3 mL anhydrous DMF, and N-hydroxysuccinimide (NHS) (83 mg, 0.72 mmol), 4,4-dimethylaminopyridine (DMAP) (12 mg, 0.10 mmol), and dicyclohexylcarbodiimide (DCC) (204 mg, 1.0 mmol) are added to the solution. The reaction mixture is stirred at 21 °C for 2 h. After, a solution of compound 3 (309 mg, 0.51 mmol) and N,N-diisopropylethylamine (DIPEA) (130 µL, 0.75 mmol) in 2 mL chloroform are added. The mixture is stirred for 16 h at 21 °C, after which the volatiles are evaporated at 65 °C under reduced pressure. The residue is dissolved in hexane: ethyl acetate (1:1) and the solution filtered off urea slurry. The residue is purified using silica gel column chromatography using chloroform/methanol (6:1) as eluent. Yield: 179 mg (52.5 %) wrt compound 2. Theoretical mass: 1634.1, found m/z 1657.1 (Mass+Na⁺). 1H NMR (400 MHz, CDCl₃), δ (TMS, ppm): 1.44 (s, 54H), 1.46 (s, 27H), 1.53 (m, 16H), 1.63 (m, 8H), 3.20-3.31 (m, 12H), 3.44 (m, 16H), 5.08 (s, 2H), 7.34 (m, 5H).

**Compound 5**

Compound 4 (179 mg, 0.11 mmol) is dissolved in 1.2 mL methanol and 10 % Pd/C (4 mg) was added under nitrogen atmosphere. The mixture is stirred vigorously for 16 h under H₂ atmosphere at 21 °C. Pd/C is filtered off and solvent of the filtrate is evaporated under reduced pressure. Yield: 135 mg (82 %) wrt compound 4. Theoretical mass: 1500.0 found m/z 761.5 (Mass+Na⁺+H⁺).

**Compound 6**

In general, for the deprotection of the NTA groups of compound 5, either before or after functionalization, the compound is dissolved in chloroform, and TFA (50 vol% compared to chloroform), and TIS (10 vol% compared to chloroform) are added. The reaction mixture is stirred at 21 °C. After 3 h, methanol (25 vol% compared to chloroform) and water (13 vol% compared to chloroform) are added to the reaction mixture. The volatiles are evaporated under reduced pressure. The residue is dried azeotropically with toluene and precipitated in anhydrous ethyl ether. The precipitate was collected and dried under high vacuum.
**Functionalization Tris-NTA**

For the functionalization of the Tris-NTA moiety, here we dissolved 50 mg (0.033 mmol) of compound 5 in 2.7 mL of chloroform with 133 µL (0.76 mmol) DIPEA. The mixture is purged with argon for 30 min after which 17.9 mg (0.037 mmol) 5/6-carboxyfluorescein succinimidyl ester is added. The reaction mixture is stirred under argon atmosphere for 20 h at 21 °C. The product is purified using silica gel column chromatography with chloroform/methanol (8:1) as eluent. Subsequently the NTA groups were deprotected using the procedure described for compound 6. Due to the low yield the compound could not be properly analyzed, therefore resynthesis of the compound is required to verify the results described here.

**7.1.13 Rhodamine-labelled PSS synthesis**

Rhodamine-labeled PSS was synthesized according to the procedure described in Chapter 4, Section 4.5.3 of this thesis.

**7.4.13 Kinetic labeling of CCMV CP**

To label the N-terminus of CCMV CP, we dialyze the protein (~1 mg mL⁻¹) to a pH 7.5 phosphate buffer (50 mM phosphate, 0.5 M sodium chloride). To 0.6 mL of CP in pH 7.5 phosphate buffer 21.5 µL of Alexa Fluor 688 succinimidyl Ester (1 mg ml⁻¹ in DMSO) is added in 10 fractions over 2 hours. After addition, the solution is stirred at 21 °C for 2 hours and dialyzed to pH 7.5 phosphate buffer for 16 h at 4 °C to remove unbound dye. Subsequently, the labeled CP is purified and analyzed using SEC. The SEC-fractions containing CP are isolated, concentrated using spin filtration, dialyzed to capsid storage buffer (50 mM sodium acetate, 1 M sodium chloride, 1 mM sodium azide, pH 5), and stored until use.

**7.4.14 PSS-templated VLP assembly**

CP (or CP-A488, 5-10 mg/mL) in PSS encapsulation buffer (50 mM Tris; 300 mM sodium chloride; pH 7.5) was mixed 1:1 into a solution of PSS (or R-PSS) in MQ water (weight ratio CP:PSS = 2.4:1). The mixture was stirred at 4 °C for at least 1 h. The VLPs that are formed were purified using size-exclusion chromatography using a 2× diluted PSS encapsulation buffer (25 mM Tris; 150 mM sodium chloride; pH 7.5) as eluent. After SEC, the VLPs were concentrated using spin
filtration and analyzed using DLS, TEM, UV-Vis spectroscopy, and fluorescence spectroscopy.

