Electrostatic Self-Assembly

From Proteins, Viruses, and Nanoparticles to Functional Materials

Ville Liljeström

A doctoral dissertation completed for the degree of Doctor of Science (Technology) to be defended, with the permission of the Aalto University School of Science, at a public examination held at the lecture hall TU1 of the school on 25th of August 2017 at 12 noon.

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School of Science
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Biohybrid Materials
Abstract

This thesis is a study on the structure and formation of materials that can be produced through self-assembly of nanoparticles in aqueous solution. Nanoparticles exhibit size-dependent properties that are modulated by the arrangement of the particles, and they are promising for the development of new functional materials. The focus is on the electrostatic self-assembly of biological and synthetic nanoparticles into hierarchical structures with properties that arise from the constituent units.

Publication I studies the electrostatic self-assembly of cocrysalis consisting of apoferritin protein cages and poly(amideamine) dendrimers. A systematic series of poly(amideamine) dendrimers with generations from two to seven were used to produce cocrysalis. The experiments demonstrated that the lattice geometry and the lattice constant of the cocrysalis depend on dendrimer generation. Ionic strength was used to control the structural formation and to reversible disassemble the cocrysalis.

In Publication II functional crystalline arrays of biological particles were formed by combining cowpea chlorotic mottle virus and avidin protein. Enzymatic activity, plasmonic, and fluorescent properties were included in the crystals, which were successfully functionalized through binding of biotin-tagged functional units to avidin.

In Publication III photoactive protein-dye crystals were produced. The crystals consisted of apoferritin protein and a photoactive phthalocyanine-1,3,6,8-pyrenetetratsulfonic acid complex that is used to generate singlet oxygen under irradiation of visible light. The incorporation of the photoactive dye into protein crystals was found to be a facile approach to immobilize the dye without losing the singlet oxygen generating property.

In Publication IV proteins and dendrimers were functionalized with azobenzene to obtain light-induced motion of the particles, demonstrating that even very large supramolecular complexes can be made to move in response to light. The light-induced motion could be used to arrange the functionalized particles into a periodic pattern on a substrate.

In Publications V and VI highly monodisperse cationic gold nanoparticles were synthesized, and used to form electrostatically self-assembled superlattice wires together with a rod-like tobacco mosaic virus. The particles assembled in a cooperative manner yielding superlattice wires with a characteristic helical twist that was also observed in the optical properties of the material.

The results of this thesis contribute to the development of new functional materials with highly ordered nanoscale structure. The thesis has especially underpinned the possibility to use nanoparticles as functional modules that can be incorporated in higher-order structures.

Keywords self-assembly, nanoparticles, proteins, viruses, functional materials
Sammandrag


I Publication II framställdes samkristaller som bestod av biologiska partiklar, vilka var pflantaviruset CCMV (eng. cowpea chlorotic mottle virus) och proteinet avidin. Eftersom proteinet avidin binds starkt till molekylerns biotin, kunde samkristallerna funktionaliseras med olika enheter som var sammanlänkade med biotin. Således kunde samkristallerna funktionaliseras med fluorescerande, plasmoniska samt enzymatiska egenskaper.

I Publication III framställdes fotoaktiva samkristaller som bestod av proteinet apoferritin och ett fotoaktivt färgmedel som används för att producera singlet syre. Således kunde det fotoaktiva färgmedlet immobileras utan att försämra dess förmåga att producera singlet syre.


De resultat som påvisas i avhandlingen stöder för sin del en fortsatt utveckling av funktionella material med väldefinerad nanostruktur. Avhandlingen lyfter fram möjligheten att använda nanopartiklar som funktionella moduler som kan ordnas till större enheter.

Nyckelord självbyggande, nanopartiklar, proteiner, virus, funktionella material

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Preface

The research presented in this thesis has been carried out at the Department of Applied Physics at Aalto University during the years 2013–2017. The starting point of this thesis work was a continuation of my supervisor prof. Mauri Kostiainens research on the electrostatic self-assembly of protein cages and synthetic nanoparticles. Self-assembly of nano-sized building blocks was, and still is, an interesting and promising strategy for producing functional materials. We had the goals set high and we aimed at demonstrating novel materials features that result from nanoparticle self-assembly. However, initially I had no idea of how to reach those goals, but the research work and the topics were intriguing, and I was carried away by the challenges and the problems that were waiting to be solved. Mauri, I am greatly indebted to you for introducing this fascinating field to me, for your guidance both on fundamental and practical level, and for giving all the support needed to conduct the highly cross-disciplinary research. You have been a great mentor during these years and supported me in growing as a scientist and in developing my professional identity.

This thesis includes methods from various fields, and the projects would never have been successful without a great community. The research included in this thesis has been carried out in close collaboration with professors Olli Ikkala, Arri Priimagi, Päivi Törmä, and Robin Ras. Thank you for sharing your enthusiasm, creativity, ambition, and critical thinking. Much of the work has been carried out in HYBER, which is one of the Academy of Finland’s Centers of Excellence. This environment has resulted in inspiring collaborations and formed a framework for successful cross-disciplinary research. I want to thank Dr. Marjo Kettunen for all her efforts to facilitate collaborations by bringing different people together. I also want to acknowledge Emil Aaltonen Foundation and the Swedish Cultural Foundation in Finland for personal funding.

This period of time has included ambiguous goals, coping with uncertain outcome of research, failing, iteration, but also many moments of success. I started the first project by learning some of the basic experimental methods. Soon the work became a systematic study of self-assembly phenomena, and finally we focused on designing the self-assembling systems. During that time I have been surrounded by incredible people who have been exclusively supportive. I want to thank Dr. Joona Mikkilä for all the collaboration and for the help and valuable hints I got especially in the very beginning of my
doctoral studies. Thank you Dr. Ari Ora. Discussions with you and your advices have helped me to manage many of the challenges I have encountered and you have helped me to identify important problems related to this thesis. Especially at a later stage of my studies I was significantly supported by Dr. Jukka Hassinen. Thank you, Jukka, for pulling me up a few levels in my knowledge of chemistry and for being around to solve problems together with me. When I have needed extended knowledge in physics, I have consulted Tuukka Verho, Mikko Poutanen, Mika Latikka, and Heikki Rekola. Thank you for your unreserved enthusiasm for solving problems together. I want to thank Salla Välimäki, and Dr. Veikko Linko and Sami Nummelin for collaboration and for being those jovial fellows at the lab, with whom I often shared thoughts with. I also want to thank Dr. Jenni Koskela for the successful collaboration that I had the chance to participate in during the first year of my doctoral studies.

In my research I have focused on structural characterization in order to describe the studied self-assembly phenomena. This thesis includes a great portion of characterization, which would not have been successful without the help and advices from prof. Janne Ruokolainen, Dr. Panu Hiekkataipale, Dr. Juuso Korhonen, and Dr. Jani Seitsonen. Thank you for all the support in materials characterization. I also want to acknowledge Dr. Nonappa, Maria Heilala, and Ville Hynynen, for kindly helping me with crucial synthesis and characterization. Dr. Christoph Hörenz deserves special thanks for reading the final draft of the thesis manuscript and giving valuable comments.

I thank prof. Trevor Douglas from Indiana University and prof. Rafal Klajn from Weizmann Institute of Science for the preliminary examination of the thesis. I thank prof. Takafumi Ueno from Tokyo Institute of Technology for accepting to act as the opponent in the upcoming defence.

Many successful ideas and plans have been initiated in our office 115a, where I have spent thousands of hours during the past four years. I have been fortunately surrounded by friendly people with whom I have discussed virtually all aspects of science, lab work, and life in in general. Those who shared the office with me have been very important to me during this period of time. Thank you Teemu Myllymäki, Lahja Martikainen, Riikka Koski, Dr. Jason McKee, Dr. Matilda Backholm, and prof. Jaakko Timonen. Many fruitful discussions took also place in the neighbouring office 114, which was the office of team Gröbling. Thank you Tina and André.


Denna avhandling skulle aldrig blivit till utan henne som alltid stöttat mig, varit med i både framgångarna och motgångarna under denna resa och delat kärleken med mig varje dag. Mina ord räcker inte till för att tacka dig Linda.
Ivar ja Arvid, olette päivittäin auttaneet, ilahduttaneet ja hauskuuttaneet iskää. Olette minun todellinen ilo ja ylpeyden aihe.

Kerava, 10 July 2017

Ville Liljeström
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This doctoral dissertation consists of a summary and of the following publications which are referred to in the text by their Roman numerals


Author’s Contribution

**Publication I:** Electrostatic Self-Assembly of Soft Matter Nanoparticle Co-crystals with Tunable Lattice Parameters

The author performed and analyzed all experimental work, excluding cryo-TEM imaging, which was conducted by Dr. Jani Seitsonen, as well as drafted the manuscript and corrected it according to comments from the other authors.

**Publication II:** Self-Assembly and Modular Functionalization of Three-Dimensional Crystals from Oppositely Charged Proteins

The author performed and analyzed most of the experimental work. EMSA and DLS measurements were carried out together with Dr. Joona Mikkilä, and cryo-TEM imaging was carried out together with Dr. Jani Seitsonen. The author drafted the manuscript and corrected it according to comments from the other authors.

**Publication III:** Hierarchical Organization of Organic Dyes and Protein Cages into Photoactive Crystals

The author participated in designing the experimental work together with the co-authors, carried out the SAXS experiments and data analysis, assisted in optical microscopy, and participated in revising the manuscript.

**Publication IV:** Light-Fuelled Transport of Large Dendrimers and Proteins

The author participated in designing the experimental work together with the co-authors, carried out the DLS, SAXS and FTIR experiments and data analysis, participated in revising the manuscript.

**Publication V:** Rapid Cationization of Gold Nanoparticles by Two-Step Phase Transfer

The author carried out the preparation of gold nanoparticle—virus assemblies and the SAXS characterization including the data analysis, and participated in revising the manuscript.

**Publication VI:** Cooperative Colloidal Self-Assembly of Metal-Protein Superlattice Wires

The author performed and analyzed most of the experimental work, except from the cryo-TEM characterization that was done by Dr. Nonappa. Dr. Ari
Ora, Dr. Jukka Hassinen, and Maria Heilala contributed in nanoparticle synthesis, UV-Vis and CD characterization, and TEM studies. The author planned the computational modelling with M.Sc. Heikki Rekola who conducted the computational modelling of the CD signal. The author drafted the manuscript and corrected it according to comments from the other authors.
Other Publications

The author has also contributed to the following related publications.


## List of Abbreviations and Symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>a.u.</td>
<td>arbitrary units</td>
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<td>aFT</td>
<td>apoferritin</td>
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<tr>
<td>AFM</td>
<td>atomic-force microscopy</td>
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<tr>
<td>AuNP</td>
<td>gold nanoparticle</td>
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<tr>
<td>B-AuNP</td>
<td>biotin-tagged gold nanoparticle</td>
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<tr>
<td>BF</td>
<td>biotinylated fluorescein</td>
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<tr>
<td>B-HRP</td>
<td>biotinylated horseradish peroxidase</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCMV</td>
<td>cowpea chlorotic mottle virus</td>
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<tr>
<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>cryo-ET</td>
<td>cryogenic electron tomography</td>
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<tr>
<td>cryo-TEM</td>
<td>cryogenic transmission electron microscopy</td>
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<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DLVO-theory</td>
<td>theory named after Boris Derjaguin and Lev Landau, Evert Verwey and Theodoor Overbeek</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPBF</td>
<td>1,3-diphenylisobenzofuran</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>EO</td>
<td>ethyl orange</td>
</tr>
<tr>
<td>fcc</td>
<td>face-centred cubic</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
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<tr>
<td>G</td>
<td>generation</td>
</tr>
<tr>
<td>hcp</td>
<td>hexagonal close packed</td>
</tr>
<tr>
<td>LSPR</td>
<td>localized surface plasmon resonance</td>
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MUTAB  (11-mercaptoundecyl)-N,N,N-trimethylammonium bromide
NMR  nuclear magnetic resonance (spectroscopy)
PAMAM  poly(amidoamine) or poly(amidoamine) dendrimer
pI  isoelectric point
PTSA  1,3,6,8-pyrenetetrasulfonic acid
RNA  ribonucleic acid
SAXS  small-angle X-ray scattering
SRG  surface relief grating
TEM  transmission electron microscopy
TMV  tobacco mosaic virus
UV-Vis  ultraviolet-visible spectrophotometry
ZnPc  zinc phthalocyanine

a  lattice constant
c  concentration
c_t  threshold concentration
D_h  hydrodynamic diameter
D_{interstitial}  interstitial spacing between two adjacent particles
D_{OV}  minimum diameter of octahedral void
D_{PAMAM}  diameter of PAMAM particle
D_{TV}  minimum diameter of tetrahedral void
E  electric field
e  elementary charge
h  Planck constant
k  Boltzmann constant
N_q  number of charges
p_{osm}  osmotic pressure
T  temperature
t  time
V_0  initial velocity (slope) of absorbance progress curve
z_{i}  valency of ion i
1. Introduction

Technological development proceeds parallelly with the development and understanding of the nature of materials\(^1\). Specific materials properties require certain structures, and the increasing miniaturization of technology also requires control over structural features on small scale\(^3\). This has led to the development of nanoscience and nanotechnology, dealing with materials or phenomena that are connected to the nanometer (10\(^{-9}\) m) scale structures\(^4\).

