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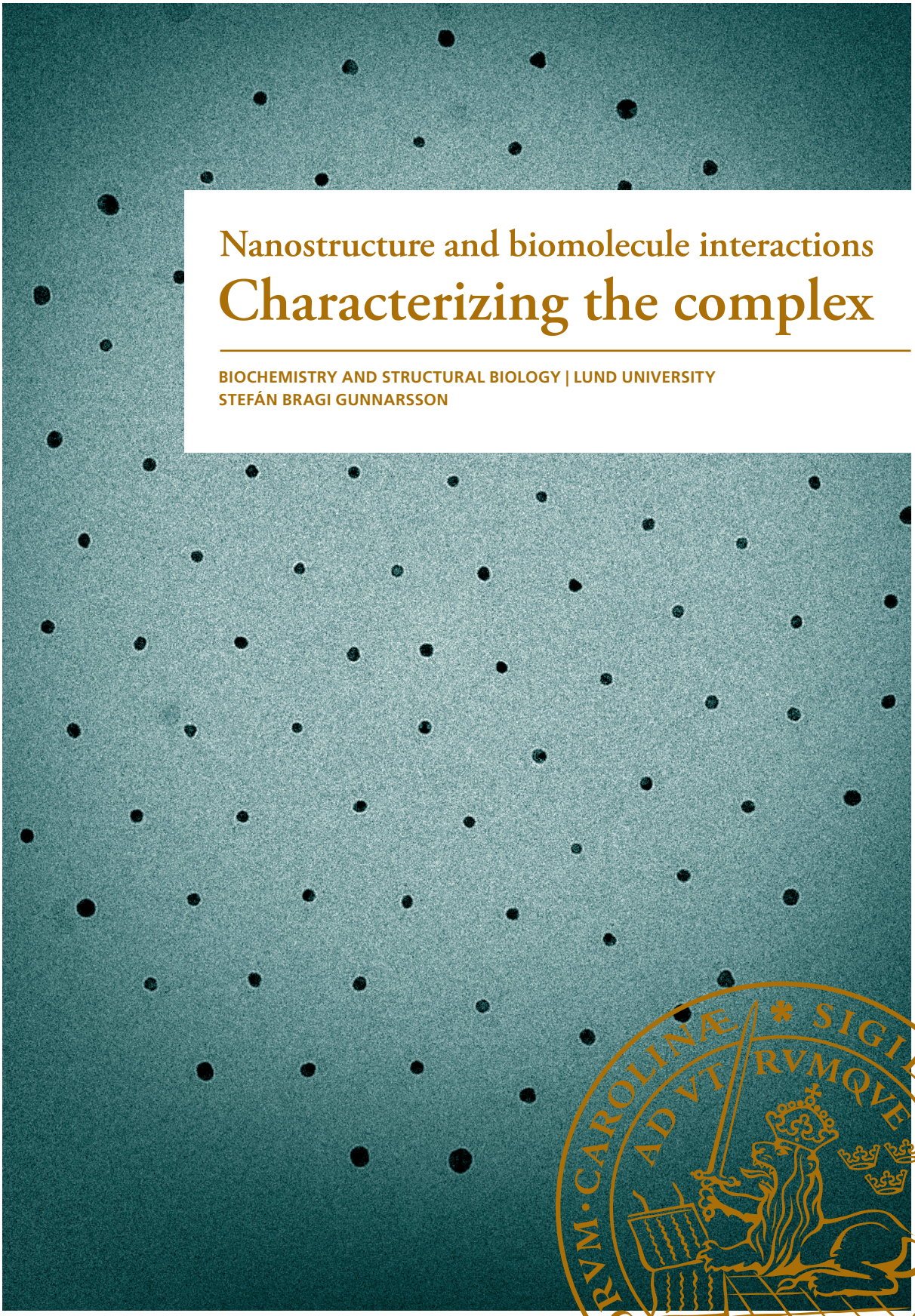
Characterizing the complex

Gunnarsson, Stefan

2018



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BIOCHEMISTRY AND STRUCTURAL BIOLOGY | LUND UNIVERSITY
STEFÁN BRAGI GUNNARSSON

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Stefán Bragi Gunnarsson

LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Science, Lund University, Sweden.
To be defended at Kemicentrum, Lecture Hall B, at 10:00 a.m. on Wednesday October 10th.

Faculty opponent
Professor Fredrik Höök
Biological Physics, Department of Physics
Chalmers University of Technology

Organization	Document name
LUND UNIVERSITY	Doctoral dissertation
	Date of issue 2018-10-10
Author Stefán Bragi Gunnarsson	Sponsoring organization

Title and subtitle
Nanostructure and biomolecule interactions – Characterizing the complex

Abstract

This thesis presents the results of studies done on various biomolecules and their interactions with nanomaterials. The biomolecule sources are everything from purified, single proteins to the complicated mixture of blood serum and cell culture media. Similarly, the nanomaterial sources vary from spherical titanium dioxide, gold, and polystyrene nanoparticles to novel nanowires of gallium arsenide or gallium phosphide. Regardless of the biomolecules or nanomaterials, they interact in ways that require careful characterization in case the environment and organisms are exposed to theses increasingly common materials. The adsorbed protein layer on a nanostructure is called the protein corona and the nanomaterial together with its adsorbed biomolecules is called a complex.

We show that when titanium dioxide nanoparticles are mixed with blood serum, a broad range of complex sizes are formed. Traditionally, the protein corona has been considered homogeneous for specific nanoparticle and serum concentration ratio. However, our results show that the corona varies with the complexes' size.

We also look at the effect protein has on very low concentration of 20 nm gold nanoparticles in cell culture medium. Using a combination of analytical techniques, we show that in protein enriched cell culture medium, the nanoparticles are stable. In cell culture medium without added protein, the nanoparticles aggregate slowly. We describe the aggregation rate and the aggregate morphology and identify arginine, an aggregation inducing amino acid in the biomolecular corona.

Furthermore, we study the binding of purified proteins on nanowires by cryo-transmission electron microscopy, X-ray based analytical techniques, and by changes in the sedimentation rate of nanowires with and without adsorbed protein layer. By rotating the electron microscope stage during imaging, we can image irregularities in the protein corona formed by the large, cross shaped protein laminin.

Finally, we study the effect of various nanostructures on the activity of the enzyme myeloperoxidase. The nanostructures' effect was highly dependent on the complexity of the environment. The effect ranged from almost total inactivation to increasing the activity up to three-fold.

Key words: nanomaterials, nanoparticles, nanowires, biomolecules, proteins, aggregation, protein corona

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language English
ISSN and key title		ISBN 978-91-7422-592-1
Recipient's notes	Number of pages 220	Price
	Security classification	

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Stefán Bragi Gunnarsson

LUND
UNIVERSITY

Cover photo by Stefán Bragi Gunnarsson.

Faculty of Science
Department of Biochemistry

ISBN 978-91-7422-592-1

Printed in Sweden by Media-Tryck, Lund University
Lund 2018

Ritgerðin er tileinkuð ömmum mínum, Snæju og Svölu.

Table of Contents

Popular science summary

Nanostructures are found everywhere in our environment, of either natural or anthropogenic origin. Volcanoes emit them, breakdown of plastic releases them, and they are even produced in candlelight. Designed nanostructures are used in sunscreen, food products, electronics and many more readily available products. Still, there are a lot of things we do not know about their behavior. To call them miniscule would be an understatement. Their size is between one and one hundred nanometers. The width of a human hair is close to the resolution limit of our eyes. Nanostructures are more than a thousand times smaller. To better emphasize their size, your fingernails grow by one nanometer every second! But why and how should we investigate them?

When we put a grain of salt in a glass of water, the grain will dissolve into its charged atoms, or ions. The grain of salt has only disappeared in the sense that our eyes do no longer detect it. Its ions are floating around in the glass of water. If we want to see objects smaller than a hair, we need some sort of equipment like a magnifying glass or a microscope. Doing so, we can see individual cells but we are still quite far from being able to see the ions. The ions are, after all, among the smallest things there are. As we know, when we eat too much salt, we feel bloated. This is because the ions are so small that they can travel more or less anywhere within our bodies and they retain water.

The size of nanostructures is closer to the size of atoms than to the grain of salt. The smallest nanostructures are only a few atoms in diameter which gives them properties very different to those of larger objects, for example a grain of sand. Similar to the grain of sand, many nanostructures do not dissolve easily. What happens when these nanostructures enter our bodies? There they meet molecules of a similar size – proteins. Proteins are responsible for most of our bodies' functions. Everything from our senses like eyesight and taste, to transport of oxygen and the structure of muscles, from our immune system to blood clotting. Thousands of different proteins, each with their unique structure and functions, maintain a properly functioning organism. When nanostructures and proteins interact, they can bind to each other and form a new structure with properties that lie between the two individual components. The nanostructure adsorbs a layer of proteins. This layer can be composed of proteins with various biological functions. By adsorbing to the nanostructure, the proteins might lose their structure and function. In order to understand the biological impact of nanostructures, it is important to know which proteins bind to nanostructures and the effect it has on the proteins.

Working with structures on this size scale comes with great challenges. In order to identify proteins bound to the nanostructures, we can isolate them by centrifugation. Doing so, we can identify single proteins adsorbed to the nanostructure out of a pool of thousands of proteins found in blood. When we have identified the proteins, we get clues about which biological mechanisms might affected by the binding. We can detect this binding by looking at how fast the nanostructure moves in solution. When proteins bind to it, it will move slower. We can even look at single nanostructures and the proteins adsorbed to them by using an electron microscope.

When we understand how nanostructures behave in the biological environment, we can ensure that their design and function is in good agreement with nature and society, thereby helping fulfil the enormous potential of this rapidly growing technology.