7.5 References


Polymerization reactions initiated in virus-like particles

Hybrid structures that combine natural and synthetic materials can have unique properties, as they include characteristics of both components. This chapter describes the work that was performed on polymerization reactions inside cowpea chlorotic mottle virus (CCMV)-based capsids, to create virus-polymer hybrids and study potential confinement effects on these reactions. To this end, we synthesized a polystyrene sulfonate-based macrorinitiator for atom transfer radical polymerization reactions and show that it can indeed initiate the polymerization of various monomers. This macrorinitiator is successfully introduced in CCMV-based virus-like particles (VLPs), which ensures that the polymerization reactions are started inside the protein capsid. However, due to VLP instability under polymerization conditions, irreproducibility of results, and hampered polymer analysis due to the protein shell, we are unable to conclude if the polymerization reactions inside the capsids are successful. We suggest several changes to the design that could be incorporated to obtain positive results.
Chapter 8

8.1 Introduction

In nature many chemical processes take place in a confined space. Cells, for example, contain various specialized subcompartments, called organelles, that are separated from the bulk of a cell by a biological semipermeable membrane. The membrane controls transport of species into and out of the organelle, creating a specific environment on the inside.\textsuperscript{1,2} Compartmentalization in a cell allows many otherwise incompatible processes to take place in a single cell simultaneously.\textsuperscript{1,3} Also in bacteria, which do not have organelles, confinement takes place. In this case, enzymes are encapsulated in proteinaceous microcompartments.\textsuperscript{4} Confinement of (cascade) reactions in nature often also leads to increased performance, for example higher efficiency or specificity.\textsuperscript{5} Improved performance due to compartmentalization is likely caused by the environmental conditions inside the confined space; shielding, for example, enzymes from the exterior environment and competing reactions. Furthermore, the effective concentration of materials in a confined space is higher than it would be in bulk, placing reactants and catalysts – in nature often enzymes – in close proximity which favors reaction kinetics.\textsuperscript{6} To utilize the advantages of confinement, scientists have tried to mimic cellular compartmentalization, for example, using liposomes,\textsuperscript{7-9} polymersomes,\textsuperscript{9,10} protein capsids,\textsuperscript{4,5} cubosomes,\textsuperscript{11} and colloidosomes.\textsuperscript{12}

8.1.1 Polymerization reactions in confinement

From both a fundamental and an industrial point-of-view, controlling polymerization reactions with regards to kinetics and polymer features are of interest. The development of highly sophisticated organometallic catalysts has allowed to gain control over many polymerization processes.\textsuperscript{13-16} Furthermore, also in polymer science reactions in a confined space have gained interest, using confinement to influence polymer characteristics such as average molecular weight, molecular weight distribution, degree of branching, or glass transition temperature.\textsuperscript{17,18} Polymerization reactions have been performed in the confinement of various types of synthetic materials, e.g. porous inorganic materials such as anodized aluminum oxide,\textsuperscript{19-21} mesoporous silica,\textsuperscript{18,22-25} and zeolites,\textsuperscript{26-29} showing effects of confinement on the polymer’s properties and the polymerization kinetics.\textsuperscript{30,31} Besides affecting the polymer, such confined
Polymerization reactions initiated in virus-like particles

polymerization reactions allow for the creation of hybrid materials with unique properties.\textsuperscript{20, 24}

Taking advantage of their highly defined structures, bio-based confinements, such as protein cages, have been applied in polymerizations as well, leading to well-defined polymers,\textsuperscript{32, 33} as well as polymer biohybrid structures.\textsuperscript{34-39} Such hybrid structures comprise characteristics of both components, showing, for example, biocompatibility and cell permeation properties of the protein component, while having the non-natural functionalities introduced by the polymer.

In our labs, research is being performed in this direction using the cowpea chlorotic mottle virus (CCMV). Earlier, free radical polymerization of sodium styrene sulfonate inside empty capsids, formed by self-assembly of CCMV’s capsid protein (CP), has been studied. In this research, photopolymerization, using mild conditions that are compatible with the virus protein, was initiated using the anionic dye Eosine Y. The dye was incorporated in the capsid by random encapsulation, but due to its anionic nature, an improved encapsulation, caused by electrostatic interactions with the positively charged capsid interior, was expected. The results suggest that the structure and size of the polymers formed under these conditions were determined by the capsid. However, due to lack of conclusive results and poor reducibility, this approach was not further pursued.

Here we describe the results when using atom transfer radical polymerization (ATRP) with a polystyrene sulfonate (PSS)-based macroinitiator, that can template CCMV CP assembly into virus-like particles (VLPs). This forces the initiator to be located on the inside of the capsid. This approach is chosen because it combines the high stability of PSS-containing VLPs with the control over the polymerization reaction that ATRP offers. In this way, we attempt to perform polymerization reactions inside CCMV capsids in a more controlled fashion.

8.2 Results and discussion

8.2.1 PSS-based macroinitiator

In our initial design, we aimed to synthesize PSS using ATRP, and use the bromine-group at the end of the chain after ATRP as the initiator in subsequent polymerization reactions. However, this approach proved unsuccessful, since no
polymerization was observed using ATRP-synthesized PSS as a macroinitiator. Possibly, the bromine-group is lost in one of the purification steps after ATRP, making the polymer unreactive towards re-initiation. Therefore, we designed a new macroinitiator, involving the coupling of thiol-functionalized PSS with a maleimide-functionalized initiator (Scheme 8.1).

**Scheme 8.1:** Synthesis of the PSS macroinitiator

Using the bifunctional disulfide-containing ATRP initiator, Bis[2-(2′-bromoisobutyryloxy)ethyl]disulfide (BiBOE₂), for the synthesis of PSS, we
synthesized PSS containing a disulfide bond. After purification, we treat the polymer with dithiothreitol (DTT) to reduce the disulfide bonds, yielding PSS with a thiol group as chain end. This thiol-functional PSS is coupled to a maleimide-bearing ATRP initiator using a Michael-addition reaction, yielding the PSS-based macroinitiator (PSS-In).

To prove that PSS-In can initiate polymerization, we tested it in ATRP reactions of several monomers under conditions that are compatible with CCMV CP. Under these conditions, we successfully polymerized 2-hydroxyethyl acrylate (HEA), N-hydroxyethyl acrylamide (HEAA), 2-hydroxyethyl methacrylate (HEMA), and 2-(Dimethylamino)ethyl methacrylate (DMAEMA). Most efficient is the polymerization of HEMA initiated by PSS-In in PBS (Figure 8.1).