Interestingly, the properties of metal or semiconductor particles are affected by the particle size\(^4–6\). For example colloidal gold particles have a ruby red appearance\(^7,8\), shifting towards blue, if the particles are grown larger. Also other materials properties, like magnetic properties, are affected by particle size\(^9,10\). The properties of nanomaterials can be further modulated and controlled by the organization of the constituents\(^3,11–15\). An example is again the colour of colloidal gold particles, which can shift to black or golden shiny, when the particles are aggregated.

Self-assembly is a process in which components spontaneously form ordered structures\(^16,17\). This is relevant for various biological systems\(^18–20\) and has been identified as an efficient way to control the organization of nanoscale building units\(^21–26\), but the process requires sufficiently strong interactions to occur. Electrostatic self-assembly relies on the electrostatic attraction and repulsion between charged particles or molecules\(^27,28\). This thesis deals mainly with electrostatic self-assembly of protein nanoparticles and synthetic nanoparticles. The major approach is to use the extremely well-defined structure of proteins and protein cages to direct the self-assembly of multicomponent hierarchical structures.

The synthesis of differently sized and shaped nanoparticles is advanced\(^29–32\), and there are strategies for their controlled assembly, which do not include the use of biological particles\(^33–38\). There are useful heuristic rules to describe the self-assembly of nanoparticles and the nature of the electrostatic interactions at the nanoscale are mostly understood\(^39\), but predictable electrostatic self-assembly remains a major challenge. For example the conventional DLVO-theory\(^i\) is inaccurate at the nanoscale\(^40\) and the theoretical models are not considered fully reliable if the theoretical results are not confirmed by experimental evidence.

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\(^i\) DLVO-theory is named after Boris Derjaguin and Lev Landau, Evert Verwey and Theodoor Overbeek. It is the most used theory for quantitatively describing colloidal stability.
Robust self-assembly approaches would accelerate the development of nanoparticle based applications. Meanwhile, experimental work is of major importance for the development of these kinds of new materials. In this thesis we study how ionic strength and properties of the building units affect the self-assembly process and the resulting structure. Obstacles resulting from the polydispersity of synthetic nanoparticles can be overcome by using perfectly uniform biological particles. The approach of using such particles to guide the self-assembly, makes it possible to study the relation between particle properties and assembly formation.

The thesis includes studies on hollow spherical (apoferritin and cowpea chlorotic mottle virus), tetrahedrally globular (avidin protein), and rod-like helical (tobacco mosaic virus) protein particles or cages. The unique feature of these genetically encoded, and hence atomically precise particles is that they carry a symmetric surface charge distribution that together with the shape of the particles define the higher-order self-assembled structure\(^\text{28,38}\). The thesis demonstrates that this approach can be used to construct three-dimensional materials by combining biological and synthetic functional units (Figure 1.1). The thesis also covers studies on the structure dependent properties of the nanoparticle assemblies. The examples discussed show that both the composition and the structure affect the properties of nanoparticle assemblies.

**Figure 1.1.** The studied approach to construct new hierarchical materials.

### 1.1 Outline of the Thesis

Publication I examines the electrostatic self-assembly of oppositely charged well-defined soft nanoparticles. The work shows that electrostatic self-assembly can be a reversible process that yields different structures depending on the particle size, surface charge density, and assembly conditions.
Publication II and Publication III demonstrate possible approaches for modular functionalization of three-dimensional structures and Publication IV shows that electrostatic binding of photoactive dye molecules can be used to translate light into mechanical motion of nanoparticles. The bound photoactive dye enables arranging the nanoparticles into a surface relief grating according to polarization and intensity modulation of light.

In Publication V, a convenient method for the production of highly monodisperse cationic gold nanoparticles is developed and in Publication VI such cationic gold nanoparticles are used to form metal-virus superlattice wires that exhibit a chiral plasmonic function.
Introduction
Colloidal particles are microscopic particles dispersed in continuous media. They include a wide range of particles from metal clusters and nanoparticles, biological and mineral particles, to polymeric nano- and micrometer sized particles. The same van der Waals, dipolar, and electrostatic interactions that act on atoms or molecules also determine the interaction between colloids. Their behavior, for example colloidal stability in different conditions, is dependent on multiple factors, such as surface charge or chemical composition, steric hindrance by structural units grafted on the particle surface, or size of the particle. For technological applications, these particles need to be assembled into larger structures. However, colloidal particles in a suspension undergo Brownian motion and are often too small and “sticky” to be conveniently manipulated by conventional means. Therefore, a major approach for structural control is that of colloidal self-assembly. Colloidal self-assembly can be achieved by either engineering the abovementioned chemical and structural properties of the particles, or by controlling the assembly conditions.

Self-assembly includes events where subunits assemble into ordered structures and it has been proposed as a convenient method for achieving nanoscale structural order. Thermodynamically stable self-assemblies are such that minimize Gibbs energy

\[ G = H - TS, \]

where \( H \) denotes enthalpy, \( T \) temperature, and \( S \) entropy of the system. In electrostatic self-assembly of nanoparticles, both entropy and enthalpy play an important role, but for strongly charged colloidal particles, the electrostatic forces dominate the interaction of the particles. This is beneficial, as the competing attractive and repulsive electrostatic interactions of charged colloids can be modulated by the ionic strength of the solvent. It has been shown that the electrostatic self-assembly of biological or synthetic nanoparticles can be used to form intricate structures and to control the properties of a material.

The nanoparticles discussed throughout this thesis are significantly charged and as it will be discussed in this chapter, the electrostatic repulsion and attraction (enthalpy of the configuration) are important for the colloidal
stability and dominate the self-assembly of these particles. However, the coalescence of oppositely charged particles into a larger complex undeniably also includes an entropic contribution. The global entropy is increased when counterions associated to the oppositely charged particles are partially released, as their charge is compensated by the surface charge of the bound oppositely charged particle. Furthermore, the counterions associated to a charged particle build up a significant osmotic pressure that contributes to a fast disassembly of the nanoparticle composite structure, when the Debye screening suppresses the electrostatic attraction enough. Finally, at a high ionic strength, it happens that even like-charged particles can aggregate, when the electrostatic repulsion is screened and the purely attractive van der Waals (dispersion) forces start to dominate18,42.

Recent studies on electrostatic self-assembly highlights the importance of the Debye screening in the formation of periodic structures consisting of oppositely charged particles16,28, where the competing repulsion and attraction is key in determining the geometry of the superlattice. Publications I-III and VI all demonstrate the effect of the ionic strength on the electrostatic self-assembly. In Publication VI, the ionic-strength-induced aggregation due to van der Waals forces is utilized to control the self-assembly of binary composite fibers.

2.1 Electrostatic Interaction of Charged Colloids

The best known description of the interaction of charged colloids is the DLVO-theory. It is considered to be the cornerstone of colloid science as it has accurately predicted the results of several experiments42. In essence, DLVO-theory assumes that colloidal stability and aggregation is a result of the competing repulsive electrostatic and attractive van der Waals forces between colloidal particles18,42,47. The theory is mostly used to describe the colloidal stability and aggregation of like-charged particles, but the formalism is general and can likewise be used to describe colloidal systems consisting of oppositely charged particles. The following introduction to the theory on electrostatic and counterion mediated nanoparticle interactions are qualitatively explaining the results included in this thesis, but might come short in quantitative description, as some of the theoretical assumptions do not hold perfectly for the studied systems.

In DLVO theory the interparticle interactions are described in terms of total energy of interaction that can be divided into van der Waals interactions and electrostatic interactions18,42,47. Here, we focus on the electrostatic contribution that dominates the interparticle interaction in the studied examples. The electrostatic interaction in aqueous solvents is affected by dissolved ions. Even in pure water, the concentrations of the H+ and OH− ions cause screening of the electric field. Often, the charge of a colloidal particle is a result of dissociation of ions. The dissociated counterions also contribute to screening of the electric field.
Figure 2.1. The electrostatic potential $\psi$ and the (monovalent) ion distribution in the vicinity of a charged particle. \textbf{a)} A dissolved charged particle is mostly surrounded by counter ions. The co- and counterion concentrations are equal at large distances from the particle. \textbf{b)} $\psi$ as a function of the distance $x$. The calculations are done both for a planar (solid lines) and a spherical positively charged surface (dashed lines). \textbf{c)} The ion density of co-ions ($\rho_+$) and counterions ($\rho_-$) as a function of the distance $x$. The calculations are according to equations (1), (2), (3), and (4).

The screening of the electrostatic interaction of charged particles is connected to the distribution of counterions (Figure 2.1). Some of the counterions are transiently bound to the surface of the particle, forming the so-called Stern layer, whereas some of the ions are freely moving in the diffuse layer. The concentration $\rho_{x,i}$ of counterions $i$ (with valency $z_i$) is high at the surface of the particle, but decreases rapidly as a function of the distance ($x$) to the surface, approaching the bulk concentration $\rho_{\infty,i}$ as

$$\rho_{x,i} = \rho_{\infty,i} e^{-z_i e \psi_x / kT},$$

where $e$ denotes the electronic charge, $\psi_x$ the electric potential at $x$, $k$ Boltzmann constant, and $T$ denotes the temperature. The bulk concentration $c_i$ of ions clearly affects the electric double layer. At higher ionic strengths, the electric diffuse layer is smaller, but the ion concentration within the layer is higher. The characteristic thickness of the diffuse layer is given by the Debye screening length

$$\kappa^{-1} = \left( \frac{e k T}{\varepsilon^2} \sum_i c_i z_i^2 \right)^{1/2},$$

which also determines the characteristic range of the electrostatic interaction. In equation (2), $\varepsilon$ is the permittivity of the solvent. The electric potential $\psi_x$ at $x$ is given approximately by $^{18,48}$
\[ \psi_x \approx \psi_0 e^{-\kappa x}, \]  

where \( \psi_0 \) is the potential at the Stern layer. This approximation is strictly valid only for \( \psi_0 < 25 \text{ mV} \) and overestimates \( \psi_x \), if \( \psi_0 \) is large. A more detailed discussion on the exact form of \( \psi_x \) for the case of high \( \psi_0 \) does not serve the purpose of the qualitative discussion presented here. A book by Hiemenz and Rajagopalan\(^\text{48}\) can be recommended for readers who wish for a more complete description of situations where \( \psi_0 \gg 25 \text{ mV} \).

**Figure 2.2.** Schematics of ion mediated interaction, the electrostatic potential \( \psi \) (black line) between the particles, and the ion density \( \rho \) (red and blue lines) between the particles. The potential and ion distributions are drawn in the same graph. \( \rho_{\text{bulk}} \) (black dotted line) denotes the ion densities at infinite distance. 