Á mannamáli

Nanóagnir má finna í umhverfi okkar, bæði náttúrulegar og manngerðar. Eldfjöll spúa þeim og þær verða til við niðurbrot plasts, þegar við kveikjum á kerti og þær eru framleiddar til notkunar í sólarvörn, matvöru og raftækjum. Þó er margt sem við vitum ekki um þær. Að segja að nanóagnir séu agnarsmáar lýsir ef til vill ekki vel hversu smáar þær eru. Nanóagnir eru á bilinu einn til hundrað nanómetrar. Mannshár er nálægt því minnsta sem augu okkar nema og nanóagnir eru um það bil þúsund sinnum minni. Neglur fingra þinna vaxa um einn nanómeter á sekúndu. Þær eru því ósýnilegar augum okkar. En hvernig og hvers vegna ættum við að rannsaka þær?

Þegar við setjum saltkorn í vatnsglas leysist kornið upp. Hlöðnu atóm, eða jónir, saltsins eyðast ekki heldur eru þær svífandi um á meðal vatnssameindanna í glasinu, jafnvel þó augu okkar nemi þau ekki. Viljum við sjá hluti smærri en hár þurfum við til dæmis stækkunargler eða smásjá og þannig getum við séð frumur. Með svoleiðis búnaði erum við fjarri því að geta séð jónir saltsins. Jónirnar eru með því smæsta sem til er. Þegar við borðum of mikið salt þrútnum við. Þetta gerist vegna þess að smáu jónirnar geta ferðast svo til hvert sem er í líkömum okkar, inn í frumur og þess háttar.

Stærð nanóagna er nær stærð atóma en saltkorns. Smæstu nanóagnir eru aðeins nokkur atóm í þvermál og því eru eiginleikar þeirra mjög frábrugðnir stærri ögnum eins og til dæmis sandkornum. Flestar þeirra leysast ekki auðveldlega upp. Hvað gerist þá ef þessar smáu agnir komast inn í líkama okkar? Þar hitta þær fyrir sameindir af svipaðri stærð – prótein. Prótein sjá um flestalla starfsemi líkama okkar. Allt frá sjón til bragðskyns, frá flutningi súrefnis til uppbyggingar vöðva og frá ónæmiskerfi okkar til blóðstorknunar. Þúsundir mismunandi próteina, hvert með sína byggingu og eiginleika, viðhalda eðlilegri starfsemi lífvera. Þegar nanóagnir og prótein komast í snertingu hvort við annað geta þau bundist saman og myndað nýja byggingu með eiginleika sem liggja á milli eiginleika byggingarefnanna. Nanóögnin hefur þannig hjúp próteina sem öll geta haft áhrif á ýmsa ferla innan lífverunnar. Við að bindast nanóögninni geta próteinin líka tapað byggingu sinni eða virkni. Til þess að skilja líffræðileg áhrif nanóagna er því nauðsynlegt að vita hvaða prótein bindast nanóögnunum og hvaða áhrif það hefur á þau.

Erfitt getur verið að vinna með svo smáar agnir. Algengast er þó að leyfa próteinum að bindast við ögnina í líffræðilegu umhverfi og einangra síðan ögnina og próteinin með skilvindu. Þannig getum við borið kennsl á einstök prótein sem bindast ögninni, oft úr blóðvökva sem inniheldur þúsundir próteina. Þegar við höfum fundið hvaða prótein það eru sem bindast ögninni getum við skoðað áhrifin á þá líffræðilegu ferla

sem þau stjórna. Þannig fáum við vísbendingar um áhrif agnanna á lífverur. Við getum einnig fylgst með stækkun agnarinnar með ljóstvístrunaraðferðum þar sem nanóögn með áföstum próteinum hreyfist hægar í lausn heldur en frjálsa ögnin. Við getum meira að segja skoðað stakar nanóagnir og próteinin sem þeim bindast með rafeindasmásjá.

Þegar við skiljum hvernig nanóagnir hegða sér í líffræðilegu umhverfi getum við breytt hönnun þeirra og virkni svo hægt verði að virkja þá möguleika sem liggja í þessari nýju tækni á ábyrgan og öruggan hátt.

Acknowledgement

When I decided to leave behind my life in Iceland and move to Sweden, a bit more than four years ago, I only knew what I hoped for but not really what to expect. From the first day I felt very welcome and have enjoyed the environment in the group. With people at various stages of their careers, friends are constantly leaving and new people arriving. It says a lot about the group how it maintains a friendly and supportive atmosphere despite constant changes. My time here has been way better than I dared hope for and I would like to thank the people that made this experience so enjoyable.

I would of course first like to thank my supervisor, **Tommy**. The word 'supervisor' does not really describe the way you have helped me during my time here. From the first week when you helped me settle into everyday routine to our trip to Iceland and all our conversations. It has been a great experience learning from you, as a scientist and as a person. I also want to thank **Sara** for providing this wonderfully encouraging environment and her infectious energy, ideas, and passion for life and science. **Anders,** thank you for your helpful insight into the physics aspect of our work. My first experience of independent work in science was under the supervision of **Hörður** at the University of Iceland. Working with you gave me many of the tools I have continued developing in the last four years. **Michael Persson** and **Akzo Nobel** who generously provided me with the opportunity to stay with the company during my secondment. **Gerda** for her patience, helpfulness and always providing an organized frame during the whole project. **Martin Hjort**, **Martin Magnusson**, and **Christelle Prinz** from physics for the insight into world of nanowires. **Fredrik** and **Ulrica** for the insight into the biological aspect of nanoscience during our long running collaboration. A special thanks to **Magnus** for all his help with maintaining a functioning computer and general friendliness. **Adine**, thank you for all your help with keeping life in the lab running. **Olof**, thank you for your company and our trip to France. You have done **Uli** proud as his successor as head of fika announcements. **Guðrún**, takk fyrir að vera hluti af Litla-Íslandi í deildinni!

Our excellent group is next. First I would like to thank **Katja** for being so helpful, friendly, and provide a happy presence. **Thom**, **Birgitta**, and **Eimantas**, thank you for keeping every aspect of the lab running smoothly and of course your company. **Anders**, thank you for all our conversations and your friendly energy and **Mikael** for all the insight into larger scale nanosafety. When I first joined I shared an office with **Elodie** who helped me so much in the beginning. Soon after I started we went to a Picasso exhibition in Malmö which turned out to be the first of very many trips we would go

on together and there will be more! In our office we also had **Martin** who I have learned a lot from and I want to thank you for all your help and guidance. When I first visited Lund for an interview, I was told about another Icelandic person joining the group soon. Having **Tinna** around every day with her lively and warm personality has been one of the highlights of my time here in Lund. Thank you for all our conversations and your friendship. Next, I moved into the most musically challenging office in the building with **Karin** and her excellent attention to Fridays. Thank you for our trip to France and Iceland, and all your invites to your and **Martin's** home. **Kalyani** is my longest serving office companion and it has been an absolute pleasure to sit one meter away from you, all week for three years. Your kindness and friendship will stay with me forever. I apologize for not being able to bring a better food culture to the office and thank you for all the delicious food you have prepared for us. **Tanja**, thank you for your company in the office and for joining me on the journey of thesis writing. One of the first things I had to do after moving to Sweden was to explain to **Xiaoting** how many people live in Iceland. It took seven attempts and her conclusion was that I had no idea how numbers work. **Tânia** and **Ricardo**, it was so nice to have other people from a major football nation in the group! **Mattias**, I hope the almonds will feel safe now. **Dev**, **Rebecca**, and **Veronica**, thank you for our short time together and good luck with your progress.

Finally, I would like to thank my family who I had to leave behind when I decided to move. My parents, **Stefanía** and **Gunnar**, for their infinite support and providing me with the opportunity to do what makes me happy. My sister **Sandra**, who I've watched becoming an adult during my time away from Iceland. I'm so proud of you! I really hope we will live somewhere close to you and **Gústi** in the near future. My grandmother who passed away from Alzheimer's and was the reason I started looking at this particular group. **Snæja** played a huge part in my upbringing and the person I turned into. During my time here I lost my other grandmother who also was a major part of my life from the beginning. **Svala**, thank you for everything. I dedicate the thesis to my late grandmothers who gave me the perfect, spoiled grandchild experience. I also want to thank my grandfather, who will always be one of my role models, we even share the same name. **Bragi**, growing up in your home and watching your calm and thoughtful approach to everyone and everything has been a privilege and I look forward to seeing you soon. **Inga**, who became my second mother after I first moved from home, thank you so much for everything you have done for me! Everything would have been so much more difficult without you. I moved to Lund for the challenge, experience, and a degree but I gained something much more valuable in my amazing girlfriend **Anna** and your family, **Lene**, **Emil**, and **Jens** for letting me in. Thank you for being such a positive and warm influence in my life. Thank you for your endless patience during the last few months and for making the experience even more rewarding. I look forward to having more time to give you the attention you deserve and our life together.

Abbreviations

ATR-FTIR	Attenuated total reflectance-Fourier transform infrared spectroscopy
BAM	N-tert-butylacrylamide
BSA	Bovine serum albumin
CCM	Cell culture medium
DCS	Differential centrifugal sedimentation
DLCA	Diffusion limited colloid aggregation
DLS	Dynamic light scattering
HDL	High density lipoprotein
HSA	Human serum albumin
IgG	Immunoglobulin G
LSPR	Localized surface plasmon resonance
MPO	Myeloperoxidase
MW	Molecular weight
NP	Nanoparticle
NW	Nanowire
NIPAM	N-isopropylacrylamide
O.D.	Optical density
PS	Polystyrene
RLCA	Reaction limited colloid aggregation
SANS	Small angle neutron scattering
SAXS	Small angle X-ray scattering
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEM	Transmission electron microscopy
TMB	Tetramethylbenzidine
UV-Vis	Ultraviolet-visible spectroscopy
XPEEM	X-ray photoemission electron microscopy
XPS	X-ray photoelectron spectroscopy

List of papers

I. **Analysis of nanoparticle biomolecule complexes**

Stefán Bragi Gunnarsson, Katja Bernfur, Anders Mikkelsen, and Tommy Cedervall. Nanoscale, 2018, 10, 4246-4257.