![Figure 8.1: NMR spectra of ATRP reactions of HEMA in PBS using PSS-In to initiate polymerization at T = 0 (black line) and T = 1 h (gray line).](image)

Figure 8.1 shows a comparison between a nuclear magnetic resonance (NMR) spectra before starting the polymerization of HEMA from PSS-In and at one hour after initiation. The broad peaks at $\delta \sim 0.75$ ppm and at $\delta = 3.25 - 4.25$ ppm that are visible in the gray spectrum (after one hour) are related to the polyHEMA that is formed. The conversion after one hour was determined using the integral of the peak at $\delta \sim 5.6$ ppm, related to the monomer, and the peaks between $\delta = 3.25$ and $4.25$ ppm, related to the monomer and the polymer. We corrected for the contribution of the monomer to the integral between $\delta = 3.25$ and $4.25$ ppm, and determined a conversion of $\sim 56\%$ under these conditions. In control reactions excluding either PSS-In or the catalyst, no polymerization is observed (data not shown).
8.2.2 Polymerization reactions inside VLPs

Having shown that PSS-In can induce the polymerization of HEMA, we want to use this macroinitiator for polymerization reactions inside a CCMV capsid. To this end, we mix PSS-In with isolated CCMV CP, and, after at least one hour of incubation, we analyze the sample using size-exclusion chromatography (SEC), dynamic light scattering (DLS), and transmission electron microscopy (TEM) (Figure 8.2).

Figure 8.2: a) SEC trace followed by UV/Vis absorbance of CCMV CP mixed with PSS-In. b) DLS measurement and c) TEM image of the fraction eluting from SEC at $V \sim 11 \text{ mL}$. For VLP assembly, CP (5-10 mg/mL) in PSS encapsulation buffer (50 mM Tris; 300 mM NaCl; pH 7.5) is mixed 1:1 into a solution of PSS-In in water (weight ratio CP:PSS-In = 2.4:1).

The SEC results shown in Figure 8.2a show a peak at an elution volume of $V \sim 11 \text{ mL}$, corresponding to VLPs. This VLP fraction is isolated for further analysis using DLS and TEM (Figure 8.2b and c). Both techniques show that the fraction contains well-defined particles with a number average particle size ($M_n$) = 16.7 ± 4.1 nm ($M_i$ = 19.0 ± 3.9 nm) according to DLS, and an average particle size of 20.3 ± 1.8 nm according to TEM. These results indicate successful encapsulation of PSS-In into CCMV CP based capsids.
After encapsulation of PSS-In, we performed experiments using the VLPs as initiators for polymerization. Since PSS-In is located inside the particles, polymerization should only be initiated inside the capsids. However, upon performing these experiments we discovered issues with our design. First of all, the stability of the VLPs proved to be poor under the polymerization conditions, causing precipitation of the particles from solution. Systematically testing the stability of the VLPs after each step in the sample preparation gave highly irreproducible results, showing precipitation in some but not all cases. Furthermore, we varied the conditions at which polymerizations were performed, tuning both pH and ionic strength to improve particle stability. These variations did not improve the reproducibility of the experiments. Differences were even observed in experiments where the same batch of reactants were used. Due to the inconsistency in these results, we are currently unable to identify the cause, or causes, of the precipitation.

A second issue is observed in the samples where no precipitation occurred. Analysis of the polymer is hampered by the protein capsid. For example, the NMR spectra before and after polymerization, using the VLPs containing PSS-In to induce polymerization of HEMA, are very similar (Figure 8.3).

![Figure 8.3: NMR spectra of ATRP reactions of HEMA in PBS using VLPs containing PSS-In to initiate polymerization before (black line) and after (gray line) reaction.](image)

However, more important than the similarity between the two spectra is that in the samples none of the peaks associated with PSS or polyHEMA can be clearly identified. Similar issues are encountered using different analytical techniques, e.g. SEC, where the presence of the capsid interferes with analysis of the polymer.
This makes it impossible to conclude if, and to what degree, polymerization is occurring.

The obvious solution to this would be to disassemble the VLPs, and separate the polymer from the CP. However, we found that PSS-containing VLPs are highly resistant to disassembly. Even at ionic strengths over 1 M, no disassembly was observed. Also, when applying protein denaturing conditions we were unable to isolate the polymer. Under these conditions undefined aggregates are observed that precipitate from the solution, containing both the polymer and the protein that were in the solution before the treatment. This is likely due to the strong interaction between CP and PSS, as determined in chapter 4 of this thesis.

8.2.3 Discussion

A technique that could prove whether polymerization occurs inside the particles, using the approach described here, is mass spectrometry (MS). Employing native MS techniques the mass of intact viruses can be measured with high accuracy.\(^40, 41\) By measuring the mass of the VLPs containing PSS-In before and after polymerization the difference between the two indicates whether, and to what degree, polymerization has occurred. This method of analysis requires that the VLPs are purified extensively, removing any randomly encapsulated impurities — e.g. unreacted monomer — to ensure that any mass change is due to polymer growth in the VLPs, and not to random entrapment of species in the capsid. Furthermore, this approach assumes that the amount of PSS inside the VLPs is constant. This assumption is not trivial; as mentioned in chapter 4, previous research has shown that depending on the assembly conditions different amounts of cargo, over a wide range of CP/cargo charge ratios, can be encapsulated.\(^42, 43\) However, based on the stability of the VLPs we observe here we deem it likely that, after formation, no transfer of PSS into or out of the capsids takes place. Neverthelesss, this aspect needs to be verified if mass spectrometry would be used to analyze the polymerization reactions attempted here.