- **a)** Two identical like-charged particles at a large distance.
- **b)** The like-charged particles at a smaller distance. The counterion clouds are overlapping, which causes an increase in the osmotic pressure and electrostatic potential at the midplane between the like-charged particles. A minor quantity of ions are released from the counterion clouds.
- **c)** Two identical but oppositely charged particles at a large distance.
- **d)** The oppositely charged particles at a smaller distance. The osmotic pressure and the electrostatic potential at the midplane remain unchanged. A significant quantity of ions are released from the counterion clouds.
Also the shape of the surface affects the electrostatic potential and the ion distribution in the vicinity of a charged surface. For example the electrostatic potential close to a charged sphere differs from the electrostatic potential close to a charged planar surface. The potential around a spherical surface is given by

$$
\psi_x = \psi_0 \left(\frac{R}{R + x}\right) e^{-\kappa x},
$$

(4)

$R$ being the radius of the sphere. From equation (4) it is clear that the effect of particle shape becomes significant when the curvature approaches or is less than $\kappa^{-1}$.

Calculating the exact electrostatic potential and ion distribution in the vicinity of a charged surface becomes complicated whenever multiple charged bodies are present in the system. Often, an analytical solution cannot be provided and computational simulations are used to quantitatively describe the electrostatic potential and ion distributions in the studied systems. Computational simulations can also take into account for example the finite size of ions, which also affects the ion distribution and electrostatic potential. The finite ionic radius becomes significant for any calculations where the details of the studied system approach the size scale of individual atoms. However, this thesis does not include computational methods and those will not be discussed in detail.

The change in the ion distribution during the assembly of charged particles can still be qualitatively understood by inspecting configurations where two identical charged particles are at a distance $R$ from each other (Figure 2.2). When two like-charged identical particles are separated by a large distance (Figure 2.2a) the diffuse layers of the particles are not interfering each other and the shape of the ion distribution in the vicinity of each particle is purely determined by the surface charge density of the particles and the electrolyte composition. When the like-charged particles approach each other (Figure 2.2b), the diffuse layers overlap leading to an increase of the osmotic pressure between the particles. The osmotic pressure is defined at the midplane between the particles. Overlap of the diffuse layers results also in an increase of the electrostatic potential at the midplane. The electrostatic potential and the ion distribution are connected, but both in terms of ion distribution (overlap of diffuse layers) and electrostatic potential, it is clear that aggregation of like-charged particles is unfavorable, as far as the van der Waals forces do not dominate.

When two oppositely charged particles are separated by a large distance they are, similar to the abovementioned case, not interacting due to complete diffuse layers (Figure 2.2c). It is, however, noteworthy that the diffuse layers are oppositely charged. When the oppositely charged particles approach each other (Figure 2.2d), the potential at the midplane remains unchanged, but the attractive electric field (the slope of the potential) increases. The relation between the electrostatic potential and the ion distribution implies that the osmotic pressure at the midplane is unaffected in such a situation and an equal number of positive and negative ions from the diffuse layers are released. The
aggregation of oppositely charged particles is thus favored both by the rearrangement of the ions (released ions increase the entropy) and the electrostatic interaction.

Ionic strength can be used to adjust the interaction between charged particles and therefore, it determines the self-assembly kinetics. Too strong attractive interactions lead to a fast assembly and kinetically trapped configurations with only short range order, whereas too weak interactions do not lead to assembly at all\textsuperscript{28} (Figure 2.3). In the formed electrostatic self-assemblies each charged particle is strongly bound to the surrounding oppositely charged particles. It should, however, be mentioned that the electrostatic repulsion of like-charged particles is crucial for the formation of periodic structures. Attraction alone often leads to poorly ordered structures, whereas a combination of attraction and repulsion yields a long range order that both maximizes the electrostatic attraction between oppositely charged particles and minimizes the repulsion of like-charged particles. This competition between attractive and repulsive forces can even lead to crystal structures that deviate from the typical close packed structures\textsuperscript{50}. Structural features of electrostatic self-assemblies will be discussed in more detail in chapter 3.1.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.3.png}
\caption{Schematics of typical electrostatic self-assemblies formed at different conditions. \textbf{a)} Strong interaction. \textbf{b)} Partial screening or weak interaction. \textbf{c)} Complete screening of electrostatic interaction. Particle-solvent interaction dominates over the electrostatic attraction between particles.}
\end{figure}

Biological particles, such as protein cages (for example, CCMV and ferritins) and avidin (Figure 2.4), have been used in nanotechnology and biotechnological applications, for example for functionalization or to constrain and template the formation of nanoparticles\textsuperscript{51–53}. Due to their uniform structure, protein cages can form ordered arrays on solid supports or interfaces\textsuperscript{54,55} and even form free-standing crystals or crystalline assemblies in solution\textsuperscript{56–57}. In Publication I and Publication II, electrostatic self-assembly of charged proteins, protein cages, and synthetic polymers at different ionic strengths was systematically studied. In Publication I, apoferritin protein cages (aFT) were assembled with poly(amidoamine) dendrimers (PAMAM) (Figure 2.4a).

Ferritins are a class of hollow iron storage proteins. They consist of 24 protein subunits that form a spherical protein cage with a diameter of 12 nm\textsuperscript{58} and they are negatively charged at neutral pH. aFT is an interesting building
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block for nanoscale structures, because it is known to be able to biomineralize inorganic nanoparticles in its cavity \(^{59-61}\). PAMAM dendrimers are uniform, strongly cationic, synthetic polymers with a sequentially branched structure, which can be synthesized in different generations with different sizes \(^{62,63}\). The molecular mass and the net surface charge of PAMAM dendrimers depend on the dendrimer generation so that both are doubled for each increasing dendrimer generation. PAMAM dendrimers can be used as spacers to control the physical properties of nanoparticle materials \(^{64,65}\). In Publication I we combined the cationic PAMAM particles with the anionic aFT particles.

In Publication II cowpea chlorotic mottle virus (CCMV) was combined with avidin (Figure 2.4b) to form electrostatic self-assemblies. CCMV consists of 180 identical coat protein subunits forming a 28 nm protein cage that encapsulates the RNA genome. The surface of CCMV is negatively charged at neutral pH. Avidin is a positively charged tetrameric glycoprotein. It has a hydrodynamic diameter of 5.4 nm and maximum cross-section of 7.2 nm. Avidin exhibits an extremely selective binding to biotin and it has therefore become an important tool in many biochemical techniques, like biomolecule detection, interaction studies, and purification.

![Figure 2.4](image.png)

Figure 2.4. Schematic presentation of the self-assembling soft nanoparticles. a) aFT and PAMAM dendrimer generations G2-G7 drawn to scale. Adapted from Publication I with permission from the American Chemical Society ©2015. b) Native CCMV and avidin drawn to scale. Adapted from Publication II with permission from Nature Publishing Group ©2014.

The electrostatic interactions on the nanoscale have previously been intensively studied \(^{39}\). The main focus of Publication I and Publication II was on the higher-order structures that result from the self-assembly of charged nanoparticles. One of the objectives was to demonstrate different nanoparticle crystals with varying lattices. Previous experimental studies on similar systems \(^{28,57}\) have demonstrated the importance of the electrolyte concentration in order to achieve well-ordered electrostatic assemblies of oppositely charged nanoparticles. In order to obtain self-assembled structures of high quality, the assembly conditions needed to be optimized and experiments at a variety of ionic strengths were carried out. The resulting structures were characterized using small-angle X-ray scattering (SAXS) and cryogenic transmission electron microscopy (cryo-TEM) (Figure 2.5). Regarding the self-assembly of
long-range periodic structures, the total outcome was that assembly at low ionic strength tends to yield low degree of order, whereas too high ionic strength often leads to a total lack of electrostatic interaction. Each combination of particles formed nanoparticle crystals with a significant long range-order by only optimazing the ionic strength, i.e. the sodium chloride concentration $c_{\text{NaCl}}$. The experimental result underlines the importance of the ionic strength as a factor that controls the electrostatic self-assembly.

![Figure 2.5. Electrostatic self-assembly at different ionic strengths.](image)

However, the ionic strength is not the only solvent condition that controls the interparticle interactions in the described systems. Electrostatic self-assembly of protein cages is also affected by pH. The primary structure of any protein cage consists of a chain of diverse amino acids that are subject to both protonation and deprotonation depending on the pH. Therefore the total charge and the interaction potential of protein cages are pH dependent and it has even been shown that electrostatic self-assembly can be induced or inhibited by controlling the pH\textsuperscript{28,46,66}. In Publications I and III the used particle solutions were not particularly buffered, which may lead to non-neutral pH. For example carbonic acid formed from atmospheric carbon...
dioxide leads to a decrease of pH. It should therefore be mentioned that minor effects of solvent pH might be present in Publications I and III. The systematic sample series presented in both publications are, however, prepared in identical conditions (temperature, protein concentration, solvents), implying that the observations and conclusions are not affected by a minor systematic shift of pH that could occur for example due to acidification of the used ultrapure water.

2.2 Reversible Action of Assembly and Disassembly

In the previous section, the basics of electrostatic self-assembly of nanoparticles were discussed. To obtain a good long-range order within the nanoparticle self-assemblies, the assembly of the nanoparticles needs to be dynamic in the sense that the particle configuration can rearrange, in order to optimize the structure. Long-range order cannot occur, if kinetically trapped structures dominate the assembly. Electrostatic interactions can indeed be dynamic, and the dynamics can be adjusted by the ionic strength. Our results in Publication I and Publication II show that a significant increase of the ionic strength shifts the total of the interactions between oppositely charged particles from attraction to repulsion. This allows the self-assembly process to be reversible.

From experiments it is clear that an increase of the ionic strength induces a rapid disassembly of electrostatic nanoparticle self-assemblies when added salt makes a turbid precipitate dissolve to a clear solution. On the other hand, decreasing the ionic strength causes reassembly of larger structures. In Publication I, this reversibility was first observed visually and demonstrated by adding a solution of the anionic aPT particles and cationic PAMAM dendrimers at the bottom of an NMR tube. The Cl of the particle solution was high enough to prevent assembly and the particle solution was clear and transparent. On top of the solution a smoothly decreasing vertical Cl gradient was created, reaching 0 mM at the top. Slowly a sharp, horizontal white line of precipitate was formed in the Cl gradient. The observed line was interpreted to result from oppositely charged nanoparticles that were transported by diffusion to the low ionic strength region, where they formed larger assemblies (the observed line) that readily scatter light (Figure 2.6a). Generally, the aPT-PAMAM assemblies undergo sedimentation, but in this experiment no sediment was observed to form. This was interpreted as the assemblies being disassembled upon sinking to high ionic strength. This phenomenon resembles the formation of atmospheric clouds that form when humid air reaches an altitude, where low temperature causes condensation of the humidity into water droplets.

The assembly-disassembly behavior of the nanoparticle assemblies was studied in more detail by dynamic light scattering (DLS), which both quantifies the total scattering from a sample and estimates the particle size distribution. The scattering count rate is a rough measure of the size of aggregates and was used to monitor the proceeding assembly and disassembly.
during decreasing or increasing ionic strength (Figure 2.6b). When $c_{\text{NaCl}}$ was gradually increased, the count rate dropped drastically at $c_t$, which denotes the threshold concentration. At $c_t$ the sample turned from turbid to clear due to disassembly of aggregates. Furthermore, at sufficiently high ionic strength, the free aFT particles were identified also from the particle size distribution (Figure 2.6c), where the measured hydrodynamic diameter $D_h$ for aFT is $\sim 12$ nm. On the other hand, when the ionic strength was decreased, the count rate of the sample increased significantly at $c_t$, due to the formation of large assemblies.

The effect of the ionic strength on the nanoparticle self-assembly was studied using a series of different dendrimer generations $G$. The reversible action of assembly and disassembly was observed for all dendrimer generation except the smallest $G_0$ and $G_1$ dendrimers, which did not efficiently form large secondary complexes together with aFT. Interestingly, the stability against increasing ionic strength was significantly higher for assemblies including larger dendrimers (Figure 2.6d).