II. **Analysis of complexes formed by small gold nanoparticles in low concentration in cell culture media**

Stefán Bragi Gunnarsson, Katja Bernfur, Ulrica Englund-Johansson, Per-Fredrik Johansson, and Tommy Cedervall. Submitted manuscript.

III. **Electron microscopy imaging of proteins on gallium phosphide semiconductor nanowires**

Martin Hjort, Mikael Bauer, Stefán Bragi Gunnarsson, Erik Mårsell, Alexei A. Zakharov, Gunnel Karlsson, Elodie Sanfins, Christelle N. Prinz, Reine Wallenberg, Tommy Cedervall, and Anders Mikkelsen. Nanoscale, 2016, 8, 3936-3943.

IV. **Protein structure and function after adsorption to nanowires**

Stefán Bragi Gunnarsson, Cesare Mellace, Katja Bernfur, Sudhakar Sivakumar, Martin Magnusson, and Tommy Cedervall. Submitted manuscript.

V. **Nanoparticle effect on neutrophil produced myeloperoxidase**

Elodie Sanfins, Alexandra Correia, Stefán Bragi Gunnarsson, Manuel Vilanova, and Tommy Cedervall. PLoS ONE, 2018, 13(1): e0191445.

My contribution to the papers

I. I designed the experiment together with TC. I performed all experiments, except mass spectrometry and X-ray photoelectron spectroscopy. Data processing, interpretation, and manuscript were done together with TC.

II. I had a major part in designing the experiment. I performed all experiments, except mass spectrometry. I processed the data, interpreted it, and wrote the manuscript draft together with TC. The final version of the manuscript was prepared together with UEJ and PFJ.

III. I did protein characterization and took part in data interpretation and writing the manuscript.

IV. I designed and performed all experiments, except running the electron microscope and nanowire production. I did data processing and interpretation, and wrote the manuscript together with TC.

V. I did nanoparticle characterization and binding studies and wrote the corresponding part of the manuscript. I took part in revision of the manuscript.

1. Introduction to the thesis

In recent decades, the emergence of nanotechnology and nanoscience has presented the scientific community with huge challenges on the smallest size scale. Nanomaterials have of course been in our environment from the beginning of time. However, it wasn't until after the industrial revolution that exposure to considerable amounts of nanomaterials of anthropogenic origin became a reality. Today, we are also exposed to considerable amounts of synthetic nanomaterials, i.e. materials specially designed to be on the nanoscale. Since the only common denominator is their size, nanomaterials are incredibly diverse and have applications in everything from chewing gum to cutting edge technological equipment. Like any emerging technology, it is the responsibility of the scientific community to ensure its safe introduction to nature and society. Due to their small size, nanomaterials need a special approach when we study their interaction with the environment. Sharing a size scale with the molecules of organisms, nanostructures can penetrate much further into the organisms than larger material. This calls for assessment of their effect from the molecular up to the ecotoxicological scale.

1.1 The research question

When a nanostructure enters the biological environment, it enters a very complicated system of biomolecules with various functions and specificities. Naturally occurring or synthetic, the nanostructure enters the biological environment as a relatively well defined construct but upon interaction with the molecules of this new environment, a variety of biomolecule and nanostructure complexes are formed. Proper characterization of these complexes is crucial for assessment of their toxicological impact. I will examine the driving forces behind the complexes' formation, the homo- or heterogeneity of their composition, their properties from both the biomolecules' and nanostructures' perspective, as well as the methods used to study these nanoscale interactions. What drives stable nanostructures and biomolecules to form complexes when they interact? Do these complexes form randomly or are there physicochemical factors that dictate the formation?

Assessing the biological impact of a nanostructure is not a simple task. To understand their effect on an organism, it is important to understand these interactions on a

molecular level in order to explain the effect on a larger scale. By describing the size of the complex, how it forms under different conditions, and its biomolecular content, we can describe what organisms are exposed to. When we can describe what the organism is exposed to, we can better understand the organism's response to it. Nanomaterials are designed and produced with various intended applications, some *in vivo*, others as components of high tech products under conditions very different from the biological environment. Pharmaceuticals, diagnostic nanostructures, or medical implants can be designed with coatings, stabilizers, or other properties that minimize interactions with their biological environment. Industrial nanomaterials, however, are not designed for *in vivo* applications and their surfaces might be paramount to their application. Regardless of the intended application of a nanostructure, its impact on an organism needs to be assessed in case of exposure. Furthermore, considering the relative infancy of nanoscience and its unpredictable future developments, the more information we have about their effects, we can better determine in which direction to steer their design. By forming a frame of safe applications, it will be easier to fulfil the enormous potential of these materials in a responsible manner.

The thesis is divided into six chapters. This first chapter introduces the research question and general structure of the thesis. The second chapter introduces nanostructures. The third chapter introduces biomolecules, with most focus on proteins. In the fourth chapter I discuss the interactions of proteins and surfaces, both bulk and nanoscale, and the physicochemical properties I consider most relevant to my work. In the fifth chapter I will describe the methods used to characterize the nanostructure-biomolecule complexes. Finally, I will discuss the results of the experimental work presented in papers I-V and finish with concluding remarks and future perspectives.

2. Nanostructures

Nanostructures have been produced in some form for more than 2,000 years.[1] Initially, spherical nanoparticles were more common but with advances in synthesis, a range of shapes are now available like rods, hollow tubes, stars, wires, and many more. Although the focus of this thesis is on the interaction of biomolecules with synthetic nanostructures, these structures also occur naturally and in pollution. In this chapter I will start by defining nanomaterials, both in official terms and in the context of this thesis, followed by description of various properties and behavior of these unique materials. Finally, I will briefly describe the nanomaterials used in my work.

2.1 Definition

Nanostructures are defined as materials that have at least one dimension between 1 and 100 nanometers. The European Union defines nanomaterials as materials that in an unbound or agglomerated state, 50% or more of the number concentration has at least one dimension in the size range between 1-100 nm. In the context of this thesis the definition is not quite as strict. However, all the materials studied have at least one dimension within the definition boundaries when they are in an unbound state. They are still considered nanomaterials if their agglomerates or complexes with biomolecules exceed 100 nm. Nanostructures can be divided into sub-groups, e.g. by chemical composition or shape as spherical nanoparticles, structures with a higher aspect ratio like nanorods, nanotubes and nanowires, and even nanoholes or two dimensional nanosurfaces like nanowires, where one dimension is closer to bulk size. Using this definition, it can be argued that biomolecules are nanostructures, and proteins with their different shapes could be called nanoparticles, nanorods, or nanowires. In the context of this thesis, only synthetic materials are referred to as nanomaterials. Spherical nanostructures will be referred to as nanoparticles, to clearly distinguish between them and nanostructures of other shapes, mainly nanowires.

2.2 Nanostructure properties

2.2.1 Size

To better understand the world of nanostructures, it is good to get a perspective of their size scale. Human fingernails grow by approximately 3.5 mm every month or roughly 1.5 nm every second.[2] A human eye can detect objects down to 50-100 µm, about 500 times bigger than the upper size limit of nanostructures. A covalently bound hydrogen atom has a diameter of 50 pm, or one twentieth of a nanometer. That means lining up twenty hydrogen atoms in a row would qualify as a nanostructure. While hydrogen atoms do not arrange themselves in such fashion, other elements do. Gold has a covalent diameter of 270 pm,[3] which means that lining up four gold atoms in a crystal structure would qualify as a nanostructure in one dimension. The properties of nanostructures, therefore, lie somewhere between the properties of atoms and those of bulk materials.

Two of the most common ways to report the size of nanomaterials are their hydrodynamic radius or diameter, and their radius of gyration. Hydrodynamic diameter or radius, D_H or R_H, also called Stokes radius, is a measure of the size of the structure, along with any adsorbed solvent molecules on its surface. The hydrodynamic size is estimated by the objects diffusion rate. Its size is assumed to be equal to the size of a solid sphere with the same diffusion rate. Dynamic light scattering (see 5.2) is a common technique to measure R_H and measures the change in scattering intensity of a structure undergoing diffusion. Radius of gyration measures the root mean squared distance of the components of a structure from its center of mass and can be measured by static light scattering, and for proteins small angle X-ray scattering (SAXS) or small angle neutron scattering (SANS).[4]

2.2.2 Optical properties

One of the main attractions of nanostructures in various applications are their optical properties. Being smaller than the wavelength of visible light (390-700 nm), nanostructures interact with light differently compared to bulk material. Metal nanoparticles like gold, silver, and copper have been used for centuries due to their fascinating colors. They can be found in purely aesthetical applications like decorations and window stains, as well as in high tech analytical sensors.[5-7] Nanoparticle color depends on its size. The smallest gold nanoparticles are transparent, but above approximately 2 nm their color turns red. With increased size, the color gradually approaches the color we normally associate with gold. The wavelength where the NPs absorb most light depends on their diameter; 5 nm gold nanoparticles have maximum absorbance around a wavelength of 500 nm which increases to 570 nm for 100 nm

particles.[8] This effect is called the localized surface plasmon resonance (LSPR).[5] When the Au NP sample is illuminated by visible light, the conduction electrons on the surface of the NPs are polarized. For small NPs up to around 20 nm, the surface electrons behave as an oscillating dipole, i.e. the electrons of the Au NP oscillate in order to position themselves away from the electric field of the incoming light. In larger NPs, the electrons cannot move in a dipole fashion since the light does not polarize them homogeneously which leads to a broadening of the absorbance peak, as well as a shift to higher wavelength of maximum absorbance.[9] This is extremely useful when studying the adsorption of biomolecules to gold and silver nanostructure surface since the adsorbed biomolecule shifts the wavelength of maximum absorbance to a higher wavelength.