Due to the complications associated with the system where polymerization is induced inside a capsid using PSS-In, we pursued this approach no further. We think that a different design is required to properly study these reactions. This could involve changing the PSS for another polyanion, such as single-stranded (ss)DNA which has a weaker interaction with CCMV’s CP. Another option is to anchor, either covalently or non-covalently, small initiator molecules to the CP,
for example using the tris-NTA ligand or the labeling technique described in chapter 7 of this thesis. Such an approach has the advantage that the polymerization is performed in capsids that are empty, rather than being partially filled with PSS. This leaves more room for a polymer to grow during reaction. Moreover, without the stabilization of the PSS, we expect that in these approaches the capsids can be disassembled, yielding either CP-polymer hybrid structures, or free CP and free polymer, using the covalent and non-covalent approach, respectively. Redesigning the system in such a way would facilitate the analysis, making it possible to determine if polymerization is indeed occurring inside the capsids.

Lastly, here we apply ATRP for polymerization reactions. In future work, another technique may be considered, for example a photopolymerization technique. Such techniques omit the need for a metal catalyst system and the removal of oxygen from the system and may decrease the likelihood that VLPs precipitate during polymerization. This may also improve the reproducibility of the results due to the reduction of process steps.

### 8.3 Conclusions

This chapter describes the work performed towards conducting polymerization reactions inside a CCMV-based capsid. The additional control over a polymer’s properties by synthesizing it in the confined space of a viral capsid is both fundamentally as well as industrially relevant. Moreover, the protein-polymer hybrid structures that are formed in such a process may be of interest from a materials science point-of-view.

We synthesized an ATRP macroinitiator comprised of a PSS chain modified with an initiating group and show that it can indeed initiate ATRP reactions. Subsequently, it was successfully used to template CCMV CP assembly into VLPs. In this way, we encapsulate the initiating group, thus ensuring that the polymerization reaction starts inside the capsid. Unfortunately, using this design, we encountered several issues concerning VLP stability and polymer analysis, making it impossible to discern if, and to what degree, polymerization inside the VLPs is occurring.

We suggest that for further research towards polymerization reactions inside CCMV capsids, the system should be redesigned, for example by covalent or
non-covalent coupling of an initiator to the inside of CCMV-based capsids. Furthermore, to improve VLP stability and result reproducibility, another polymerization technique instead of ATRP could be used. With these modifications, we think polymerization reactions inside CCMV-based capsids are feasible.

8.4 Experimental section

8.4.1 Materials

All chemicals were purchased from Sigma Aldrich and used without further purification unless stated otherwise. The wild-type CCMV virus is obtained according to literature procedures. Solutions were prepared using Milli-Q water (MQ, Millipore, 18.2 mΩ).

8.4.2 Size-exclusion Chromatography

Analysis and purification by size-exclusion chromatography (SEC) is performed using a Superose 6 10/100 GL column on a fast protein liquid chromatography (FPLC) system (GE), eluting with 2x diluted PSS encapsulation buffer (25 mM Tris; 150 mM NaCl; pH 7.5).

8.4.3 Dynamic light scattering

The particle size distribution of the VLPs in the various buffers is determined by dynamic light scattering (DLS), using a Microtrac Nanotrac Wave W3043. The viscosity and refractive index of water and the refractive index of native CCMV (1.54) were used in data processing.

8.4.4 UV-Vis spectroscopy

UV-Vis spectra are measured in a quartz cuvette using a PerkinElmer Lambda 850 UV–vis spectrometer.

8.4.5 Transmission electron microscopy

For Transmission electron microscopy (TEM), 5 μL of sample is drop cast onto a Formvar carbon coated copper grid. After 1 min of incubation, the remaining liquid is removed using filter paper (Schleicher & Schuell). The samples are stained using 5 μL of a 1% uranyl acetate in MQ which is removed after 20 s using
filter paper. Samples are imaged using a Philips CM300ST-FEG TEM or a Zeiss Merlin (S)TEM.

8.4.6 Nuclear magnetic resonance

\(^1\)H Nuclear magnetic resonance (NMR) spectra are recorded using a Bruker 400 MHz NMR.

8.4.7 Mass spectrometry

Mass spectra are recorded with a Waters electrospray ionization time of flight mass spectrometer operated in positive ion mode (ESI(+) -ToF, Micromass LCT).

8.4.8 Wild-type capsid protein isolation

The CP of CCMV was isolated according to procedures described in the literature.\(^{44, 45}\) Wild-type CCMV is disassembled by dialyzing against protein isolation buffer (50 mM Tris-HCl, 500 mM CaCl\(_2\), 1 mM DTT, pH 7.5) using a 12-14 k MWCO dialysis membrane with 2 times buffer replacement. The high Ca\(^{2+}\) concentration causes the viral RNA to precipitate, and it is removed by 2 h centrifugation at 40,000 RPM (179,200 \(\times\)g) and 4°C using a Sorvall WX80 ultracentrifuge. The supernatant containing CP dimers was dialyzed against cleaning buffer (50 mM Tris-HCl, 500 mM NaCl, pH 7.5), followed by dialysis, with 3 times buffer replacement, against capsid storage buffer (50 mM NaOAc, 1 M NaCl, 1 mM NaN\(_3\), pH 5) and stored at 4 °C for a maximum of 2 weeks until further use. To ensure the purity of the protein, only CP solutions with a 280/260 nm absorbance ratio of at least 1.5 was used.