A qualitative explanation for the observed trend in stability against ionic strength can be provided by simple considerations. The number of charges $N_q$ per dendrimer is

$$N_q(G) = 2^{G+2} \propto 2^G.$$  \hspace{1cm} (5)

The dendrimers can be approximated as spherical objects, with roughly homogenous mass distribution. Hence, the surface charge concentration of a dendrimer is

$$\sigma(G) \propto 2^{G/3}.$$ \hspace{1cm} (6)

The electrostatic attraction of oppositely charged spheres in an electrolyte scales with the surface charge density $\sigma$, which implies that higher $\sigma$ requires a higher ionic strength to be efficiently screened. The efficiency of Debye screening scales with ionic strength. The observed $c_t \sim 2^{G/3}$ trend in Figure 2.6d would be explained, if the mentioned scaling effects would be bravely approximated as linear, which is a too coarse approximation. Therefore it should be emphasized that the observed relation between $\sigma$ and $c_t$ remains a heuristic rule. It is known that even the generalized DLVO formalism fails in describing ion mediated interactions in nanoconfined systems\textsuperscript{49}. Also the counterion distribution of the porous PAMAM dendrimers is more complex than the counterion distribution of a hard sphere\textsuperscript{70} and no quantitative predictions of the behavior of other nanoparticle systems can be made on the basis of this observation.
In Publication II, similar studies were carried out using CCMV and avidin. These also demonstrated the concept of ionic strength induced reversible electrostatic self-assembly of charged nanoparticles. As mentioned previously, the role of dissolved ions is twofold in electrostatic nanoparticle self-assemblies; ions screen the electrostatic attraction between oppositely charged particles and the overlap of the diffuse counterion layer increases osmotic pressure at the midplane between the oppositely charged particles. By considering the number of charged groups per PAMAM G7 dendrimer (512 primary amines) and its size (8.4 nm), it can be estimated that the average charge density per dendrimer corresponds to a 2700 mM concentration of monovalent ions. This can be considered to be an order-of-magnitude estimate of the average ionic strength within the aggregate, at the point when all the electrostatic interactions between the oppositely charged nanoparticles are screened by dissolved ions. Such a high counterion concentration implies an osmotic pressure that favours a fast disassembly.

Finally, it should be mentioned that a good solubility of the nanoparticles is needed in order to achieve the reversible action of assembly and disassembly as reported in Publications I and II. The used particles were highly water soluble due to their chemical composition. For charge stabilized lyophilic particles, increasing ionic strength induces aggregation due to the attractive van der Waals forces between the like-charged particles. In such systems, similar reversible action cannot be achieved.

### 2.3 Stepwise Cooperative Assembly of Building Units with Low Mobility

Achieving a high degree of order by means of electrostatic self-assembly is more demanding for colloidal particles with a low mobility or high aspect ratio, if compared to the spherical and globular particles discussed previously. Spherical particles rearrange more easily than for example rod-like particles,
which cannot rotate after being locked in a poorly ordered aggregate. In some
cases, as mentioned in the previous chapter, particle configurations can
dynamically rearrange and nanoparticle assemblies can assemble and
disassemble as a function of the ionic strength. This approach that includes a
control over the interparticle interactions can indeed be used to gradually
assemble the particles into larger structures. Such a stepwise assembly
approach that includes mixing of the particles before initiating the assembly
improves the order and alignment of rodlike colloids.

Both Publication V and Publication VI are part of a study on the formation of
three-dimensional (3D) plasmonic gold nanoparticle (AuNP) superlattice
structures, where the structural formation is guided by the rod-like tobacco
mosaic virus (TMV). In Publication V we developed a scalable method for
producing highly monodisperse cationic nanoparticles with a strong plasmonic
resonance and narrow size distribution for the purpose of electrostatic self-
assembly of nanoparticles71. In Publication VI, we demonstrated the assembly
formation and characterized the superlattice structures.

In Publication VI, we used AuNPs with a narrow size distribution ($D_{\text{core}} = 12.4 \pm 0.9$ nm, $D_{\text{H}} = 15$ nm), which were functionalized with a covalently linked
(11-mercaptopundecyl)-N,N,N-trimethylammonium bromide (MUTAB) ligand
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(Figure 2.7a,c). The used AuNPs were positively charged at all pH values due to the ligands carrying quaternary ammonium groups. Because of the highly cationic surface, the particles had an excellent colloidal stability at a range of ionic strengths. At sufficiently high ionic strength ($C_{NaCl} \sim 500$ mM) the AuNPs were observed to aggregate, as have been shown also for other like-charged nanoparticles\(^{72}\) (Figure 2.7d). However, they reversibly dissolved upon decreasing the ionic strength.

Native TMV (Figure 2.7b) is a negatively charged protein cage consisting of 2130 coat proteins, which are helically wrapped around the viral RNA\(^ {73}\). TMV has a diameter of 18 nm and length of 300 nm. It has been used as a model particle in material science especially for its well-defined rod-like shape. It has for example been used to guide the assembly of small functional molecules\(^ {74,75}\) or inorganic materials\(^ {76,77}\). Still, studies demonstrating that TMV can be used as a template for highly ordered AuNP superlattices, have been lacking.

It was noticed that simple mixing of the cationic AuNPs and anionic TMV did not form a periodically ordered structure as neatly as the previously discussed spherical nanoparticles. Therefore, the assembly pathway needed further adjustment, which included reversible aggregation of the like-charged AuNPs. The strength of the attractive van der Waals dispersion forces that causes aggregation of like-charged colloids is determined by the size of the colloid and also by the polarizability and number density of the elements of the colloids\(^ {18,78}\). The composition dependent particle-particle interaction strength is described by the Hamaker constant \(^ {ii}\). Crystalline metal nanoparticles typically have a relatively high Hamaker constant and are in that sense more affected by van der Waals attractions than soft organic colloids\(^ {79–81}\).

We made use of the ionic strength dependent aggregation-dissolution behavior of the AuNPs, which is caused by attractive interparticle van der Waals forces that dominate when the electrostatic interaction is screened. Importantly, the ionic strength dependent aggregation-dissolution behavior of like-charged nanoparticles is opposite to that of the electrostatic assembly-disassembly of oppositely charged particles and can be used to keep the oppositely charged particles separated in different phases before initiating the assembly. Finally, dissolution of the AuNPs and subsequent assembly of AuNP-TMV structures can be initiated by decreasing the ionic strength. In order to avoid kinetically trapped structures, we chose to mix the AuNPs and TMV at high ionic strength, which ensured the aggregation of the like-charged particles due to efficient screening of the electrostatic attraction. To guarantee a gradual decreasing ionic strength, the samples were sequentially dialyzed against solutions of decreasing ionic strength, which caused the AuNPs to dissolve and form assemblies with TMV (Figure 2.8a).

\(^ {ii}\) An accurate estimation of the Hamaker constant of colloids requires sophisticated calculations and detailed data of the materials properties\(^ {174}\).
Figure 2.8. Release of AuNPs and subsequent electrostatic self-assembly. **a)** The AuNPs are aggregated at a high ionic strength, but are released when decreasing the ionic strength. Free AuNPs form electrostatic self-assemblies with TMV. **b)** Time lapse of the dialysis studied with optical microscopy. Adapted from Publication VI.

Figure 2.8b shows a time lapse of a AuNP-TMV sample with high ionic strength, which is dialyzed against low ionic strength buffer (10 mM sodium acetate). From the time lapse it is clear that AuNPs are released (red colour indicates free AuNPs) from the black aggregates after ~1 minute of dialysis. The AuNPs are subsequently bound to TMV, which is observed as a colour change towards violet and the formation of a fibrillar network. At an optimized nanoparticle stoichiometry, this kind of stepwise assembly path leads to well-defined superlattice structures with a long-range order (Figure 2.9).

Figure 2.9. A highly ordered AuNP-TMV superlattice formed at optimized conditions. Adapted from Publication VI.
3. The Structure of Nanoparticle Assemblies

The arrangement of molecular constituent units has a significant effect on the material properties. This is clear when comparing the physical properties of diamond and graphite, which are identical to the elemental composition but radically different to their appearance. Similar principles apply also for materials consisting of nanoparticles. The physical properties are modulated by the arrangement of the nanoparticles. Hierarchically ordered molecular clusters, nanoparticle superlattices, and colloidal crystals have been studied in order to develop materials with tuneable optical, magnetic, electronic, and catalytic properties. Highly organized 3D nanoparticle structures can only be achieved by self-assembly approaches. So far the progress of the experimental research of the structure dependent properties of nanoparticle self-assemblies is hampered by the obstacles in controlling the self-assembly process sufficiently well to be able to conduct systematic studies on different structures. Hence, controlling the arrangement of aggregated nanoparticles has been declared to be one of the most significant objectives of present nanoscience. As the field of nanoparticle self-assembly is rapidly progressing, more examples of structure dependent functions can be expected. Publications I, II, IV and VI focus on the structural formation and characterization of periodic 3D nanoparticle structures and different lattice geometries are demonstrated. The functionality of these structures will be discussed in chapter 4.

3.1 Size and Shape of Particles Affect the Lattice Geometry

For aggregated uniform building units a periodic lattice structure is often the minimum energy configuration and therefore it is not surprising that protein cages and highly monodisperse synthetic counterparts can form periodic structures. Such binary and ternary crystals have received focused attention because they provide an approach to integrate different components to yield multifunctional materials. The arrangement of the self-assembled nanoparticles is determined by the particle properties and the external conditions. However, reliably predicting and controlling the structure of multicomponent nanoparticle crystals remains a major challenge.
A common type of colloidal crystals include only one type of nanoparticles that are aggregated into periodic structures by drying or by changing solvent conditions. Even single component crystals can exhibit a rich variety of crystal structures and the structural diversity can be further increased by combining particles with different size or shape. In addition to the size, size ratio, and shape of particles, the lattice geometry of multicomponent nanoparticle crystals can also be controlled by the interaction between particles. These interactions can be either specific or more general to their nature. A well-known approach to utilize specific binding for lattice engineering includes specific binding via complementary DNA linkers, which in combination with well-defined particle sizes and shapes yield control over the self-assembled nanoparticle crystal. On the other hand, approaches that utilize competing interactions include both electrostatic and solvophobic interactions. Depending on the particle size and solvent conditions, electrostatic interactions can be used to regulate the lattice symmetry of nanoparticle crystals. Since colloidal stable particles often carry a surface charge, the particles can be used in electrostatic self-assembly. This enables the use of a wide variety of different building units, both biological and synthetic. An additional degree of structural diversity of electrostatic self-assembly is provided by patchy electrostatic interaction, which denotes the interaction of particles with anisotropic surface charge distribution. Patchy interaction adds a significant contribution to the arrangement of oppositely charged particles.

Many of these examples include biological building blocks, which are ideal model particles for studies on electrostatic self-assembly due to their uniform properties. The main approach in this thesis is to utilize uniform biological building blocks to achieve and study well-defined periodic structures. This chapter summarizes the observed differences in lattice geometries that result from combining anionic protein cages with cationic nanoparticles. Especially the effect of particle shape and size, competing repulsion and attraction, and patchy charge distribution are discussed.

Apoferitin (aFT) is a well-known protein cage. It is a model particle that has been used as a building block to form a variety of nanostructures. Apoferritin is known to crystallize in various salt conditions yielding a face-centered cubic (fcc) crystal structure. In Publication III the anionic aFT was combined with a cationic, polyvalent, planar macroion complex that interacted strongly with the anionic aFT (Figure 3.1). The complex consisted of zinc phthalocyanine (ZnPc) and 1,3,6,8-pyrenetetrasulfonic acid (PTSA). Similar to previous studies on aFT crystallization, also our results showed that the ensuing structure, where the planar macroion binds aFT particles together, is a close packed fcc structure with remarkably high long-range order. It was concluded that the observed crystal structure was mostly a result of the features of aFT, whereas the macroion complex acted as a molecular glue between the aFT particles.
The Structure of Nanoparticle Assemblies

Figure 3.1. Scheme of the aFT-(ZnPc-PTSA) crystallization. a) Chemical structures of ZnPc and PTSA, where sulfate and sodium are the respective counterions, and the self-assembly of tetracationic ZnPc--PTSA supramolecular complex. b) Illustrations of the 12 nm sized aFt cage and its further cocristallization with ZnPc-PTSA complex driven by electrostatic interactions. Illustrations of aFt show the folded protein subunits (left) and the calculated electrostatic surface potential (right) with negatively charged patches (in red) covering the cage surface. Note that the cartoons of 1–2, aFt and the crystals are not in the same scale. Adapted from Publication III with permission from the American Chemical Society ©2016.