Another example of optically interesting nanostructures are nanowires. Nanowires have applications as light guides, both in light emitting diodes as light source and in solar cells as light absorbers. Nanowires will be further discussed in 2.3.3. These unique optical properties are due to the high number of surface atoms, out of the total number of atoms of each structure.

2.2.3 Colloidal stability

Even if the high surface area to volume ratio of nanostructures is one of their biggest advantage, it can also be a challenge. When nanostructures are in solution they undergo random Brownian motion due to collisions with the molecules of the solvent. The presence of a large surface area in relatively low volume, combined with the fast diffusion of small particles, leads to high frequency of collisions between the nanostructures. Given the right conditions, this can lead to irreversible association of two or more nanostructures, called aggregation, and will reduce the material's available surface area. If the aggregates reach a certain size threshold, their movement will be governed by sedimentation rather than diffusion, i.e. they start to precipitate.

Boris Derjaguin and Lev Landau, and Evert Verwey and Theodor Overbeek developed a theory on the behavior of colloidal particles and their stability in solution, commonly referred to as the DLVO theory.[10] The theory has been extended to account for the behavior of different ions with the same charge (extended DLVO theory).[11] Under the non-ideal, physiological conditions multiple components act on each other in very complicated ways. However, the general behavior described is still very useful. Biomolecules and nanostructures can both be considered as colloidal dispersions. Colloidal dispersions consist of two different phases, a dispersed phase and a continuous phase. The particles are the dispersed phase and the continuous phase is a liquid, an aqueous solution in the case of biological systems. A requirement for a system to qualify as colloidal dispersion is that the dispersed phase does not sediment rapidly. For a system to be colloidally stable, it is therefore important that its dispersed phase does not form large aggregates rapidly.

The charge of a nanostructure surface will attract opposite charges, often ions, from the continuous phase, that will form an electrical layer of opposite charge. That layer will again attract ions of opposite charge. This electrical double layer will effectively determine the surface charge the nanostructure presents to its environment, measured as the structure's zeta potential. If this electrical charge is sufficiently large, it will repel other particles of the same charge and maintain the colloidal stability. In solutions of high ionic strength, the electrical double layer will become more compact, its thickness referred to as the Debye length, since the availability of ions is high. This will shorten the range of electrostatic interaction the particle has and other particles can approach it more easily.

Opposing electrostatic repulsion forces are van der Waals attractive forces. When two molecules approach each other, their electric dipoles will interact. The dipoles can be both permanent and induced. A permanent dipole is the result of asymmetrical electron distribution within the molecule, when an electronegative atom pulls the electrons away from a less electronegative atom, e.g. in hydrogen bonds. These interactions are called dipole-dipole interactions. Even in the absence of permanent polarity of a molecule, the electrons of a molecule can affect the electrons of a molecule it approaches, effectively pushing them away, forming an induced dipole in the two molecules. These interactions are called London dispersion forces.[10]

A system's colloidal stability can, in simplified terms, be described by the sum of these components.[12] Aggregation of nanostructures happens when attractive forces overcome repulsive forces. The easiest way to do so is by reducing the electrostatic repulsion by adding electrolytes to the solution. By adding electrolytes, the Debye-length, or the thickness of the electrical double layer, decreases leading to reduced electrostatic repulsion. When the repulsion decreases, other molecules, even if they have the same charge, can more readily come close enough together for the van der Waals forces to keep them together. Measures can be taken to avoid aggregation in some cases. The hydrophobicity or hydrophilicity, surface charge, and stabilizing surface functionalization can be chosen to increase stability. Charges can be induced by varying the storage solution pH, surfactants can be added, and charged polymers can be added to the structure's surface if its native surface is not necessary for it to carry out its function. Ultimately, the intended application of the nanostructure dictates the measures available to increase its colloidal stability.

2.2.4 Mechanism of aggregation

As has been described, nanostructures maintain their colloidal stability when their repulsive forces are larger than their attractive forces. Two particles with the same surface charge are kept at a certain distance by electrostatic repulsion. For gold NPs, this is often achieved by storing the particles in a few mM citrate solution. At basic pH, citrate has a negative charge due to its three carboxyl groups. The negative charges on two approaching NPs provide a large enough energy barrier for the NPs to repel each other. When the pH of the solution is lowered, the carboxyl groups of citrate become protonated and the repulsion is decreased. Similarly, when electrolytes are added the charges are shielded, the Debye length shortened, and repulsion reduced. When the energy barrier maintaining repulsion has been reduced sufficiently, the collision of two approaching NPs can bring the surfaces of the NPs close enough for the attractive van der Waals forces to result in permanent binding. The NPs have now formed an aggregate and will diffuse at a slower rate and continue to take part in aggregation events with both single NPs and other aggregates of various sizes.

In a two component system, i.e. NPs and a destabilizing agent, the mechanism of aggregation can be described in great detail.[13-15] The rate of aggregation and the aggregate morphology can be described according to the affinity the two NPs have for each other and the energy barrier, difference between attractive and repulsive forces, of the collision. If the energy barrier is similar to the collision energy, several collisions might be needed for the NPs to bind. This results in what is called reaction-limited colloid aggregation (RLCA).[14] However, if the energy barrier is much lower than the energy of collision, every collision will result in a binding event. This process is called diffusion-limited colloid aggregation (DLCA).[15] These two mechanisms of aggregation result in different degrees of packing. This can be explained by looking at RLCA, where the probability of binding upon collision is small. Early on in the aggregation process, the surface area of colliding NPs or small aggregates will require many collisions before they bind. As the aggregate size increases, larger surface contact can make up for the low probability of binding. This results in a more compact aggregate and an exponential increase in the aggregate size over time due to the increasing efficiency of collisions as the interacting surface areas increase with aggregate size. In the DLCA scheme, almost every collision results in binding, forming less compact aggregates with various shapes and morphologies. Since every collision results in binding, the growth is linear and early on, faster than in the RLCA scheme. The compactness of the aggregates, called fractal aggregates, is quantified by their fractal dimension, d_f, which can have values between 1 and 3, where 1 is the lowest compactness and 3 is a tightly packed spherical aggregate.[16] Typical values for DLCA and RLCA are 2.1 and 1.8, respectively.[14,15]

Lin *et al.* studied the aggregation mechanism of three different colloidally stable NPs. Aggregation of citrate stabilized gold NPs was induced by addition of different concentrations of pyridine. Pyridine's structure is similar to benzene, the simple hexagonal aromatic compound, except one carbon has been exchanged for nitrogen.

Primary amines have been shown to interact with gold surfaces by forming a bond somewhat covalent in nature.[17-19] Pyridine, however, has a tertiary amine but has been shown to interact with the gold surface through its nitrogen in a perpendicular orientation, exposing the carbon part of the ring to the bulk.[20] By replacing the citrate, pyridine reduces the negative charge on the gold surface and lowers the energy barrier. In low concentration, pyridine induces RLCA, while at higher concentrations the energy barrier becomes even lower and the mechanism is described by DLCA.

Doyen *et al.* used amino acids to induce aggregation of gold NPs. By systematically studying the effect of twelve out of the twenty natural amino acids, they found that two amino acids, arginine and histidine, formed fractal aggregates very efficiently. As mentioned earlier, amines bind directly to the gold surface and upon binding expose their zwitterionic tail to the bulk. At physiological pH, both arginine and histidine have positively charged side chains. The third most efficient aggregating amino acid was lysine, which also has a positively charged side chain.[21] Studying the fractal aggregation of gold NPs is made somewhat convenient by the appearance of a second absorbance band in the 650 nm range caused by interaction of the surface electrons of two or more NPs as the distance between them approaches zero.[22] This second band is easily distinguishable from the characteristic LSPR band for monomer gold between 500 and 570 nm.

When NPs form aggregates in the presence of a destabilizing agent, their physicochemical properties change. Adsorbed surface molecules can alter the NPs' charge, aggregates diffuse slower than single NPs, and may even move by sedimentation rather than diffusion. When studying NP aggregation, the NPs' concentration is very important. When the main focus of an experiment is the nature of fractal aggregation, any convenient NP concentration can be chosen. These concentrations are not necessarily biologically relevant. In the previously mentioned studies, Doyen *et al.* used gold concentration that gave absorbance of 0.75 O.D. while Lin *et al.* used a volume fraction of 2.8×10^{-6}.[14,15,21] In paper II, we used gold concentration with absorbance of 0.05 O.D. and converted to volume fraction, 1.5×10^{-7}, or fifteen and nineteen times lower, respectively. By lowering the concentration, aggregation will be slower due to the reduced probability of collisions. The presence of many different aggregating or stabilizing agents in the complex biologically relevant solutions also complicates the aggregation process. As not all NPs have the benefit of a second absorbance band in ultraviolet-visible spectroscopy, different techniques are required to assess their morphology, e.g. electron microscopy and dynamic or static light scattering.

2.3 Synthetic nanostructures

2.3.1 Nanostructure synthesis

Nanostructures can be produced by various methods with different degrees of sophistication, both under wet and dry conditions and even by mechanical breakdown of larger structures. Many different types of materials are available, like pure metal, metaloxides, elements from groups III and V of the periodic table, pure carbon, hydrocarbon polymers or plastics, and many more.

When nanostructures are produced in solution, the process often involves supersaturation of the liquid, often by heating to increase solubility, followed by cooling which results in the formation of small crystals due to the decrease in solubility.[23] Gold nanoparticles are usually produced in an aqueous solution where citrate is added to a solution of gold chloride, and plays a role in both the synthesis and in maintaining a stable solution of the formed particles. Silica nanoparticles are also produced in solution on an industrial scale for applications in various coatings. During the production process, it is important that the nanostructures do not interact with each other in an irreversible manner, losing their unique properties due to reduced surface availability or diffusivity. By changing time, temperature, concentrations and other properties of the chemical reaction of the syntheses, different sizes of nanostructures can be produced. Adding surfactants and adding more reactant to a synthesis can result in the formation of shapes other than spherical, like rods, wires, tetrapods, and stars.[23,24]

2.3.2 Spherical nanoparticles

The following examples of NPs are chosen due to their relevance for the work presented in the thesis. Gold NPs have already been discussed quite extensively and will not be described here.