8.4.9 Disulfide-containing PSS synthesis

4.9 g of sodium 4-vinylbenzenesulfonate (23.7 mmol) and 106 mg of Bis[2-(2'-bromoisobutyryloxy)ethyl]disulfide (0.24 mmol) are dissolved in 21 mL of MQ. This solution is purged with N\(_2\) gas for 45 min. An excess amount of methanol is purged with N\(_2\) gas for 45 min as well. After, 7 mL of purged methanol is added to the aqueous solution using a N\(_2\)-purged syringe. The solution is purged with N\(_2\) gas for an additional 5 min before adding 68 mg copper(I) bromide (0.47 mmol) and 150 mg 2,2'-Bipyridyl (1.0 mmol) as a solid while maintaining a N\(_2\) gas purge. After addition of the catalyst, the reaction mixture turns brown, and is stirred under N\(_2\) atmosphere for 24 h at 21 °C. After 24 h, the reaction is terminated by opening the flask, causing the reaction mixture to turn from brown
to blue indicating oxidation of the Cu(I) catalyst to Cu(II). The copper is removed by running the mixture over a silica gel column (eluent 1:1 H2O:MeOH). Next, the polymer was precipitated from THF. The precipitated solid was filtered off, redissolved in 1:1 H2O:MeOH and again precipitated from THF. The purified polymer was dried for 12 h at 60 °C before analysis using NMR. Based on the conversion determined by NMR the molecular weight of the PSS is ~20 kDa, meaning two times 10 kDa per disulfide.

8.4.10 Maleimide-functionalized initiator synthesis

The maleimide-functionalized initiator is synthesized according to Scheme 8.2. We performed this two-step synthesis according to procedures described in literature.46

Scheme 8.2: Synthesis of maleimide-functionalized ATRP initiator

![Scheme 8.2: Synthesis of maleimide-functionalized ATRP initiator](image)

**Compound 1**

2-(2-aminoethoxy)ethanol (1.35 g, 13 mmol) is dissolved in saturated NaHCO3 (65 mL) and cooled in an ice bath. N-(methoxycarbonyl)maleimide (2.0 g, 13 mmol) is added in fractions, and the solution is stirred for 20 min. After, the ice bath is removed and the solution is stirred at 21 °C for 30 min. The solution is extracted with chloroform (3 × 50 mL) and the combined extracts are dried using sodium sulfate. Compound 1 is obtained as an oil upon removal of the solvent under reduced pressure. Yield: 1.39 g (58 %) Theoretical mass: 185.2, found m/z 183.2 (Mass+H`). 1H NMR (400 MHz, CDCl3), δ 2.32 (t, 1 H, OH), 3.54 (m, 2 H, CH2), 3.64 (m, 6 H, CH2), 6.72 (s, 2 H).
**Compound 2**

All glassware was dried at 120 °C for at least 16 h before use. Compound 1 (0.11 g, 0.59 mmol) is dissolved in 15 mL of dry methylene chloride in 2-neck round bottom flask equipped with an addition funnel and is cooled to 0 °C. N,N-diisopropylethylamine (DIPEA, 124 μL, 0.71 mmol) is added via syringe. A solution of 2-bromoisobutyryl bromide (73 μL, 0.59 mmol) in 5 mL of dry methylene chloride is added dropwise over 10 minutes. The reaction mixture was warmed to 23 °C and stirred under argon for 12 h. Next, the solvent is removed in under reduced pressure and the crude product is purified by silica gel column chromatography (50:50 hexane:ethyl acetate; Rf = 0.6). Yield: 0.12 g (61%). Theoretical mass: 334.2, found m/z 356.2 (Mass+Na⁺). 1H NMR (400 MHz, CDCl3), δ 1.92 (s, 6H, CH₃), 3.60 (m, 6H, CH₂), 4.21 (m, 2H, CH₂), 6.70 (s, 2H, maleimide), 13C (400 MHz, CDCl3): δ 171.6, 170.6, 134.2, 68.2, 67.9, 65.0, 55.7, 37.2, 30.7.

8.4.11 Macroinitiator synthesis

Disulfide-containing PSS and dithiothreitol (DTT) are dissolved in MQ such that the final PSS concentration is ~3 mg mL⁻¹ and the PSS : DTT molar ratio is 1:10. This solution is stirred for 16 h at 21 °C, after which the DTT is removed by washing three times with MQ using spin filtration. Subsequently, the MQ is replaced by washing two times with phosphate buffered saline (PBS, 10 mM sodium phosphate, 137 mM NaCl, 3 mM KCl, pH 7.4). The PSS solution is concentrated to the initial volume, giving a concentration of ~3 mg/mL, and 10 equivalents of compound 2 – the maleimide-functionalized initiator – are added. Note that the number of PSS chains has doubled upon reduction of the disulfides in the original PSS. The solution is stirred for 16 h at 21 °C, followed by extensive washing with spin filtration to remove the unbound compound 2. By comparing the integrals of the NMR signals at ~7.5 ppm, related to the PSS, and at ~3.5 ppm, related to compound 2, we determined ~86 monomers NaSS per initiator group. Based on the initial number of monomers per thiol group (~54), this gives a degree of functionalization of 63%.

8.4.12 General polymerization protocol

Polymerization reactions using PSS-In, either encapsulated or free in solution, are generally performed according to the following protocol. Initiator (~1 μM) and monomer, at a molar ratio of 1:100, are dissolved in PBS in a Schlenk flask. To
remove oxygen from the system, the solution is frozen in liquid N\textsubscript{2} under vacuum, followed by thawing under N\textsubscript{2} atmosphere. These freeze-thaw cycles are performed five times. In the fifth cycle, copper(I) bromide (2 eq. compared to initiator) and 2,2'-Bipyridyl (3 eq. compared to initiator) are added to the frozen solution under N\textsubscript{2} gas flow, and dissolve during thawing starting the polymerization reaction. To end the reaction, the flask is opened which causes deactivation of the copper catalyst. After, the products are purified using spin filtration or SEC and are analyzed using techniques such as NMR, UV/Vis spectroscopy, DLS, and TEM.