In Publication I, highly ordered aFT structures were produced in similar manner as in Publication III by using cationic PAMAM dendrimers. Low generations of PAMAM dendrimers are comparable to polyvalent macroions due to their size and valency, but the higher generation PAMAM dendrimers are better described as monodisperse soft nanoparticles with a defined positive charge. It was observed that the smaller dendrimer generations yield aFT-PAMAM cocrystals where PAMAM acts as a spacer or molecular glue between the aFT protein cages. The observed fcc lattice was similar to those previously reported for aFT. However, higher-generation PAMAM particles occupy a larger volume and hence, the packing of particles becomes a more significant factor to determine the lattice geometry. The lattice has to adjust in order to allow efficient packing of different sized particles. Importantly, one of the results of the study was that the high PAMAM generations yielded a hexagonal close packed (hcp) cocrystal structure instead of the typical fcc structure observed for small PAMAM generations (Figure 3.2).
The Structure of Nanoparticle Assemblies

The formation of different aFT-PAMAM structures can be understood in terms of particle size and interparticle interactions. PAMAM diameter $D_{\text{PAMAM}}$, net charge, and surface charge density $\sigma$ are dependent on the dendrimer generation. None of these variables is solely responsible for the formed crystal structure. The role of particle size and interparticle interactions in the observed shifting from an fcc structure for low PAMAM generations to an hcp structure for high PAMAM generations, are hard to describe with full confidence. However, when considering the geometry of the cocrystal structures $D_{\text{PAMAM}}$ becomes the dominant variable, as Debye screening compensates to some extent for the variations of the net charge and $\sigma$. Still, the role of particle charge is significant, not only for the attraction between oppositely charged particles, but also for the repulsion between the like-charged particles.

In most of the cases, where highly crystalline assemblies were observed, the Debye screening length was adjusted to $\sim 2$ nm. Our results showed that the interstitial distance between adjacent aFT particles was found to be in the range 2.5-4.5 nm (Figure 3.2c), if the diameter of aFT is assumed to be 12 nm. This finding is significant, because it indicates that the electrostatic repulsion between adjacent aFT particles in a crystalline arrangement is high enough to hinder an ultimate close packing of aFTs and hence, affects the final structure.
To back up this argument, it is useful to further inspect the lattice and to consider the most probable lattice voids occupied by PAMAM dendrimers.

Unfortunately, a major challenge in the experimental characterization of the aFT-PAMAM cocrystal structures was the poor contrast of the soft PAMAM dendrimers in comparison to aFT that consists of a dense and rigid protein shell. The organization of aFT can be determined from the SAXS and cryo-TEM data, whereas the arrangement of PAMAM particles cannot. However, once the arrangement of aFT particles is solved, an inspection of the possible arrangement of PAMAM dendrimers in the voids of the aFT lattice can be done. Close packed fcc and hcp structures include certain voids that are favorable for PAMAM dendrimers of different size. These can be divided into three categories, which are listed in increasing order as 1) the interstitial spacing $D_{\text{interstitial}}$ between two adjacent aFT particles (Figure 3.3a), 2) the tetrahedral void with a diameter $D_{\text{TV}}$ that is formed between four aFT particles (Figure 3.3b), and 3) the octahedral void with a diameter $D_{\text{OV}}$ that is formed between six aFT particles (Figure 3.3c).

Small dendrimers occupy preferably the interstitial voids. Revisiting the data presented in Publication I shows that the fcc cocrystals that included PAMAM generations 2-4 had a structure with $D_{\text{interstitial}}$ that matches well with the size of the PAMAM dendrimers (Figure 3.4a). This strongly suggests that $D_{\text{interstitial}}$, and thus the lattice constant, is defined by the size of the PAMAM dendrimer that acts as a spacer between adjacent aFT particles. In these fcc structures, PAMAM dendrimers are probably also present in the tetrahedral and the octahedral voids, which are large enough to leave free space for the dendrimers. In fact, the tetrahedral and octahedral voids are large enough to host even more than one dendrimer. However, such dendrimers probably do not act as spacers that define the cocrystal geometry. It should be emphasized that if PAMAM generations 1-3 would not occupy the interstitial void in the fcc structures, the structure would be more compact and adjacent aFT particles would be packed closer to each other (Figure 3.4b), which would increase the electrostatic repulsion of the aFT particles.

Intermediate dendrimers prefer the interstitial or the tetrahedral void depending on the ionic strength. Interestingly, PAMAM G4 can either form an fcc or an hcp structure in combination with aFT. When aFT-PAMAM G4
cocrystals are formed at a higher ionic strength, the resulting structure is an hcp lattice with a significantly smaller interparticle distance than would be expected for a structure with dendrimers in the interstitial voids. On the other hand, the observed hcp structure includes tetrahedral voids with a $D_{TV}$ that coincides with the PAMAM size, whereas $D_{interstitial}$ in this structure is too small to host PAMAM G4 particles. The tighter packing of aFT in this structure is possible due to the stronger Debye screening causing a smaller repulsion between adjacent aFT particles.

Large dendrimers occupy the tetrahedral voids. For dendrimer generations 5-7 only hcp structure was observed. As seen from Figure 3.4a, $D_{TV}$ matches well with the sizes of the PAMAM dendrimers, indicating that the cocystal lattice parameters are defined by the PAMAM dendrimer located in the tetrahedral void. It is clear that $D_{interstitial}$ in all hcp structures is too small to be occupied by the corresponding PAMAM dendrimers (Figure 3.4). However, the electrostatic repulsion of adjacent aFT particles is not as significant in these structures, as $D_{interstitial}$ is large enough for efficient Debye screening. It can be added, that the octahedral void, which is the largest, probably hosts one or more dendrimers, but it seems that the structures observed here are defined either by the dendrimers that occupy the interstitial void (fcc), or by the dendrimers that occupy the tetrahedral void (hcp).
Figure 3.4. a) A comparison between the experimentally obtained void sizes and dendrimer size. The comparison shows that for fcc structures (blue region) the interstitial void size is defined by the dendrimer size. In hcp structures (yellow region) the dendrimer size defines the size of the tetrahedral void. b) An aFT-PAMAM structure, where PAMAM occupies the interstitial void, is favourable for small dendrimer generations. A structure with unoccupied voids leads to a narrow or no spacing between adjacent aFT particles, and is hence unfavorable. c) A structure with occupied tetrahedral voids and unoccupied interstitial voids is allowed for higher PAMAM generations and favorable due to efficient packing.

In Publication II, the electrostatic self-assembly of CCMV in combination with avidin or PAMAM G6 dendrimer, was studied. Avidin and PAMAM G6 are similar in size, but the structural features are different. PAMAM is a spherical particle with a smooth surface charge distribution. Avidin is a rigid tetrameric protein particle that has a tetrahedral surface charge distribution. The results showed that packing of the anionic CCMV is dependent on the choice of the cationic counterpart. Previous studies show that fcc packing is favored for electrostatic self-assemblies that include CCMV combined with soft or smoothly charged building blocks. This study showed that the combination of CCMV and PAMAM G6 also resulted in the typical fcc structure, but in contrast to previous results, the combination of CCMV and avidin resulted in a body-centred cubic (bcc) structure.

For the interpretation of the result of differing lattice geometries it is important to mention that CCMV has an uneven surface charge distribution (Figure 3.5a). In fact, the patchy electrostatic surface potential map of CCMV forms a truncated icosahedron symmetry, also known as the “C60 structure”, “bucky-ball structure”, or “football structure” with an edge length of ~6 nm. The distinct structure of the 28 nm CCMV capsid results from the assembly of the 180 chemically identical protein subunits. Previous studies have shown that the patchiness of CCMV is sufficiently distinct to direct the assembly of small AuNPs into a unique superlattice structure. Here, the resulting untypical bcc structure is similarly attributed to the patchiness of CCMV, but also to the patchiness and rigidity of avidin.
It should be emphasized that, as in the case of aFT-PAMAM structures, where only aFT particles could be localized unambiguously from structural characterization, here, only the CCMV particles could be unambiguously localized from SAXS and cryo-TEM data (Figure 3.6). Thus, further analysis on the location of PAMAM G6 and avidin is based on geometrical considerations on the particle structure versus lattice geometry. The tightly packed CCMV-PAMAM structure with an fcc lattice (lattice constant \( a_{\text{CCMV-PAMAM}} = 40.5 \text{ nm} \)) is almost identical to what was previously reported for a CCMV-AuNP superlattice (lattice constant \( a_{\text{CCMV-AuNP}} = 40.6 \text{ nm} \)), for which the AuNPs (\( D_{\text{AuNP}} = 8.5 \text{ nm} \)) within the AB\(_8\) fcc-type (CCMV–AuNP\(_8\))\(_{\text{fcc}}\) superlattice could be localized\(^{28}\). Both the AuNPs with long ligands and the dendrimers are considered to be soft particles and the diameter of PAMAM G6 (\( D_{\text{PAMAM G6}} = 6.7 \text{ nm} \)) is comparable to \( D_{\text{AuNP}} \). Hence, it was concluded that the most probable structure adapted by CCMV and PAMAM G6 is the AB\(_8\) fcc-type (CCMV–PAMAM G6\(_8\))\(_{\text{fcc}}\) lattice.

In contrast to PAMAM G6, avidin carries a patchy surface charge distribution that forms a tetrahedron with an edge length of \(~ 5.1 \text{ nm}\) (Figure 3.5b). The interaction between oppositely charged patches of CCMV and avidin causes avidin particles to align in order to maximize the electrostatic attraction. Avidin is also more rigid than the soft dendrimer, which implies
that the arrangement of CCMVs need to be such that both the electrostatic binding and the alignment of avidin is optimized. The less common open bcc packing of spherical particles is an inefficient way of packing, but in the case of CCMV-avidin cocrystals the open structure leaves more space for optimal arrangement of the rigid tetrameric avidin. From Figure 3.7a and Figure 3.7b it is clear that truncated icosahedra can be packed in an fcc lattice such that they (CCMV particles) are connected by the hexagonal faces. The bcc arrangement of CCMV particles is coordinated by avidin. The edge length of the truncated icosahedron symmetry of CCMV matches quite well with the edge length of avidin. Aligning avidin according to the patches of CCMV like in Figure 3.7c can be energetically favorable even though it would lead to an open bcc structure. The proposed models of CCMV-avidin cocrystal structural details are not perfectly conclusive, but in total the differing lattice symmetries of CCMV-PAMAM G6 cocrystals and CCMV-avidin cocrystals are attributed to the different surface charge distribution and rigidity of PAMAM G6 and avidin.

![Figure 3.7. The proposed bcc structure. a) An fcc unit cell with truncated icosahedrons at the lattice points. b) The (111) directions are indicated with yellow bars that go through the center of connecting hexagonal faces of the icosahedron, c) Schematics of a possible arrangement of avidins (red tetrahedron) that bind two CCMV particles (blue truncated icosahedron).](image)

The particle shape affects the lattice symmetry of nanoparticle self-assemblies. In Publication VI, the electrostatic self-assembly of rod-like and spherical particles was studied. Periodic structures were formed by combining the negatively charged rod-like tobacco mosaic virus (TMV) with positively charged synthetic nanoparticles, i.e. small or large cationic AuNPs, and PAMAM G5 dendrimers. In contrast to the previously described periodic structures, the chosen particle combination of particles should not lead to a 3D
lattice, as rod-like building blocks are prone to form a two-dimensional (2D) lattice.

2D hexagonal close packing is the most efficient way of packing uniform rods. However, our results showed that also in the case of a binary 2D lattice, the lattice symmetry is dependent on the size ratio ($D_{NP}/D_{TMV}$) of the building blocks (Figure 3.8a). We observed that small cationic nanoparticles and TMV (size ratio 0.3) formed the typical 2D hexagonal lattice (Figure 3.8b), whereas a combination of large cationic AuNPs and TMV (size ratio 0.86) formed a 2D square lattice (Figure 3.8c).