2.3.2.1 Polystyrene

Polystyrene is produced in enormous amounts every year. The styrene polymer is also one of the most common materials used for nanoparticle preparation. Polystyrene's attractive properties are its low cost, chemical stability, and light weight. In 2005, it was estimated that 2.6 million tons of polystyrene waste were discarded in the USA alone.[25] In nature, the polymer accounts for a large part of marine pollution, with debris of different sizes floating in the oceans, constantly breaking into smaller pieces, down to the micro- or 1 µm to 1 mm, and nanoscale.[26] In biological applications, a nanoparticle that breaks down too easily upon exposure to the biological environment

is not an efficient carrier of drugs or imaging contrast agent. However, too high stability can also lead to accumulation of the material within organisms. In that sense, polystyrene's greatest benefit during its early life-cycle, i.e. its stability, becomes its greatest threat at the end of that cycle.

2.2.1.2 Titanium dioxide

Titanium dioxide, TiO_2, like polystyrene, is produced in huge amounts each year. Out of the millions of tons manufactured, around 70% is used in paint. In 2005, approximately 2,000 tons of nanoscale TiO_2 were produced. Five years later, the amount had increased to 5,000 tons. The most common use of nanoscale TiO_2 is in sunscreens.[27] In food, TiO_2 is a common additive and can be recognized by the European food additive number E171.

In a study of TiO_2 exposure to humans from food sources, Weir *et al.* found that children are exposed to up to 2 mg/kg/day of food additive TiO_2 and in typical consumer products, 36% of the total TiO_2 content is on the nanoscale.[27] Generally considered safe for consumption, the NPs were the topic of investigation by Bettini *et al.* and were administered orally to rats. The NPs were found to affect the immune system and indications of the formation of tumors were found after chronic exposure to dietary relevant concentrations of TiO_2 NPs.[28] A second potential exposure pathway for TiO_2 NPs is through the skin. When particles become smaller than half the wavelength of light, they appear transparent. This is extremely important in cosmetics, since it offers protection against ultraviolet radiation without giving a white color after application to skin.[29,30] The third way of exposure to NPs is through the pulmonary system. Exposure to airborne TiO_2 NPs is a concern for many workers, both during production of the particles as well as after their application.

2.3.3 Nanowires

Nanowires, like nanorods, are elongated nanostructures. No official definition exists for how elongated the structure needs to be to be considered a nanowire, but the convention seems to be somewhere around length-to-diameter, or aspect ratio, of 10 or greater. NWs are typically from 20-100 nm in diameter and up to a few micrometers in length. Semiconducting nanowires have gained a lot of attention due to their waveguiding properties, with applications in extremely important fields like solar cells, light emitting diodes, and in electronics.[31] Vertical nanowire arrays are also under investigation as support for cell cultures,[32-34] and nanowires are also an attractive option for brain implants since their elongated structure allows for their integration into the neuronal network of the central nervous system.[35]

2.4 Nanostructures in the environment

Not all nanostructures are synthetic. In our everyday environment, we are constantly exposed to nanostructures. As mentioned earlier, according to the definition, biomolecules can be classified as nanostructures. Our food contains a lot of material of the nanoscale, milk contains micelles and fibres,[36] during fires and volcanic eruptions, large amounts of nanomaterials are released,[37] and weathering of rocks produces huge amounts of nanoscale materials.[38] Nanoparticles are not only synthesized on purpose or by nature, a large part of the nanoparticles in our environment are the by-product of other processes. These processes can be on the domestic level, even in our homes like cooking and candle light,[39] up to global scale like the breakdown of plastics,[40] fuel combustion,[41] and from waste containing nanostructures like cosmetics and detergents.

3. Proteins

3.1 Introduction to proteins

Most functions of a living organism are performed by the biopolymer called proteins. Proteins are chains of various lengths, composed of different combinations of twenty small and relatively simple molecules, amino acids. Proteins perform functions as delicate as detecting the taste of the food we eat to crude breakdown of the bonds holding our food together. Proteins transport oxygen from our lungs to muscles, where a different set of proteins catalyzes the release of energy that is used by yet another set of proteins to move our muscles. Our defense mechanisms are also governed by proteins. Blood coagulation is performed by a series of proteases, protein cleaving enzymes, ever present in our blood but kept inactive until they are required.[42] Our immune system consists of different proteins, both the innate immune system that acts immediately upon exposure to an intruder in the body, and the adaptive immune system that is highly specific towards antigens. The diversity of molecules formed by these twenty different amino acids is enormous and their proper function is vital for a healthy individual. It is therefore very important to understand the effect nanostructures have on proteins. Some of the aspects of protein properties and behavior, relevant to their interactions with nanostructures, will be described in the following paragraphs.

3.2 Protein properties

3.2.1 Amino acids

The twenty naturally occurring amino acids are all L-isomers and contain a central carbon atom, the α carbon, linked to an amine and a carboxyl group, as well as a variable R-group. The amine and carboxyl groups of two different amino acids can form a bond, the amide bond, resulting in a peptide. The R-group determines the chemical nature of the amino acid. The twenty different R-groups, or side chains, can be divided into groups depending on their nature. The groups are determined by polarity or non-polarity, and positive or negative charge. These positive and negative

charges dictate the isoelectric point of the protein, pI, which is defined as the pH value where the protein has no net charge. In the aqueous, biological environment, non-polar amino acids prefer to interact with other non-polar amino acids rather than the water of the environment. This is referred to as the hydrophobic effect, as the release of water molecules from the hydrophobic part of the chain is entropically favorable, and is one of the driving forces in protein folding and stability.[43,44] Positively and negatively charged amino acids can form salt bridges that contribute to the overall stability of the protein, as well as take part in surface interactions as they interact favorably with the aqueous environment.[45]

3.2.2 Protein structure

The amino acid sequence is referred to as the primary structure of the protein. Secondary structure describes the structure of α-helices and β-sheets, as the amino acids order themselves into their energetically favored shapes.[46-49] The tertiary structure describes the order of secondary structure components into three dimensional structures. Finally, the quaternary structure further describes the construct of different tertiary structure components into more complicated structures with subunits, dimers, etc. Proteins have a slightly higher density than water, usually estimated to be between 1.3-1.4 g/cm^3 and their texture is best described as hard, dry plastic.[50,51] Proteins come in many shapes and only a few are spherical and most are somewhat elongated. Fibrinogen, for example, is very elongated and has a length of 45 nm,[52] while immunoglobulin G has a Y-shape,[53] serum albumin has a heart shape,[54] and laminin is in the shape of a cross with its longest dimension around 90 nanometres.[55]

3.2.3 Protein size

When assessing the interactions between nanostructures and biomolecules, it is quite important for physicochemical characterization to understand the size of the biomolecules. Conventionally, protein size is given by their molecular weight (MW) in Daltons (Da). The unit is named after the chemist, physicist, and meteorologist John Dalton (1766-1844), who was one of the pioneers of atomic theory and in defining the MW of elements. One Dalton is approximately 1 g mol^{-1}, or one twelfth of the MW of carbon twelve. Proteins usually weigh thousands of Daltons so the unit kDa is the conventional way of describing a protein's MW. However, the MW does not fully describe the size of a protein molecule.

As mentioned, proteins' shape can vary greatly and is often directly related to its function. Fibrinogen, for example, is very elongated which makes it so efficient in the formation of fibrillary network required for blood clotting.[52] This property became very apparent in the work of Theodor Svedberg, a Swedish chemist who won the Nobel Prize in chemistry in 1926. Svedberg worked extensively on the relationship between

protein size and shape and its sedimentation rate under centrifugal force. The Svedberg formula can be used to calculate the sedimentation coefficient, S, by equation 1.[50]

$$S = \frac{M(1-\nu\rho)}{N_0 f} \quad \text{Eq. 1}$$

Where M is the molecular weight of the protein, ν is the partial specific volume for proteins (inverse of the density), ρ is the density of the solvent, N_0 is Avogadro's number, and f is the frictional coefficient. The frictional coefficient depends on the shape of the protein, its surface roughness and the water molecules bound to its surface, with the lowest value obtained for a perfectly smooth sphere. Proteins do not at all have the surface of a smooth sphere and this causes considerable deviations from sedimentation coefficient values that assume smooth surfaces.[50]

As discussed in 2.2.1, nanostructure size is often measured by dynamic light scattering. It can also be used to measure the hydrodynamic size of proteins while small angle X-ray or neutron scattering (SANS and SAXS) can be used to measure the radius of gyration. Another method to measure the size of a protein is by gel filtration.

3.2.4 Protein colloidal stability

Despite the distinction between nanostructures and proteins in the context of this thesis, they of course obey the same principles of chemistry and physics. As discussed in 2.2.3 on the colloidal stability of nanostructures, in a two phase system, where the continuous phase is an aqueous solution of electrolytes, the dispersed phase can be a biomolecule. There are still some differences to consider. The surface of a nanoparticle is usually fairly homogenous. A protein, on the other hand, can have different surface chemistry in different patches.[56] To further complicate their behavior, proteins can shift their conformation according to the environment. Hydrophobic, electrostatic, and van der Waals interactions as well as crowding can be affected when the system is perturbed, and the properties of ionic molecules in the solution can also influence the protein stability.[57] These properties of proteins are important to keep in mind when studying their interactions with nanostructures. Colloidal stability of proteins is also very important in the pharmaceutical industry where extremely high concentrations of antibodies and other therapeutic proteins are often needed.