### 8.4.13 PSS-templated VLP assembly

CP (5-10 mg/mL) in PSS encapsulation buffer (50 mM Tris; 300 mM NaCl; pH 7.5) is mixed 1:1 into a solution of PSS-In in MQ water (weight ratio CP:PSS-In = 2.4:1). The mixture is stirred at 4 °C for at least 1 h. The VLPs that are formed are purified by SEC using a 2× diluted PSS encapsulation buffer (25 mM Tris; 150 mM NaCl; pH 7.5) as eluent. After SEC, the VLPs were concentrated using spin filtration and analyzed using DLS and TEM.

### 8.5 References

Polymerization reactions initiated in virus-like particles

Polymerization reactions initiated in virus-like particles


Summary

Viruses provide a whole new set of building blocks for the development of new materials and as such have found application in, for example, materials science, nanotechnology, and medicine. Their monodispersity and high degree of symmetry surpass any synthetic nanoparticle currently available, and due to the huge amount of different viruses found on Earth there are many shapes and sizes to choose from. In particular the capsid of viruses, that surrounds their genome, is commonly studied. This protein shell transports and protects the genome, and releases the viral genetic material once it has entered a host cell to allow reproduction of the viral components. After reproduction, these reassemble into full viruses which are released from the host. This assembly and disassembly behavior requires specific inter-subunit interactions to provide stability of the virus during transportation while allowing disassembly to infect a host. Both for the purpose of understanding viral reproduction and treating viral infections as well as for using viral components for various applications and being able to predict the structures that will be formed from them, gaining insight in these interactions is crucial.

The work described in this thesis extends our knowledge on viral assembly, by studying the assembly and confinement conditions of the cowpea chlorotic mottle virus (CCMV). By introducing microscale thermophoresis (MST) as a new way to study (self-)assembly, we were able to study and compare the assembly behavior of native CCMV capsid protein (CP) with that of two genetically modified versions of this protein over a wide range of conditions (Chapter 3). MST was also used in combination with isothermal titration calorimetry (ITC) to study CCMV CP assembly into virus-like particles (VLPs) templated by polyanionic species at neutral pH (Chapter 4). Continuing the study of polyanion-templated assembly, we determined the minimum length of single-stranded (ss)DNA, correlating to a minimal electrostatic interaction, required to induce viral assembly at neutral pH (Chapter 5).

To gain insight into the physio-chemical conditions inside a protein cage, we studied the pH conditions inside CCMV-based capsids, using a negatively charged, pH-responsive fluorescent probe (Chapter 6). These results, combined with a theoretical model, show that the pH inside a protein cage is not necessarily
the same as in the bulk solution, which may be of interest for catalytic purposes or the development of new virus-based materials. To aid in the development of new CCMV-based materials and applications, we developed new methods for VLP assembly and functionalization, and studied polymerization reactions inside CCMV-based capsids (Chapters 7 & 8).

Overall, we see great potential for the application of viruses in various fields, however, to meet this potential, further research towards the assembly and confinement properties of virus(-like) particles is required. The studies presented in this thesis extend our understanding of these structures, however building on the research shown here new questions arise. Instead of studying the assembly of virus particles and the energies involved by looking at an average behavior of many particles in a solution, it might be interesting to follow separate components, e.g. a single CP or a single polyanion chain. This potentially provides new information on viral assembly on a smaller scale, rather than giving an average of millions of particles, and may allow for the development of a more accurate understanding of assembly pathways. Furthermore, more knowledge on the interactions between various components is required to be able to accurately design specific structures. Also, knowing the pH inside protein cages can deviate from it surrounding, what other differences in physiochemical properties occur inside such structures? And how is the cargo affected by this? A difference in pH causes changes in the protonation state of the cargo, while molecular crowding may affect the mobility or even the mechanical properties of the cargo. These are issues to consider when designing new materials based on viruses. Lastly, the data obtained for CCMV as a model virus and the techniques used to obtain them are potentially applicable over a wider range of viruses. Future research using other viruses is required to determine if these data are a general trend for virus assembly and confinement properties in general.
Samenvatting

Met virusdeeltjes openen we een heel arsenaal aan bouwstenen voor het ontwikkelen van nieuwe materialen. Ze kunnen bijvoorbeeld worden toegepast in materiaalkunde, nanotechnologie, of geneeskunde. De hoge mate van monodispersiteit en symmetrie in deze structuren overschrijdt elk synthetisch nanodeeltje dat momenteel beschikbaar is, en doordat er zoveel verschillende virussen op Aarde te vinden zijn, met allerlei verschillende vormen en formaten, is de keuze vrijwel eindeloos. Vooral het capsid van virussen, die het virale genetisch materiaal omsluit, wordt onderzocht. Deze eiwitschil transporteert en beschermt het genetisch materiaal totdat het zich in een cel bevindt waaraan het virus zich kan voortplanten. Eenmaal in de cel komt het genetisch materiaal vrij zodat de cel kan worden aangezet tot productie van de verschillende componenten van het virus. Als ze gevormd zijn, assembleren deze componenten zichzelf in nieuwe virusdeeltjes die weer uit de cel vrijkomen. Deze reversibele demontage en assemblage vereist specifieke interacties tussen de verschillende componenten van een virus om ervoor te zorgen dat het deeltje stabiel genoeg is om cellen te kunnen bereiken, maar ook fragiel genoeg is om uit elkaar te kunnen vallen in de cel om infectie te bewerkstelligen. Zowel voor het begrijpen van virus reproductie en het behandelen van virale infecties, als voor het gebruik van virussen of hun componenten, met voorspelbare uitkomst, is het van belang om de interacties tussen de verschillende componenten van een virus te begrijpen.