TMV confines the cationic particles in linear voids in both geometries. In a favourable periodic structure, the cationic particles can bind efficiently to a maximal number of TMVs. By inspecting the geometry of binary 2D lattices with different particle size ratios, it is clear that large cationic particles can bind to four TMVs without resulting in an overlap of the TMVs. TMV particles start to overlap at $D_{NP}/D_{TMV} \sim 0.41$, which means that a nanoparticle combination yielding a smaller size ratio will favour the 2D hexagonal packing, where nanoparticles are confined in a trigonal void. Our experimental data supports this simple consideration. However, it can be added that even smaller cationic particles ($D_{NP} < 2.7$ nm, $D_{NP}/D_{TMV} < 0.15$) cannot bind efficiently to three TMVs, if they are located in the trigonal void. The most favorable location for such small particles would probably be between two adjacent TMVs.
Figure 3.8. The nanoparticle-TMV lattice geometry depends on the size ratio $D_1/D_2$. a) The size ratio of the spherical and rod-like building blocks determines the favorable packing of the particles. b) Combining the PAMAM G5 and TMV leads to a 2D hcp lattice. The lattice constant $a$ obtained from SAXS data is 19.6 nm. c) Combining large cationic AuNPs and TMV leads to a 2D square lattice. The lattice constant $a$ obtained from SAXS data is 23.2 nm. Adapted from Publication VI.

There are multiple strategies to obtain a variety of periodic multicomponent nanoparticle structures. In Publications I, II, III, and VI particle size ratio, rigidity, charge, and patchiness were identified as variables that can be tuned to obtain different structures. In summary, all the cocrystal structures that included smoothly charged spherical nanoparticles could be related to space filling that optimizes the electrostatic attractive and repulsive interactions. In contrast, the combination of patchy rigid particles leads to an open bcc structure that proposedly results from patchy interaction that leads to suboptimal space filling.

### 3.2 Macroscopic Habit of Multicomponent Self-Assemblies

When self-assemblies form, they eventually adapt a macroscopic shape. The shape can be determined by confinement, the assembly mechanism, or by an intrinsic property of the self-assembling units. Also external fields can affect the macroscopic shape of nanoparticle assemblies. In the previous
chapter, the lattice symmetry of periodic nanoparticle structures was discussed. It was concluded that a specific lattice results from nanoparticle shape and interparticle interactions. The same interactions that determine the lattice symmetry also affect the macroscopic habit of nanoparticle self-assemblies. Hence, the topic deserves a cursory discussion, even though macromolecular crystallization in general is far too complex to be described in detail in the scope of this thesis\textsuperscript{112}.

The shape of an ideal crystal in thermodynamic equilibrium is determined by the specific surface free energy $\sigma$ of different crystal facets, as initially stated by Wulff\textsuperscript{113}. Later, the growth of faceted crystals has been described in terms of growth rate in different lattice directions\textsuperscript{114–116}. The fundamental idea is that the crystallographic arrangement implies a different amount of formed bonds (or specific binding energy) for interfaces formed in different lattice plane directions, which leads to a growth of faceted crystals. Initially, the theory was developed to describe molecular crystals, but recent work by Mirkin \textit{et al.} shows that similar rules can be applied to some nanoparticle crystals\textsuperscript{110,117}. From the aspect of crystallization, colloidal particles can be compared with atoms and therefore they are sometimes referred to as “colloidal atoms”\textsuperscript{118,119}. Especially the growth of nanoparticle crystals resemble that of the growth of molecular crystals, including “colloidal adatoms”, vacancies, and growth \textit{via} proceeding step edges\textsuperscript{117,119,120}. For nanoparticle crystals, surface energy tends to be smallest for facets with the closest packing of NPs. It has been reported that such minimum energy facets dominate Wulff polyhedra assembled from nanoparticles\textsuperscript{117}.

In Publication I and Publication III, the relation between crystal structure and crystal habit was directly observed for samples with fcc structure. For the fcc crystal structure, the closest packing is found in the $\{111\}$ lattice plane (Figure 3.9a,b). Previous studies on aFT crystallization show that the crystallization proceeds in the $\{111\}$ plane by the formation of step edges\textsuperscript{120–122} (Figure 3.9c). Such crystal growth should result in octahedral crystal habit, as the fcc lattice includes eight $\{111\}$ lattice planes. The crystallization was not studied \textit{in situ} in Publications I and III, but cryo-TEM and optical microscopy showed that crystallites exhibited an octahedral Wulff polyhedron habit that is in line with a crystal growth similar to conventional aFT crystallization.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.9.png}
\caption{Scheme demonstrating the relation between fcc structure and the observed octahedral crystal habit. \textbf{a)} Fcc unit cell (left) and an octahedron with internal fcc structure (right) (adapted from Publication III with permission from the American Chemical Society ©2016). \textbf{b)} $\{111\}$ plane with hexagonal close packing of particles. \textbf{c)} Proceeding step edge.}
\end{figure}
Interestingly, cryo-TEM studies revealed directly the relation between crystal symmetry and crystal habit. Figure 3.10 shows an octahedral aFT-PAMAM crystallite viewed from the [100] direction. However, the fast formation of aFT-PAMAM cocrystals did not yield crystallites large enough to be readily characterized by optical microscopy. On the other hand, almost millimeter sized crystals (Figure 3.11a-c) were produced in Publication III by a slower self-assembly process. The large crystallites also showed an octahedral habit and fcc crystal structure that was confirmed by SAXS (Figure 3.11d).

Nucleation and growth is significant for the shape and size of the crystallites. Crystallization is typically controlled by initiation and growth\textsuperscript{123}. Thus, describing the macroscopic habit of crystallites as Wulff polyhedra is strictly speaking not necessarily correct, as Wulff polyhedra are defined as structures that are in thermodynamic equilibrium\textsuperscript{113}. Growing structures are not in thermodynamic equilibrium. Thus, also the resulting final structures might remain slightly out of equilibrium, especially if the growth is terminated before an equilibrium structure is reached.

The observations from optical microscopy suggest that the observed octahedral crystal habit is mostly related to the crystal growth mechanism. Importantly, the crystals typically exhibit vertices that are not fully developed (Figure 3.12). Many of the octahedra remain unregularly truncated and \{111\} facets are observed to “bend” towards the vertices. Further support for the mechanism of nucleation and growth comes from the observation, that slowing down the assembly by a higher \(c_{\text{NaCl}}\) the crystals grow large due to decreased nucleation rate and thus, fewer but larger crystals (Figure 3.11a-c).
Figure 3.11. aFT-(ZnPc-PTSA) crystals formed at different \( c_{NaCl} \). a) \( c_{NaCl} = 0 \) mM. b) \( c_{NaCl} = 20 \) mM. c) \( c_{NaCl} = 30 \) mM. Scale bars in all images are 50 \( \mu \)m. d) SAXS profile from an aFT-(ZnPc-PTSA) sample (\( c_{NaCl} = 20 \) mM). Adapted from Publication III with permission from the American Chemical Society ©2016

Publication VI demonstrates radically different habit of nanoparticle superstructures that are formed by the self-assembly of rod-like TMV and spherical nanoparticles. The structure is described as a 2D superlattice, but the superlattices exhibit a wire-like macroscopic habit. Also here, the habit was explained by an assembly mechanism dominated by nucleation and growth. Superlattices nucleation occurs when TMVs are crosslinked by AuNPs (Figure 3.13a,b). The crosslinked TMVs attract AuNPs to a higher degree than single TMVs. Adjacent TMVs become aligned, and free AuNPs are subject to a focused attractive field between the TMVs. Therefore, the interstitial channel
between TMVs is an energetically highly favorable position for AuNPs, leading to a zipper-like growth, where superlattices grow as nanoparticles crosslink TMVs (Figure 3.13c). The superlattice wires form even larger bundles that can be observed with optical microscopy (Figure 3.13c-e). Although additional TMVs are incorporated both in the TMV axis and the normal-to-axis direction, the zipper-like assembly mechanism with the rod-shape of TMV provides fast growth in the TMV axis direction, leading to a high aspect ratio of the formed superlattices.

Figure 3.13. Cooperative self-assembly leads to highly ordered superlattice wires that arrange into macroscopic bundles. a) Schematics of the cooperative self-assembly. b) Cryo-TEM images of the cooperatively formed structures at different stages of assembly. c) Cryo-TEM image of a bundle of superlattices, (close-up from d) d) Optical microscope image of the macroscopic habit of superlattice bundles. a)-d) Adapted from Publication VI.
Even though the studies presented in this thesis do not include in situ experiments on the formation mechanism of multicomponent crystals, Publications I, III, and VI include interesting observations of large nanoparticle structures, where the habit of the assemblies can be directly related to the assembly mechanism. In all cases, it was concluded that a directionally anisotropic growth rate leads to the observed habit. In cases that involved aFT, the crystal habit was related to the fast growth of \{111\} planes, which yielded regular octahedral crystal habit. TMV-AuNP superlattices formed superlattice wires due to the fast zipper-like growth of superlattices in the direction of the TMV main axis. These studies underline the possibility of using well-defined protein cages as building units that direct the assembly of a specific superstructure habit.

3.3 Light Induced Patterning of Nanoparticle Assemblies

External fields and energy sources can be used to induce patterns in cases where particles do not spontaneously form regular structures. The challenge in such cases is to transfer the external energy into directed particle motion and deformation of the material. In Publication IV cationic nanoparticles were functionalized with an anionic light-responsive azo dye to induce formation of surface relief gratings (SRG) directed by visible light.

Azobenzene is an aromatic molecule that responds to visible light by a reversible conformational change; illumination by light leads to trans-cis photoisomerization of azobenzene\textsuperscript{124} (Figure 3.14a). The high quantum yield of azobenzene photoisomerization leads to rearrangement of molecules in azobenzene functionalized materials\textsuperscript{123}. Illuminating such material with an interference pattern of polarized light induces pressure gradients and mass transport within the material, and leads ultimately to an SRG defined by the interference pattern\textsuperscript{124–126} (Figure 3.14b). Azobenzene containing molecules and polymers have been used to coat surfaces, in order to prepare SRGs\textsuperscript{124}.

![Figure 3.14. Photoisomerization induces mass transport. a) Trans-cis photoisomerization of azobenzene. b) Illuminating an azobenzene functionalized material with a polarized interference pattern leads to the formation of an SRG, where the mass transport (dashed arrows) is directed from high intensity towards low intensity.](image)

The aim of Publication IV was to demonstrate that large dendrimers and biologically relevant protein cages can be arranged into SRGs by light. aFT and large triazine dendrimers with a molecular weight up to 528 kDa\textsuperscript{127,128} were functionalized with ethyl orange (EO) (Figure 3.15), which is an anionic water
soluble azo dye with a hydrophobic tail. The functionalization was carried out in dilute aqueous solution by the electrostatic attraction between the positively charged amine surface groups of the dendrimers and the negative sulfonic acid group of EO. Moreover, the functionalization of aFT was carried out at a pH below the pI of the protein cage, where it is on average cationic and was thus found to bind to EO.

The electrostatic complexation was characterized by DLS and AFM, which showed that titrating EO into triazine dendrimer or apoferritin solution lead to aggregation and phase separation of the complexes (Figure 3.16a,b). The complexation was also confirmed by UV-Vis and FTIR measurements (data not shown here). Aggregation of the cationic particles and the anionic dye resembled more a dye concentration dependent phase separation than electrostatic self-assembly, where like-charged particle are crosslinked by oppositely charged counterparts. This conclusion was based on the observation that a plateau in the size evolution of the complexes was not reached before the sample included a significant excess of EO in respect to the number of the dendrimer end groups or the amount of aFT. Neither did SAXS characterization of aFT-dye complexes indicate any periodic arrangement of the aggregates (Figure 3.16c), even though aFT is prone to form periodic self-assemblies, as discussed in chapter 3.1.
The Structure of Nanoparticle Assemblies

Figure 3.16. Phase separation induced by electrostatic complexation. a) DLS scattering intensity and particle size evolution as a function of the EO/G7 ratio. At low EO concentrations the dendrimers remain isolated in the water solution (1), but increasing the amount of EO results in ionic complexation and aggregation of the complexes (2), which then swell at excess amount of EO (3). b) AFM height and phase images (500 nm × 500 nm) taken from drop-cast samples of pure G9 dendrimer (top), 0.75 m(EO)/m(G9) ratio (middle) and 1.5 m(EO)/m(G9) ratio (bottom). c) The SAXS profiles measured from aqueous aFT-EO precipitate. The profiles are vertically offset for clarity. Adapted from Publication IV with permission from the American Chemical Society ©2014

The electrostatic binding of EO can be utilized to gain light-fuelled mechanical motion of the nanoscale building blocks. In order to prepare SRGs, thin films with thicknesses ranging from 300 nm to 600 nm were prepared, by spin coating of glass substrates (Figure 3.17a) with aqueous solution of complexes. Polarized laser light with wavelength of 488 nm and intensity of about 300 mW/cm² in a Lloyd’s mirror setup was used to create an interference pattern that induce grating formation. The formed gratings were imaged with AFM (Figure 3.17b). Surface patterns with modulation depths of ~50–150 nm were successfully inscribed in thin films of all complexes. Figure 3.18a shows the grating cross-section profiles for all dendrimer samples, while the AFM height images are shown in Figure 3.18b and c. Perhaps the most striking observation of the study was that the modulation depths of the SRGs were not negatively affected by increasing dendrimer size, but instead it increased for larger dendrimer generations. Even the aFT sample was clearly patterned, even though the inscription efficiency was higher for dendrimer samples (Figure 3.18d).
The Structure of Nanoparticle Assemblies

Figure 3.17. a) A photograph of a triazine dendrimer G5-EO sample film with two perpendicularly inscribed SRGs. b) The corresponding AFM images from (1) a flat (unilluminated) film, (2) a single grating, and (3) from the intersection of the two gratings. Adapted from Publication IV with permission from the American Chemical Society ©2014

Figure 3.18b and Figure 3.18c reveal rough surface textures on the SRGs of all complex generations, which likely originate from the complex aggregates present in the solution state. Such aggregates are not found on the unilluminated areas of the sample films. It is reasonable that the coarse grains visible at the crests result from a mass transport, in which the structure of the complex aggregates is at least partly remained; the complexes do not fully break apart during the mass transport.