3.3 Protein interactions

In the biological environment, both inside and outside of cells, proteins and other biomolecules are in high concentration. Biological functions, such as binding between a signaling molecule and a receptor, depend on the appropriate level of binding and

dissociation. Similar to nanoparticles suspended in a solvent, protein stability is determined by a net effect of electrostatic attraction and repulsion, van der Waals forces, and hydrophobicity. Proteins, in general, have a more irregular shape and surface heterogeneity than nanostructures, allowing them to interact with each other in a more delicate manner. For two proteins to interact, they have to have a good surface and electrostatic complementarity. Flexibility of the protein structure also plays a part, as well as the surface availability. Few cases of interaction with contact surfaces smaller than 600 $Å^2$, or approximately 2.4 x 2.4 nm, have been reported.[58-60] Despite hydrophobic forces being important for protein folding, as hydrophobic amino acid side chains interact with other hydrophobic side chains to form the hydrophobic core of the protein, hydrophobic patches are still found on the protein's surface. These hydrophobic patches are often the hot spots, where binding is strongest, of protein surfaces when it comes to ligand binding[61] with some exceptions.[59] Even if many protein interactions are very selective and specific, many promiscuous proteins exist that bind to many ligands.[62] Studying these interactions, and in fact their interactions with nanostructures, is therefore extremely complicated due to the diversity of interactions in these dynamic systems.

3.3.1 Protein-ligand interactions

Most biological processes are governed by protein interaction with a second molecule, referred to here as a ligand. When studying a foreign molecule, e.g. a nanostructure, in the vicinity of proteins, it is important to understand the mechanisms of protein-ligand interactions. For proteins to interact, a certain threshold of interaction complementarity needs to be reached. This threshold is determined by a sum of energetically favorable interactions which need to be greater than the sum of energetically unfavorable interactions. The favorable interactions are hydrophobic and charge complementarity.[63] For strong interaction, hydrophobic patches within the contact surface of the two molecules need to be in contact, while opposite charges need to be favorably positioned. The affinity between a protein and a ligand is often described as the ratio of the rate constants of dissociation and association. The ratio is called the equilibrium dissociation constant given in equation 2 and the Gibbs free energy can be calculated by equation 3.

$$K_d = \frac{k_{off}}{k_{on}} \qquad \text{Eq. 2}$$

$$\Delta G = -RTln(K_d) \qquad \text{Eq. 3}$$

K_d is the equilibrium dissociation constant, k_{off} the dissociation rate constant, and k_{on} the association rate constant. ΔG is the change in Gibbs free energy, R the gas constant, and T the temperature.

The hydrophobic effect and its role in ligand binding is a whole research field in itself. With ever increasing resolution in structural analysis of proteins, the nature of the hydrophobic effect is under debate. The classical hydrophobic effect describes the interaction of water with non-polar amino acid residues as more ordered than bulk water. The non-polar entity can be thought of as a cavity in the space available for water molecules, and it does not contribute to the hydrogen bond network in the bulk water[64] and the release of these ordered water molecules entails an entropic gain upon binding. Other sources describe a gain in enthalpy as water molecules with one, out of possible four, hydrogen bond partners in the non-polar vicinity gives a high enthalpy gain when moved to the bulk.[65] As expected, there are conflicting conclusions as it is very difficult to describe universal rules for the extremely complicated and diverse situations encountered when working with proteins.[66]

The ability of proteins to bind strongly to highly specific targets is the basis of our immune system. Seemingly simple and limited structures on the recognition part of antibodies are able to discriminate between similar surface features of an antigen. This specific binding is to a large extent dependent on interactions of aromatic amino acids, particularly tyrosine and tryptophan, of the antibody to the backbone atoms and amino acid side chain carbons of the antigen.[67]

Avidity is another factor of protein interactions that is relevant in the scope of this thesis. When at least one of the two binding components has more than one binding site towards the second component, an increase in affinity is observed.[68] The explanation for this is that when one binding site binds, it brings the second binding site into the vicinity of the ligand. When the protein and its ligand dissociate, the probability of reattachment by one of the two binding sites is increased.

Another important phenomenon is the crowding effect. Under biological conditions, e.g. inside cells, extremely high concentration of biomolecules is encountered. The concentration of hemoglobin in red blood cells is approximately 330 mg mL^{-1}, and the average distance between the centers of two molecules is 6.9 nm and their diameter is 5 nanometres.[50] In blood serum, protein concentration is approximately 70 mg cm^{-3} and assuming a protein density of 1.37 g cm^{-3}, roughly 5% of the total volume is occupied by proteins. When a large part of the available space is occupied by large 'solid' molecules like proteins, less solvent volume is available for dissolved compounds, effectively increasing their concentration which can increase kinetic rates.[69]

3.4 Blood serum and plasma

For animal welfare and ethical reasons, studying blood serum or plasma *in vitro* is a valuable tool to mimic the biological environment a nanostructure enters. Blood consists of red and white blood cells and platelets that make up approximately 45% of the total volume, the remaining 55% is the blood plasma. Blood plasma contains coagulation factors, proteolytic enzymes responsible for the blood coagulation cascade and fibrinogen. After blood coagulation, the remaining liquid is called blood serum. The major component of both plasma and serum is water, but it also contains high concentration of biomolecules – proteins, lipids, nutrients, and amino acids – and various electrolytes.[70,71] Of the proteins, serum albumin is the most abundant and in fact it makes up more than 50% of total protein content. Albumin is a 65 kDa promiscuous protein, with many different binding partners such as fatty and amino acids, and calcium.[54] It also plays a role in maintaining osmotic blood pressure.[72] The second most abundant protein is immunoglobulin G, a 150 kDa protein of the immune system. It consists of two 50 kDa heavy chains and two 25 kDa light chains. The four subunits are linked together by disulfide bonds[53] and appear as two strong bands in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, the heavy chain slightly below the strong serum albumin band. The third most abundant protein in serum is transferrin.[73] Of similar concentration to transferrin is the blood clotting protein fibrinogen.[52]

An interesting group of proteins, as they are often found in the biomolecular corona of nanostructures, are the lipoproteins. In order to transfer lipids in blood, it is necessary to shield their hydrophobic fatty acid chains from the aqueous bulk. This is achieved by forming small particles consisting of a core of lipids coated by proteins and other amphiphilic molecules.[74,75] As the size and relative amount of lipids to proteins increases, the lower their density becomes. The high density lipoproteins (HDL), are in the range of 10 nm, while chylomicrons can be up to 1 µm in diameter and have a density lower than the density of water.

4. Nanostructure and protein interactions

For more than fifty years, the phenomenon of proteins binding to surfaces has been a topic of interest for many researchers. In the 1960s, Leo Vroman studied the binding of blood plasma proteins to hydrophobic and hydrophilic surfaces. He noticed that immediately after exposure to plasma, surfaces are covered with proteins. First by proteins in high abundance but of low affinity that over time are replaced by less abundant proteins of higher affinity. This phenomenon is called the Vroman effect.[76,77] Ever since Vroman's discovery, the topic has remained relevant in a number of research fields. From the large scale in food industry,[78] down to the molecular level of research, protein adsorption to surfaces can cause unexpected problems. Efficiency of medical implants depends on proper integration of the implant into surrounding tissues, facilitated by the connecting protein layer.[79] The presence of a foreign surface can activate the blood coagulation cascade through binding of the zymogen factor XII.[42,80] Efforts have therefore been made to minimize undesirable adsorption by surface coating, e.g. in low-binding consumables in research. This is not always possible since the functions of many materials depend on their surfaces.

In chapters 2 and 3, the properties of nanostructures and proteins were discussed. In this chapter, I will introduce the main mechanisms that control the result of combining the two systems. Vroman studied the interaction of proteins with flat, immobile surfaces. Many of the principles still apply to nanostructures while the mobility and subsequent colloidal stability of the nanostructures in the complex environment of biological fluids provides both challenges and possibilities.

4.1 Proteins on surfaces

Among the main functions of proteins in biological systems is to serve as transport, signaling, and detection molecules. All these mechanisms depend on the protein's ability to bind to various molecules. It is therefore not surprising that they also bind to foreign, solid surfaces. Like proteins, the solid surfaces can be hydrophobic, hydrophilic, positively, negatively, or neutrally charged. Compared to the complex surfaces of proteins, solid surfaces can be considered quite homogeneous. The solid

surface properties depend on its chemical composition and environmental factors at the solid-liquid interface. In general, the mechanisms governing adsorption are the same as for protein-protein interaction and colloidal stability. However, due to the homogeneity of solid surfaces, they have more than one binding site for a protein with an affinity for its surface. This results in the formation of a protein layer on the surface.

When a pristine surface is exposed to proteins, the events that follow depend on the surface and protein properties, as well as microenvironmental factors. The main environmental factors are temperature, pH, and ionic strength of the solvent. The frequency of collisions between proteins and the surface increase at higher temperatures due to faster diffusion rates. As the frequency of collisions increases, the probability of a favorable binding event also increases.[56] The charge of the surface and protein will depend on the environment pH, and charge shielding by salt ions will increase with higher ionic strength, reducing the effect of electrostatic interactions.

4.2 Proteins on nanosurfaces

In the beginning of the 21st century, with rapid advances in nanotechnology, the focus was turned to the possible impact of nanostructures on organisms and the need to ensure their safe application.[81,82] Despite the fact that nanoscale materials have been present in our environment from the beginning of time, it wasn't until the industrial revolution that people were exposed to large amounts of nanostructures of anthropogenic origin.[81] Although nanostructures share some of the surface properties of bulk materials, they have unique properties that call for a different approach to assess their interactions with proteins. Their small size enables them to penetrate organisms and brings the complex study of surface-protein interactions into biological fluids. Given the novelty of many of the materials produced today, biological functions of an organism have not evolved in the presence of these materials. This calls for thorough assessment of their effect on the delicate equilibrium of biological systems.