Het onderzoek dat is beschreven in deze thesis verbreedt de kennis omtrent virus assemblage, door de assemblage- en insluitingseigenschappen van het cowpea chlorotic mottle virus (CCMV) te bestuderen. Door microschaal thermoforese (MST) toe te passen als nieuwe techniek om (zelf)assemblage te bestuderen hebben we de assemblage van natuurlijke CCMV capsid eiwitten (CPs) kunnen bestuderen en vergelijken met de assemblage van twee genetisch gemodificeerde versies van het eiwit onder veel verschillende condities (Hoofdstuk 3). We hebben MST ook gebruikt in combinatie met isotherme titratie calorimetrie (ITC) om de assemblage van CCMV CP om polyanion ketens tot virus-gelijkende deeltjes (VLPs) te bestuderen (Hoofdstuk 4). Doorbouwend op de assemblage om polyanionen hebben we gekeken naar de minimale lengte van enkelstrengs DNA
(ssDNA), wat gerelateerd is aan een minimale elektrostatische interactie, die nodig is voor de vorming van virusdeeltjes bij neutrale pH (Hoofdstuk 5).

Om meer inzicht te krijgen in de fysio-chemische eigenschappen aan de binnenkant van een eiwit kooi hebben we de pH condities in een op CCMV gebaseerd capside bestudeerd door middel van een negatief geladen, pH gevoelige sensor (Hoofdstuk 6). De resultaten hiervan, in combinatie met het theoretische model dat hierbij is gebruikt, laten zien dat de pH condities in een eiwit kooi niet noodzakelijkerwijs hetzelfde zijn als in de oplossing waarin het zich bevindt. Dit kan interessant zijn voor katalytische eigenschappen of voor de ontwikkeling van materialen die op virusdeeltjes zijn gebaseerd. Om de ontwikkeling van op CCMV gebaseerde materialen te bevorderen hebben we nieuwe methodes ontwikkeld om VLPs te assembleren en te functionaliseren en hebben we polymerisatiereacties in een op CCMV-gebaseerd capside onderzocht (Hoofdstuk 7 & 8).

Er zijn potentieel veel mogelijkheden voor het gebruik van virussen in allerlei toepassingen, maar om dit potentieel te verwezenlijken is meer onderzoek naar de assemblage- en insluitingseigenschappen van virus (gelijkende) deeltjes nodig. Met het onderzoek in deze thesis verbreden we onze kennis van deze structuren, maar doorbouwend op deze resultaten zijn er nieuwe vragen te stellen. In plaats van naar een gemiddelde assemblage gedrag van een groot aantal deeltjes in een oplossing en de bijbehorende energieën te kijken, is het interessant om te focussen op een losse component, bijvoorbeeld een enkel CP of één polyanion keten. Mogelijk geeft dit informatie op een veel kleinere schaal dan een gemiddelde van miljoenen deeltjes en kan hiermee een preciezer beeld van assemblage mechanismen worden gegeven. Daarnaast is er meer kennis van de interacties tussen losse componenten nodig om structuren accuraat te kunnen ontwerpen. Verder, nu we weten dat de pH in een eiwitkooi niet noodzakelijk hetzelfde als erbuiten is, is de volgende vraag: Welke andere afwijkingen zijn er in de fysiochemische eigenschappen van een eiwitkooi? En hoe beïnvloeden deze de lading die zich in de eiwitkooi bevindt? Een verschil in pH verandert de protonering van de lading, terwijl ophoping van moleculen mogelijk de mobiliteit of zelfs de mechanische eigenschappen van de lading beïnvloedt. Dit zijn enkele van de aspecten die moeten worden overwogen bij het ontwerpen van nieuwe, op virus gebaseerde materialen. Tenslotte, mogelijk zijn de data die we hebben verkregen door gebruik te maken van CCMV als een modelvirus, en de
technieken die we hierbij hebben gebruikt, van toepassing op andere virussen. Verder onderzoek waarbij gebruik wordt gemaakt van andere virussen is nodig om te bepalen of de huidige data een trend is voor de assemblage- en insluitingseigenschappen voor virussen in het algemeen.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>10-mer</td>
<td>Segment of 10 negative charges</td>
</tr>
<tr>
<td>A488</td>
<td>Alexa Fluor 488</td>
</tr>
<tr>
<td>AEMA</td>
<td>2-aminoethyl acrylate</td>
</tr>
<tr>
<td>AGE</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>ARM</td>
<td>Arginine rich motive</td>
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<tr>
<td>ASE</td>
<td>Amplified spontaneous emission</td>
</tr>
<tr>
<td>ATRP</td>
<td>Atom transfer radical polymerization</td>
</tr>
<tr>
<td>AuNPs</td>
<td>Gold nanoparticles</td>
</tr>
<tr>
<td>BBMV</td>
<td>Broad bean mottle virus</td>
</tr>
<tr>
<td>BiBOE2</td>
<td>Bis[2-(2’-bromoisobutyryloxy)ethyl]disulfide</td>
</tr>
<tr>
<td>CCMV</td>
<td>Cowpea chlorotic mottle virus</td>
</tr>
<tr>
<td>CP</td>
<td>Capsid protein</td>
</tr>
<tr>
<td>CPMV</td>
<td>Cowpea mosaic virus</td>
</tr>
<tr>
<td>CPΔN26</td>
<td>CCMV CP lacking 26 amino acids on its N-terminus</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3-Diaminobenzidine</td>
</tr>
<tr>
<td>DCC</td>
<td>Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMAEMA</td>
<td>2-Dimethylaminoethyl methacrylate</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELP</td>
<td>Elastin-like polypeptide</td>
</tr>
<tr>
<td>ELP-CP</td>
<td>Elastin-like polypeptide modified capsid protein</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>F-3NTA</td>
<td>Fluorescently labeled tris-nitrilotriacetic acid</td>
</tr>
<tr>
<td>FMA</td>
<td>Fluorescein methacrylate</td>
</tr>
<tr>
<td>FMA-PSS</td>
<td>Fluorescein-containing polystyrene sulfonate</td>
</tr>
<tr>
<td>F-NTA</td>
<td>Fluorescein-modified nitrilotriacetic acid</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>GE</td>
<td>Gel electrophoresis</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HEA</td>
<td>2-Hydroxyethyl acrylate</td>
</tr>
<tr>
<td>HEAA</td>
<td>2-Hydroxyethyl acrylamide</td>
</tr>
<tr>
<td>HEMA</td>
<td>2-Hydroxethyl methacrylate</td>
</tr>
<tr>
<td>His-CP</td>
<td>Hexahistidine modified capsid protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-pressure liquid chromatography</td>
</tr>
<tr>
<td>I</td>
<td>Ionic strenght</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LbL</td>
<td>Layer-by-layer</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography and mass spectrometry</td>
</tr>
<tr>
<td>LCST</td>
<td>Lower critical solution temperature</td>
</tr>
<tr>
<td>LS</td>
<td>Light scattering</td>
</tr>
<tr>
<td>M_i</td>
<td>Intensity average diameter</td>
</tr>
<tr>
<td>M_n</td>
<td>Number average diameter</td>
</tr>
<tr>
<td>MPS-PPV</td>
<td>Poly(5-methoxy-2-(3-sulfopropoxy)-1,4-phenylenevinylene)</td>
</tr>
<tr>
<td>MQ</td>
<td>Milli-Q water</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MST</td>
<td>Microscale themorphoresis</td>
</tr>
<tr>
<td>Mw</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccimide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitrilotriacetic acid</td>
</tr>
<tr>
<td>N-term</td>
<td>Peptide consisting of 26 amino acids corresponding to the 26 N-terminal amino acids of CCMV CP</td>
</tr>
<tr>
<td>OEGMA</td>
<td>Oligo(ethylene glycol) methacrylate</td>
</tr>
<tr>
<td>P(r)</td>
<td>Pair-distance</td>
</tr>
<tr>
<td>P4VP</td>
<td>Poly(4-vinylpyridine)</td>
</tr>
<tr>
<td>PAA</td>
<td>Poly(acrylic acid)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered silane</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PFS</td>
<td>Polyferrocenylsilane</td>
</tr>
<tr>
<td>polyU</td>
<td>Polyuracil</td>
</tr>
<tr>
<td>PSS</td>
<td>Polystyrene sulfonate</td>
</tr>
<tr>
<td>PSS-In</td>
<td>Polystyrene sulfonate-based macroinitiator</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>R_g</td>
<td>Radius of gyration</td>
</tr>
<tr>
<td>R_h</td>
<td>Radius of hydration</td>
</tr>
<tr>
<td>R_{model}</td>
<td>Radius determined using a homogeneous sphere form factor fit</td>
</tr>
<tr>
<td>R_{P(r)}</td>
<td>Radius determined by pair-distance distribution</td>
</tr>
<tr>
<td>R-PPS</td>
<td>Rhodamine-containing polystyrene sulfonate</td>
</tr>
<tr>
<td>SANS</td>
<td>Small-angle neutron scattering</td>
</tr>
<tr>
<td>SAXS</td>
<td>Small-angle X-ray scattering</td>
</tr>
</tbody>
</table>
List of abbreviations