In conclusion, the results show that globular molecules can move in response to light via complexation to an azo dye. Very large complexes, including functionalized triazine dendrimers or aFTs, were able to undergo mass transport and form SRGs under illumination. The concept was, for the first time, applied to induce surface patterns in a film of an azobenzene-functionalized native protein. The study is important in the context of this thesis, because it demonstrates an additional approach to control protein cage and nanoparticle arrangement, and allows for specific control over surface topography.
Figure 3.18. a) SRG line profiles for all dendrimer−EO complexes. b) AFM height images the SRGs (5 × 5 μm). c) 3D AFM height image (left) and phase image (right) of the G9-EO grating surface. d) A 10 × 10 μm AFM height image of an SRG on the aFT-EO film, with the corresponding phase image and a 3D presentation. Adapted from Publication IV with permission from the American Chemical Society ©2014
4. Modular Functionalization of Nanoparticle Assemblies

Materials that possess intrinsic properties and functions of their own are often considered as functional materials. Such functionality might arise from chemical composition, structural features, or due to a combination of these. A variety of functionalities would be achieved in a combinatory manner, if functionalization could be carried out in a modular fashion, which would include that the functionality of a building unit would be retained after combining it to other building units.

Multicomponent nanoparticle structures naturally provide a much broader range of compositions and functionalities as compared to the assemblies of one type of nanoparticles\(^3\). The collective physical or chemical properties of assembled nanoparticles often differ from the properties of dispersed particles, a feature which might be beneficial or unwanted. Beneficial collective properties are especially such that cannot be generated by other means\(^3,11,129\). An example of an unwanted collective feature is the decreased catalytic activity of an aggregated catalyte\(^3,130,131\).

Traditional materials science routinely deals with designing materials properties by means of chemical composition, crystal structure, and crystallinity. Producing functional materials by self-assembly approaches is in this sense similar to traditional materials science. However, the synthesis of inorganic nanoparticles\(^3\) has developed at a fast pace parallely with the field of biological particles\(^51,52,132–134\), leading to a rich library of functional particles that can be combined to yield materials with unforeseen properties. Developing self-assembly approaches, and exploring the possibilities of combining the available building blocks into functional materials, is a major task that will probably lead to both fundamentally and technologically significant findings\(^3,135\).

Self-assembly also enables the formation of structures that can be used as nanofinements for chemical reactions and catalysis. Such structures can be used for example to immobilize catalytic components\(^136,137\), and it has been shown that confining chemical reactions into such structure enhances the reaction rate and selectivity of the reaction product\(^138\). A range of different approaches to arrange functional building blocks are established. These include crystallization\(^99,139,140\), crosslinking\(^136,137\), electrostatic\(^69,97\) and solvophobic interactions\(^83,88\), coordinated binding\(^141\), DNA technology\(^142,143\), and
selective interactions between proteins\textsuperscript{144–146}. From a structural point-of-view, this thesis focuses on the electrostatic interaction, which is nonspecific, and can be used in binding charged functional units to oppositely charged building blocks in aqueous environment. In addition, specific binding is used to extend the range of components available for functionalization. In this chapter, three examples of modular functionalization are discussed.

4.1 Singlet Oxygen Generating Protein Crystals

Publication III covers a study on immobilizing zinc phthalocyanine (ZnPc) by assembling it with aFT into cocrystals with a long-range order. Phthalocyanines are organic photosensitizers that are widely used in materials science and various technological applications\textsuperscript{147–149}. Phthalocyanines are especially known for their ability to generate highly reactive singlet oxygen upon illumination by visible light\textsuperscript{150}. However, immobilizing ZnPc into solid state is not trivial, because ZnPc derivatives tend to stack in aqueous solution losing their singlet oxygen (\(1^O_2\)) photogenerating property\textsuperscript{130,131}. aFT, on the other hand, is well-known for its biological function to sequester metal ions from solution by biomineralizing nanoparticles in its interior cavity\textsuperscript{151}. This makes it a generally interesting building block for functional crystals.

Before crystal formation ZnPc was complexed with 1,3,6,8-pyrene-tetrasulfonic acid (PTSA), in order to avoid the disadvantageous stacking of ZnPc. The ZnPc-PTSA complex is a cationic tetravalent macroion (Figure 3.1) that was bound to the anionic aFT particles, leading to aFT-(ZnPc-PTSA) cocrystal formation, which was discussed in chapter 3.1. However, regarding the structure of the cocrystals, it is essential to note that packing aFT (\(d_{\text{aFT}} \approx 12\) nm) into an fcc lattice with a lattice constant of 19.6 nm results in a porous structure with a packing fraction less than 50\%. This leaves significant voids between the aFT particles (Figure 4.1a). Such a porous structure allows diffusion through the structure and it was found to yield efficient \(1^O_2\) photogeneration.

The \(1^O_2\) quantum yield of the cocrystals was determined using the relative method\textsuperscript{152}. In short, the relative method estimates via absorption the rate of photodegradation of a chemical scavenger (1,3-diphenylisobenzofuran, DPBF), which is directly proportional to the formation of \(1^O_2\). The quantum yield values given here indicate the measured \(1^O_2\) quantum yield relative to a reference sample in optimal conditions (ZnPc dissolved in DMSO, relative value: 1). The technical details of the experiment are described elsewhere\textsuperscript{131}, but the results (Figure 4.1b) of the measurements can be recapitulated as follows: Dispersed ZnPc showed the highest \(1^O_2\) quantum yield (relative value: 0.70), which was decreased by complexation to PTSA (relative value: 0.55). Importantly, the \(1^O_2\) quantum yield of the ZnPc-PTSA complex was clearly improved (relative value: 0.63 and 0.68) when immobilized to the porous cocrystals, which shows that the crystalline arrangement of the building units does not only retain the photogeneration of ZnPc-PTSA, but even enhances it.
This result is in line with the conclusion that protein crystals can be used to form porous structures that support chemical functionality.\(^99\)

**Figure 4.1.** ZnPc-PTSA immobilized in a porous structure yields efficient $^1\text{O}_2$ photogeneration

**a)** Schematics of the aFT-(ZnPc-PTSA) fcc structure. The particles are drawn to scale, but the number and arrangement of ZnPc-PTSA complexes within the unit cell are only suggestive. A realistic number of ZnPc-PTSA complexes within the unit cell is an order of magnitude higher than visualized here. **b)** Scheme of $^1\text{O}_2$ photogeneration followed by degradation of DPBF to o-dibenzoylbenzene (ODB).\(^{153}\). Normalized absorption ($\ln(A_0/A_t)$) of DPBF over time, showing its photodegradation rate in the presence of different photosensitizer systems. Solid lines present linear fits from which the slope is directly proportional to the photoinduced generation of $^1\text{O}_2$. ZnPc accounts for the nonsubstituted derivative used as reference compound. Adapted from Publication III with permission from the American Chemical Society ©2016.

### 4.2 Modular Functionalization by Means of Biotin-Avidin Binding

Porous 3D structures in aqueous solution can be efficiently loaded with functional moieties and enable high efficiency in diffusion dependent reactions, as mentioned in the previous section. However, bigger functional units require larger frames for immobilization. In Publication II, CCMV with a diameter of ~28 nm was used as the major component to form cocrystals in combination with avidin. As discussed in chapter 3.1, the patchy electrostatic attraction between CCMV and avidin leads to an open bcc structure with a low packing fraction. Avidin is a tetrameric glycoprotein (Figure 4.2) that has an extremely high selective affinity to biotin, better known as vitamin B7 and vitamin H. This ability has allowed avidin and its derivatives to become important in many biochemical techniques, including biomolecule detection, interaction studies and purification.
In this study, avidin was used to bind uncharged biotin tagged components in order to include different functionalities to cocrystals. Our intention was to demonstrate, that the chosen building units (CCMV and avidin) can be used as a platform for developing biobased materials with specific functionalities. The aim was to study if functional units can be readily incorporated in the open bcc crystals, if the incorporation will retain the initial functionality of the moieties, and whether the functionalization will affect the structure of the CCMV-avidin crystal. This proof-of-concept was demonstrated by incorporating biotin (5-fluorescein) conjugate (BF), biotin-tagged horseradish peroxidase enzyme (B-HRP) and biotinylated 5 nm AuNPs (B-AuNP) into the crystal structure. The functionalization was carried out by two approaches (Figure 4.2): First, by pre-functionalizing avidin with the biotinylated functional unit and then adding the virus particles to form the crystals (Method 1). Second, by first forming the crystals followed by the addition of biotinylated agent (post-functionalization, Method 2).

Method 1 and 2 were first carried out using biotinylated fluorescein (BF) (Figure 4.3a). BF is small ($d_{\text{max}} \sim 1.5$ nm) compared with the unit cell of the CCMV-avidin crystal and did not affect the structure of the functionalized crystal. Both pre- and post-functionalized samples were characterized by SAXS, which confirmed that the structure was identical to the pristine non-functionalized crystal structure (Figure 4.3b).

BF has a fluorescence emission maximum at $\lambda_{\text{em}} \sim 525$ nm, making the functionalization straightforward to confirm by fluorescence measurements. The initial amount of BF in the samples was slightly less than one BF per
avidin. The functionalized crystals were separated from the unbound BF by centrifugation. Both the supernatant (sup.; 80% of the sample volume) and the sediment (20% of the sample volume) were diluted to the same volume and the net amount of BF in both fractions was measured using fluorescence spectroscopy. In order to avoid interfering scattering from the large crystal aggregates during fluorescence measurements, concentrated NaCl solution was used to disassemble the crystals. The integrated fluorescence intensities of pre-functionalized sediment and supernatant were 87 % and 16 %, respectively, of the pure BF sample fluorescence intensity (Figure 4.3c,d). For post-functionalized samples, the respective values were 82 % and 16 %. The result shows that the BF is readily absorbed in the CCMV-avidin crystal regardless of how the BF functionalization is carried out. This implies that the crystal structure is porous enough to allow the diffusion of BF.

Figure 4.3. Functionalization with fluorescent dyes. a),b) Crystals (cryst.) functionalized with biotin (5-fluorescein) (BF) show high degree of crystallinity in SAXS measurements irrespective of preparation method (Methods 1 or 2). c),d) Fluorescence spectra and integrated fluorescence intensities show the presence of BF predominantly in the crystals but not in the supernatant (sup.). Adapted from Publication II with permission from Nature Publishing Group ©2014.