Two important properties of nanostructures, compared to immobile flat surfaces, are their high curvature and the possibility of surface-surface interactions as described in 2.2.4 on aggregation. Since the size scale of nanostructures is between 1 and 100 nm, it means that the smallest nanostructures are smaller than proteins, and the largest approximately ten times the size of an average protein. Not only do the smaller NPs have less surface area, curvature can reduce the accessible surface even further. While proteins compete to bind to the surface, bare surfaces of diffusing nanostructures are susceptible to aggregation.[83] Aggregation can also be protein induced, i.e. one protein molecule can serve as a bridge between two NPs. This effect is often concentration dependent and is related to protein avidity, discussed in 3.3.1, as proteins can have more than one binding site for a nanostructure. At intermediate concentration where the protein partially covers the NP surface, the coverage is high enough for the

probability of the collision of a bound protein and bare surface of a second NP to come in contact.[84] Proteins that denature upon adsorption can also induce aggregation by binding to other, denatured proteins on neighboring NPs.[85] An interesting group of nanostructure surfaces are immobile and therefore not prone to aggregation. They can be produced with high precision, e.g. as vertical nanowire arrays[33] and nanoholes.[86]

4.3 The protein corona

The adsorbed protein layer on a nanostructure is called the protein corona[87,88] and the combined nanostructure with its adsorbed biomolecules is called a complex. The corona can consist of anything from a single protein, up to thousands of different proteins, depending on the complexity of the protein source, proteins' affinity towards the surface, and the available surface. In blood serum or plasma, thousands of different proteins are present in highly differing concentrations.[89] Following exposure of a pristine surface to proteins and the subsequent protein adsorption, the adsorbed protein layer serves as the complexes' biological identity.[90] This new identity will determine the NPs' fate within an organism, its location, residence time, and the organism's biological response.[91] NPs exposed to cells in the absence of a protein corona tend to be more readily internalized by the cells, possibly due to direct adsorption to cell membrane by non-selective internalization. However, proteins in the corona can induce selective internalization by binding to receptors on the cell's surface.[92]

In nanomedicine, the adsorbed protein corona can often interfere with the surface design of the NP. That has led to the development of NPs with signal molecules incorporated into the corona itself.[93] In order to understand the biological impact of nanostructures, it is therefore important to understand their interactions with the environment on a molecular level.

4.3.1 Protein corona composition

Given the importance of the protein corona composition on the fate of nanostructures in an organism, its analysis has received a lot of attention. Protein identification and quantification is usually done by SDS-PAGE, followed by mass spectrometry. To illustrate the variations in corona composition, three samples from the literature have been chosen based on the NPs' properties. Tenzer *et al.* studied the effect of silica NP size on the protein corona formed in blood plasma.[94] Dobrovolskaia *et al.* studied 30 and 50 nm Au NPs in blood plasma,[95] and Cedervall *et al.* studied N-isopropylacrylamide and *N-tert*-butylacrylamide (NIPAM-BAM) of 70 and 700 nm diameter with varying hydrophobicity.[87] In all studies, a variety of proteins were found. Tenzer found that significant differences are in the corona composition for 20, 30, and 100 nm diameter NPs. However, no correlation was found between NP size and the

size of proteins in the corona. In total, they identified 125 different proteins, albumin the most abundant followed by apolipoprotein AI, the most abundant protein in high density lipoproteins.[75] Coagulation factors, lipoproteins, and complement system components were found in high concentration compared to their concentration in plasma, while immunoglobulins were found in lower abundance. Dobrovolskaia identified fibrinogen as the most abundant, followed by inter-α-trypsin inhibitor heavy chain H4 precursor, and gelsolin on the 50 nm NPs, while plasma serine protease inhibitor precursor was also among the most abundant in 30 nm NPs. On hydrophobic NPs, however, Cedervall found that apolipoprotein AI was the most abundant. Albumin, the most abundant protein in plasma, was only found in minor amounts. Interestingly, the lipid binding protein apolipoprotein AI was found in both hydrophilic and hydrophobic NPs, which can be explained by its biological function of binding to hydrophobic molecules and making them soluble in the aqueous environment. We also see that even though albumin is the most abundant protein in blood, it is not necessarily the main protein of the corona due to the surface affinity of other proteins. Considering the infinite possible combinations of protein sources and concentrations, NP material, size, and solution conditions, it is very difficult to predict the composition of the protein corona.

4.3.2 Lipids in the corona

Most of the research focus has been on the protein content of the biomolecular corona. However, it does contain other biomolecules. Hellstrand *et al.* were the first to report on the lipid content of the protein corona of the same NIPAM-BAM NPs described above. These NPs offer the possibility of tuning their hydrophobicity by altering the ratio of NIPAM and BAM. The study shows that the NPs bind intact lipoprotein particles with a specificity towards high density lipoproteins (HDL).[96] The binding of intact HDL has also been found on silica NPs.[97] The most abundant protein of HDL is apolipoprotein AI, found in the protein corona of all the NPs discussed above, as well as in the corona of TiO_2, gold, and on GaAs nanowires in our own work. Raesch *et al.* studied the biomolecular corona formed on NPs in pulmonary surfactant, the liquid layer of the lung. This layer is composed of four surfactant proteins together with lipids. Phosphatidylcholine was the most abundant lipid found in the corona.[98]

4.3.3 Analyzing the corona

The methods used to measure proteins on flat, solid surfaces are sometimes not applicable when the surface is not flat, like ellipsometry[99] which measures the change in refractive index upon adsorption and gives information about the thickness of the adsorbed layer. Other methods can be used for both bulk and nanosurfaces, like neutron scattering.[100,101] The high mobility of nanostructures, especially spherical NPs,

offers the possibility of different analytical methods compared to immobile surfaces. As mentioned in 2.2.3, the two modes of nanostructures' movement are diffusion and sedimentation and depend on the NPs mass. Changes in size and mass upon adsorption and aggregation are detectable by light scattering techniques that measure the diffusion rate (further discussed in 5.2 on Dynamic light scattering).[102] By introducing a fluorescent label into the system, usually on a NP, fluorescence correlation spectroscopy detects the slower movement of a NP-biomolecule complex compared to the free NP.[103,104] Sedimentation rate is affected by changes in both size and density and will be further discussed in 5.1.1 on differential centrifugal sedimentation.[105,106]

Formation of the protein corona in blood serum or plasma is very rapid, usually on the scale of seconds.[107,108] Proteins are therefore known to somewhat stabilize NPs in solution depending on their concentration ratio.[83] During incubation, the protein corona develops.[94,108-110] In order to study the composition of the corona, the complex needs to be extracted from the solution. Experimentally, this is quite challenging. The most common method for isolating the complex from unbound molecules is centrifugation, followed by repeated washing steps. Removing the complex from its environment can lead to increased rate of dissociation for some proteins. To pellet small NPs, long centrifugation steps may be required. When studying the time evolution of the corona, this is not optimal since the complex is not removed from the solution rapidly enough. To overcome this problem to some extent, Docter *et al.* introduced a simple system where the NPs and biological fluid mixture is loaded on top of a sucrose cushion of higher density than the mixture. By centrifugation, given that the NPs' density is high enough, only the complex sediments through the cushion, reducing the exposure of the complex to the biological fluid.[110]

4.4 Proteins on nanostructures

As has now been established, protein-ligand interactions are a delicate system that maintain many of the biological functions of healthy organisms. The effect of adsorption on a protein's structure and ability to function is therefore of great interest. Many of these interactions are very specific. For proteins to function properly, it is very important that the interacting patches on their surface remain free and intact. This can be compromised upon adsorption, e.g. if an important protein-ligand binding site is bound to or blocked by the nanostructure surface. Protein structure might also be affected by the adsorption with effect on both itself and other molecules. Furthermore, the interface between a nanostructure surface and the aqueous, biological environment is not very well understood.

4.4.1 Protein orientation and residence time

In addition to protein composition, the thickness and packing density of the corona can vary. At pH close to the protein's pI, electrostatic repulsion between adsorbed protein molecules is minimized and the adsorbed protein layer becomes denser.[111] In high protein concentrations, the packing also tends to be denser since many proteins arrive and bind to the surface simultaneously, giving the protein molecules less time to spread out to maximize their surface contact. Neighboring protein molecules provide steric hindrance and the proteins undergo less structural changes upon binding than when proteins are in low conentrations.[101] Due to the patchy nature of a protein's surface, different parts of the surface have different affinities towards the solid surface.

This is demonstrated well by the intrinsically disordered protein α-synuclein which has a very distinct charge distribution as its C-terminal tail is very negatively charged. It interacts very selectively with Au NPs, depending on the NPs' surface functionalization, as the C-terminal tail binds to positively charged, and the N-terminal tail to negatively charged NPs.[112] However, most proteins have a more even charge distribution so they might have more than one possible binding orientation. Energetically less favorable orientations will dissociate more rapidly than favorable ones, leading to increased probability of finding proteins with the most energetically favorable binding with time, which explains Vroman's observation. Röcker *et al.* studied the adsorption of human serum albumin to FePt NPs and found that the protein resides on the NP surface for approximately 100 seconds before it dissociates and another protein molecule can replace it. Albumin has a triangular, heart-shape, with the edges of the triangle approximately 8 nm in length and its thickness is approximately 3 nm. In an orientation maximizing surface contact, each albumin molecule takes up approximately 32 nm^2.[113]

This number differs quite significantly from the 6 nm^2 average of interacting area in protein-protein interactions, mentioned in 3.3. However, considering the homogenous nature of the solid surfaces, it provides multiple binding sites for proteins and protein adsorption is limited by the available solid surface area. Proteins can bind very tightly, almost irreversibly, to the NP surface. Lundqvist *et al.* found that incubating NPs in plasma, then moving the NP-protein complex to cytosolic fluid, the NPs retained some of its plasma protein corona, and the final corona was composed of proteins from both biological fluids.[114]

4.4.2 Protein function on nanostructure surfaces

An interesting and related field of research is enzyme immobilization on surfaces. Flat, solid surfaces, porous materials, and more recently nanostructures, have been used as the support material. Porous and nanomaterials provide more surface area than the flat, solid surfaces, while nanostructures can eliminate the diffusion problems posed by the porous materials.[115] In engineered enzyme immobilization, the enzyme can be

designed to contain a binding tag with affinity for the surface. Doing so, the orientation of the enzyme can be controlled. The engineered enzyme is designed so it exposes its active site towards the bulk, minimizing steric hindrance and increasing its availability of the active site to its substrate.