SDS-PAGE  Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEC    Size-exclusion chromatography
siRNA   Small interfering RNA
ssDNA   Single-stranded DNA
ssRNA   Single-stranded RNA
T       Triangulation
TCV     Turnip crinkle virus
TEG     Tetraethylene glycol
TEM     Transmission electron microscopy
TFA     Trifluor acetic acid
TIS     Triisopropylsilane
TMV     Tobacco mosaic virus
VLP     Virus-like particle
WT      Wild-type
WT-CP   Wild-type capsid protein
ZnPc     Zinc phthalocyanine
Acknowledgements

Nu het wetenschappelijke gedeelte van mijn thesis voorbij is, volgt er nog een stuk dat minstens zo belangrijk is. Want de thesis die je nu leest was er nooit geweest zonder de hulp die ik tijdens, maar ook al voor, mijn PhD tijd heb gehad. Ik wil iedereen die hierbij betrokken is geweest heel hartelijk bedanken voor jullie hulp, advies, en steun, maar zeker ook voor de leuke tijd! Er is een aantal mensen dat ik iets nadrukkelijker wil bedanken.

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About the author

Stan Maassen was born on September 27, 1990, in Nijmegen. After obtaining his VWO and International Baccalaureate diploma at the Kandinsky College in Nijmegen, he moved to Enschede to study Chemical Engineering at the University of Twente. After obtaining his bachelor degree in 2012, he continued his studies in Chemical Engineering following the Molecules & Materials master track at the University of Twente. During his master’s, he followed an internship at the Institute of Material Research and Engineering, a research institute of the Agency for Science, Technology and Research, in Singapore towards anti-biofouling surfaces. The topic of his master thesis, which was performed at the Molecular nanoFabrications group at the University of Twente, supervised by Prof. Jurriaan Huskens, was “Multivalent interactions on surface density gradients for (bio)sensing applications”. His master thesis was awarded second best chemical master thesis of the year 2014 by the Royal Dutch Chemical Society (KNCV).

In June 2014, Stan started as a PhD candidate in the Biomolecular NanoTechnology group under supervision of Prof. Jeroen Cornelissen. The results of this research are presented in this dissertation.
List of publications