Biotin-tagged AuNPs (B-AuNPs) with a metal-core diameter of ~5 nm (Figure 4.4a), were used to show that also larger components can be incorporated into the CCMV-avidin crystals. The stoichiometric ratio of AuNP to CCMV was roughly 1:10, which did not affect the crystalline structure, according to SAXS. Both pre- and post-functionalization method led to efficient binding of B-AuNPs into the crystal aggregates, which had a dark appearance, whereas the supernatant was clear. From SAXS and TEM data, it could be concluded that the B-AuNPs are evenly distributed within the crystals, without clear spatial correlation or clustering (Figure 4.4b-d). This observation highlights that the uncharged B-AuNPs are not essentially
contributing to crystal structure, but are passively coordinated by random binding to avidin. In contrast, electrostatic binding of AuNPs to CCMV leads to a distinct superlattice structure shown in Figure 4.4e and f.

**Figure 4.4.** Functionalization with AuNPs. **a-b)** Biotinylated gold nanoparticles (B-AuNPs) with a gold core diameter of ~ 5 nm can be incorporated to crystals according to the measured SAXS curves. Form factor FF of the AuNPs dominates the scattering pattern indicating non-correlated distribution of AuNPs in the crystal. **c)** TEM image of the AuNP functionalized crystals. Scale bar, 150 nm. **d)** An AuNP cluster in the absence of protein crystals compared with a magnified view of the AuNP-functionalized crystals. Dimensions for the images are 150 nm × 150 nm. A comparison to a previously obtained AB₈ fcc-type CCMV–AuNP binary crystals viewed along the [110] projection axis **e)**. Here the AuNPs occupy defined lattice points. **f)** Fourier transform from image and inverse Fourier transform calculated with selected Fourier components. Adapted from Publication II with permission from Nature Publishing Group ©2014.

Both BF and B-AuNP modulate only the optical properties of the CCMV-avidin crystals, but the range of possible applied functionalities is much wider. Catalytic activity was implemented to the crystals by biotinylated horseradish peroxidase (B-HRP), which has a maximum diameter of ~ 6 nm and is frequently used in clinical diagnostic kits and for immunoassays (Figure 4.5a). B-HRP was successfully immobilized to the crystal aggregates by both pre- and post-functionalization. Post-functionalization preserved the highly ordered crystal structure, but pre-functionalization resulted in a more disordered structure, which is observed as broad diffraction peaks in the SAXS data (Figure 4.5b). However, the decreased degree of order was likely an effect of the presence of bovine serum albumin (BSA) in the commercial B-HRP product. BSA is a protein with a maximum diameter of ~ 14 nm and it cannot fit the crystal voids without affecting the structure. Nevertheless, B-HRP was in both cases efficiently immobilized to the CCMV-avidin crystals and retained its catalytic activity.
B-HRP catalyses the one-electron oxidation of 3,3’5,5’-tetramethylbenzidine in the presence of hydrogen peroxide (Figure 4.5), which allows straightforward spectrophotometric quantitation. Progress curves indicate the increasing concentration of the intermediate product, the electron transfer complex (Figure 4.5). The initial velocity $V_0$, which is the interpolated slope of the progress curve at $t = 0$, is directly proportional to the enzymatic activity of the sample. The conclusion based on the measured progress curves (all data is not shown here) was that roughly 90 percent of the enzymatic activity of all B-HRP added to the samples was immobilized to the CCMV-avidin crystals that were centrifuged from the solution. Residual activity of the supernatant was likely due to unbound B-HRP, B-HRP that was bound to free avidin units, and B-HRP bound to very small CCMV-avidin crystallites that did not sediment properly. After washing the sample four times (washing includes centrifugation and replacing the supernatant with fresh buffer) the enzymatic activity of supernatant virtually disappeared, whereas the activity of the sediment remained (Figure 4.5).
Importantly, the finite crystallite size and nanoscale porosity of the crystals allowed diffusion of substrate. This can be concluded from the results, which did not show that the immobilization of B-HRP into CCMV-avidin crystals would yield a clear negative effect on the enzymatic activity. As a reference, aggregating B-HRP–avidin units with poly(acrylic acid) (Mw 250 000 Da) yielded amorphous but tight complexes with relatively low enzymatic activity.

In total, the electrostatic assembly of CCMV and avidin was found to be a versatile platform for specifically immobilizing a range of functional units. The porous structure of the crystal allows diffusion of relatively small molecules, which is essential as far as chemical or catalytic functionalities are concerned. The electrostatic self-assemblies are also sensitive to ionic strength, which can be used for releasing the functionalized avidin units from the crystal aggregates.

### 4.3 Plasmonic Superlattices from Gold Nanoparticles and Protein Rods

Nanoparticles exhibit size-dependent magnetic and plasmonic properties that can be modulated by arranging the nanoparticles into larger superstructures. Gold nanoparticles (AuNPs) have been extensively studied because of their high stability and exotic optical properties, dominated by the localized surface plasmon resonance (LSPR). In Publication V we presented a scalable synthesis of cationic AuNPs with tunable sizes between 8–20 nm and narrow size distribution. Compared to previously established synthesis of cationic AuNPs with sizes only up to 5 nm these nanoparticles offer a stronger light absorption in the visible range because of the approximately cubic dependence of the LSPR intensity on particle size.

The collective properties of nanoparticle assemblies can be changed by arranging plasmonic nanoparticles into different configurations. Tuning materials properties by modulating the structure is of fundamental interest in materials science. For example, asymmetric absorption of left- and right-handed circularly polarized light (circular dichroism) can be affected by arranging AuNPs in a chiral configuration. Such findings have led to intensive studies on materials that exhibit chiral plasmonic features. In Publication VI AuNPs were combined with TMV resulting in highly ordered superlattice wires exhibiting circular dichroism. The assembly mechanism was discussed in chapter 3.3.

It was observed that the right-handed helical symmetry of TMV led to right-handedness of the superlattice wires (Figure 4.7). This was explained in terms of electrostatic forces applied on a helical charge distribution in a spherically symmetric electrostatic field, which on average implies bending (trivial) and torsional (nontrivial) forces that strives to deform the helical construct. It should be emphasized that simultaneous pointwise bending and torsion of a rod-like particle yields a helical deformation. Cationic AuNPs give rise to a spherically symmetric electrostatic potential and the electrostatic surface potential map of TMV exhibits a helical symmetry (Figure 4.7a). Thus, the
A qualitative electrostatic description was found to be in good agreement with the known structural details of the building blocks and the resulting structure.

Figure 4.7. The helical charge distribution of TMV leads to a helical superlattice structure. 

a) The electrostatic surface potential map of TMV in aqueous solution shows that TMV surface charge distribution has a helical symmetry. 
b) 3D density isosurface of a selected volume of the cryo-ET reconstruction showing the lattice twist on a nanoparticle level. 
c) A schematic of the superlattice including TMVs. 
d) Cryo-ET reconstruction of a single superlattice wire. 
e) SEM image of a plunge-frozen and freeze-dried superlattice wire. Adapted from Publication VI.

Importantly, the efficient packing of AuNPs in the superlattice led to plasmonic coupling. This implies that electromagnetic fields, related to the LSPR of the tightly packed AuNPs, interfere. A consequence is the observed redshift of the plasmonic absorbance peak, which is visible as a violet appearance of the sample (Figure 4.8a). In contrast, free unbound AuNPs have a ruby red appearance. The plasmonic coupling also resulted in plasmonic circular dichroism (CD). The measured CD spectra showed a peak-dip feature at visible wavelengths, which occurs as the superlattice wires form (Figure 4.8b).

Several studies show that CD spectra of helical plasmonic constructs exhibit a peak-dip or dip-peak feature depending on the handedness of the structure. Simulations based on the coupled dipole approximation have sufficiently well reproduced CD spectra measured from well-defined helical structures, even though simulated spectra typically show peaks and dips that are narrow in comparison to the features in the measured spectra. Such studies typically show that a peak-dip feature corresponds to a left-handed and a dip-peak feature corresponds to a right-handed structure.

In order to explain the shape of the CD signal we also carried out simulations of a theoretical nanoparticle configuration based on the structural details that were available from SAXS and cryo-ET characterization. The simulations
confirmed that the measured CD spectra correspond to a wire-like right-handed helical superlattice structure, even though the peak-dip feature typically correspond to left-handed structures. Importantly, details of the simulations reveal that the circular dichroism of the helical superlattice is direction dependent. A simulated CD spectrum of light propagating along the helical axis (direction of the superlattice wire) shows a dip-peak feature typical to right handed helical structures. However, the CD in the axial direction (Figure 4.8d) is weak in comparison to other directions of propagation. In addition, practically all light propagating in the direction of the superlattice wire will attenuate due to the strong absorbance of the AuNPs. On the other hand, as seen from Figure 4.8c, the superlattice structure exhibits left-handed characteristics when viewed from the transverse direction. Simulations confirmed a peak-dip feature in the CD spectrum of light propagating in the transverse direction. What is more, light propagating in the transverse direction does not attenuate as significantly as light propagating in the axis direction due to the high aspect-ratio of the superlattice wires.

Figure 4.8. Optical properties of the superlattice depends on the structure. a) UV-vis data (scaled for clarity) at different salt concentrations showing the effect of AuNPs assembling tightly in the superlattice as a function of the ionic strength. The plasmonic resonance peak at 524 nm corresponding to free AuNPs disappears completely only at low ionic strength. b) CD data that is Savitzky–Golay filtered for clarity (original data in Publication VI). CD features appear as the superlattices form upon decreasing ionic strength. Inset shows the simulated CD
spectrum for different directions of light propagation and taken as an average of all directions. (c), (d) The superlattice model including 400 AuNPs that was used to model the CD spectrum. The figure indicates that the lattice undergoes a left-handed (c) or a right-handed (d) twist depending on the viewing direction. Adapted from Publication VI.

In conclusion, the studies included in Publication VI, coherently show how the combination of rod-like particles with a right handed helical charge distribution and plasmonic spherical particles leads to a superlattice that exhibits left-handed plasmonic characteristics, which is a property that cannot directly be related to either of the particles alone. In total, the work underlines that novel materials properties can be achieved in a combinatory manner via relatively complex mechanisms. This reveals the potential of hierarchical structuring where each structural hierarchy modulates the materials properties.
The results presented in this thesis demonstrate that electrostatic self-assembly guided by biological particles is a viable tool in nanoparticle superlattice engineering. Electrostatic self-assembly depends on the interplay between competing repulsive and attractive forces, which ultimately leads to well-defined periodic minimum energy structures. Achieving highly ordered structures via electrostatic self-assembly can be tedious work, but the examples that were discussed show that electrostatic interaction can be tuned by ionic strength. In addition, varying the self-assembling building blocks gives a rich diversity of superlattice structures with properties depending on both the particle combination and the arrangement of the constituent particles. Properties of the superstructures, such as lattice geometry, structural stability against ionic strength, crystal habit, and chemical and physical properties were all traced back to the properties and interactions of the constituent units.

This thesis work is ultimately based on the cornerstones of supramolecular chemistry, structural biology, colloid science, the early work on nanoparticle chemistry, and template based assembly of nanoparticles. In that sense, the included research could be assumed to be outdated. Still, during the timespan of this thesis work, the field of nanoparticle synthesis and bioengineered particles, and self-assembly has developed significantly. This work has been a part of the scientific dialogue that is constantly looking for new approaches to develop materials with exquisite structural features and functionalities.

During these four years the fields of polymer self-assembly, field driven nanoparticle self-assembly, template based self-assembly, DNA based structures, and bioengineered hierarchical structures have demonstrated unforeseen control over nanoscale structural details. In the context of this thesis, it is remarkable that genetic engineering and ab initio design of amino acid design of amino acid and nucleic acid based particles with virtually atomically precise structure has developed in a rapid pace.

This thesis is a study on self-assembly of well-known nanoparticles. In principle, modern computational and biotechnological methods allow de novo design of protein cages and DNA origami structures on demand. It is not too far fetched to claim, that future research on self-assembling proteins and protein cage based materials will increasingly rely on ab initio tailor made structures that are designed for specific purposes. Therefore, the crossdisciplinary work included in this thesis will hopefully for its own part
bridge the gap between hierarchical self-assembly and designed particles and thus ultimately contribute to the development of *ab initio* designed hierarchical functional materials.
References


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"Materials scientists increasingly look to nature for design principles for new materials and draw inspiration from the many examples of self-assembly within hierarchical structures, which result in complex coordinated biological function. The long-term goal of the work presented in this thesis is to establish effective strategies to harness the self-assembly of biological and synthetic building blocks into hierarchically ordered structures with spatial control."

- Prof. Trevor Douglas, Indiana University