By the simplest approach, immobilization of an enzyme on a NP would lead to somewhat slower diffusion of the NP-enzyme complex, compared to the free enzyme. This would reduce the frequency of collisions between the enzyme and its substrate. If the enzyme's structure is intact, this should lead to only a small decrease in activity. However, the opposite is often observed. Activity of enzymes has been shown to increase by up to a factor of four, when immobilized on a NP.[116] Breger *et al.* studied the kinetic efficiency of phosphotriesterase after immobilization on NPs. Similarly, they found an increase in catalytic activity by a factor of four and the catalytic efficiency (k_{cat}/K_M, a measure of activity of each enzyme molecule divided by the substrate concentration required to reach half the maximum catalytic rate).[117]

The main difference between this designed immobilization approach and nanosafety is that in nanosafety studies, the enzyme will adsorb in its favored orientation. After that, both fields are faced with the same problem. What happens on the nanostructure surface? At the solid-liquid interface, conditions are not very well understood. Breger *et al.* discuss this at length. Different hypotheses are presented, mainly emphasizing this complex environment on the surface. Among the phenomena suggested are localized increase in concentration of the enzyme and the effect it has on the charge distribution at the interface. The concentration, diffusion, binding, and dissociation of the substrate are also discussed without a conclusion. From a nanosafety perspective, this becomes even more complicated due to the presence of various biomolecules from the biological environment that might also be present in localized, high concentrations. This complicated behavior will be very clear in papers IV and V.

4.4.3 Protein structure

Upon adsorption, proteins can also undergo structural changes.[83,85] Norde and Giacomelli studied the effect of hydrophobic polystyrene NPs on bovine serum albumin (BSA). The protein was incubated with the NPs, allowing the protein to bind and dissociate freely. After incubation, dissociated proteins showed differences in their secondary structure and a lowered enthalpy of denaturation, compared to the native protein, indicating that the protein was irreversibly denatured by its interaction with the particles' surface. No such difference was detected for hydrophilic silica particles.[118] Lundqvist *et al.* studied the effect of NP curvature on the structural stability of human carbonic anhydrase I in the presence of 6, 9, and 15 nm silica NPs. They found that the effect on secondary structure of the protein increased for the larger NPs, indicating that the 6 nm NP did not provide a large enough surface area for the interaction.[119]

At physiologically relevant conditions, proteins do not necessarily behave like they do in dilution in a test tube. Under the crowded, physiological conditions inside the cell, parameters describing protein behavior are altered. van den Berg *et al.* studied refolding of the highly positively charged protein lysozyme in the presence of different crowding agents. Using polysaccharide, the effect on the enzyme's refolding was less than when proteins were used instead of the polysaccharide. This indicates that under crowded conditions, protein-protein interactions play a role in the behavior of some proteins.[69] Although the concentration of biomolecules in most studies involving nanostructures does not reach similar levels as inside the cell, localized concentrations of biomolecules on the nanostructures' surface can be much higher than in the bulk.

Proteins are, to various extent, prone to undergo structural rearrangement to form the cross-β-sheet protein aggregates called amyloid fibrils. Cabaleiro-Lago *et al.* studied the effect of NPs on the fibril formation of the protein monellin and five of its mutants. The mutants differed in stability and, in the presence of NPs, showed a correlation between decreased stability and propensity to form fibrils.[120] Linse et al. studied the effect of NPs on fibrillation of β_2-microglobulin and found that in the presence of NPs, the lag phase (the time before significant fibrillation takes place) was significantly shortened.[121] In both studies it was concluded that the localized, high concentration of proteins as they adsorb on the nanostructures' surface was crucial for the formation of the nuclei from which the fibrils grow.

4.4.4 Protein induced nanoparticle aggregation

Not only do nanoparticles induce protein aggregation, proteins can also induce nanoparticle aggregation. The events that take place when nanoparticles and proteins come together in solution are difficult to predict and depend on various factors. As has been described, when repulsive forces are larger than attractive forces, nanoparticles remain colloidally stable. Colloidally stable NPs can, however, aggregate in the presence of proteins. Bharti *et al.* studied silica NPs' interaction with lysozyme at different pH values. Lysozyme's isoelectric point is around pH 11 which means it's positively charged at physiological pH. Silica, on the other hand, has an isoelectric point between pH 1 and 3, which makes it negatively charged at pH levels above that. In the absence of lysozyme, the NPs were stable, due to their negative charge. However, in the presence of lysozyme, they aggregated. In order to aggregate, lysozyme needs to form bridges between the NPs, since their attractive van der Waals forces are not sufficient to overcome electrostatic repulsion between the bare surfaces.[122]

Lysozyme is not a typical protein given its high isoelectric point. In fact, most proteins are negatively charged at physiological pH. Furthermore, the ionic strength was kept very low in order to not induce aggregation by shielding the negative charges of the NPs' surface. These results still show that proteins can serve as bridges between NPs. In another study, Cukalevski *et al.* found that immunoglobulin G and fibrinogen

induce NP aggregation in a concentration dependent manner. At low and intermediate protein concentration, i.e. when the proteins do not completely cover the NPs' surface, they can form aggregates by multiple binding sites on one protein to more than one NP. At lower concentration, the aggregates are smaller since more free NP surface is present once a protein molecule has bound.[84]

4.4.5 Thermodynamics of corona formation

In thermodynamic terms, the adsorption is driven by the enthalpy gain from favorable van der Waals, hydrophobic or electrostatic interactions and entropy gain from released surface water molecules and ions.[123,124] More hydrophobic proteins undergo structural changes upon adsorption to somewhat hydrophobic surfaces, due to the entropy gain of increased backbone flexibility.[125] Proteins generally adsorb more to hydrophobic than hydrophilic surfaces and the adsorption to hydrophobic surfaces tends to be irreversible.[56] In general, negatively charged proteins adsorb more onto surfaces than positively charged ones, regardless of the surface charge.[108] When negatively charged proteins adsorb to negatively charged surfaces, the entropy gain by increased flexibility in the protein structure needs to be sufficient to make up for the electrostatic repulsion.[118]

5. Methods

In this chapter, I will describe the methods used in my work. In order to observe the interaction of such miniscule objects, like nanostructures and biomolecules, various methods are available. Most of them distinguish between nanostructures and biomolecules by some differing physicochemical properties. The methods have various robustness, some of them can measure thousands of samples in a day, others can only look at a few molecules. All the methods have strengths and weaknesses. Using them in combination is crucial to prevent misinterpretation of data and gives a better image of these complicated events.

5.1 Sedimentation methods

A useful way to study changes in nanostructures' properties is to study their sedimentation rate. Sedimentation rate depends on the centrifugal force applied to the structures, their size, shape, and the difference in density of the nanostructure and the solvent it is suspended in.[126]

5.1.1 Differential centrifugal sedimentation

Differential centrifugal sedimentation (DCS) is a very efficient separation technique that relies on different sedimentation rates of different components of a sample. The technique can be applied in various degrees of sophistication, depending on the sample volume, size, and the equipment available. A common application of the technique is for isolation of different cell organelles from cell lysates. Depending on the centrifugal force and time, different organelles will sediment and form a pellet in the centrifugation tube.

A more sophisticated apparatus is illustrated in figure 1. The instrument, called a disc centrifuge, uses centrifugal force to sediment a sample through a density gradient in a spinning disc. By controlling the centrifugal force in a gradient of well-defined density, the sedimentation rate of the sample is measured by a detector, shown as a black square. As the sample travels past the detector, light is scattered or absorbed by the sample, which leads to reduced intensity in light that reaches the detector. This

reduced intensity can be converted to weight, surface area, or number of particles in the sample, according to user defined parameters.

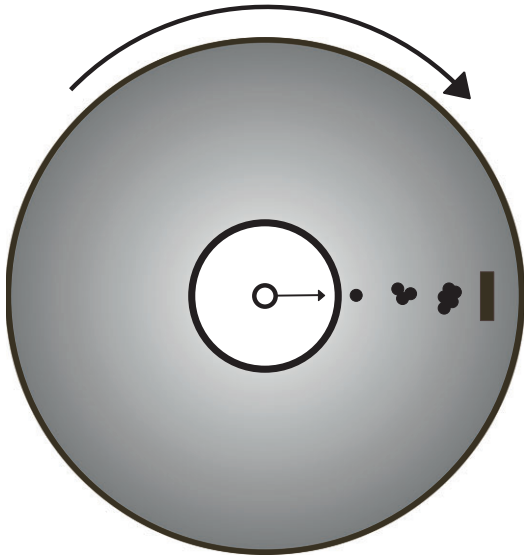

Figure 1. Illustration of a disc centrifuge. The sample is injected into the centre circle of the rotating disc. Particles of the sample will then migrate towards the outer, big circle due to the centrifugal force. The largest particles migrate fastest. The black square represents the light detector. As the sample moves past the detector, it scatters or absorbs light, producing the measured signal.

The raw data is absorbance over time. In order to transform the raw data to size, number, or surface area, it is necessary to know the shape and density of the sample. For relatively pure NP samples, these parameters are often known and deviation from a spherical shape can be accounted for to a certain extent. However, in biological samples, the interactions of nanostructures and biomolecules often lead to complexes that are far from spherical. Furthermore, the density of the complex is not known and will be an average of the density of the NP and the biomolecular corona. To further complicate the data processing, complexes of different sizes and densities are formed.[127]

$$D_{st} = \sqrt{\frac{18\,\eta \ln\left(\frac{R_f}{R_0}\right)}{(\rho_p - \rho_l)\omega^2 t}} \qquad \text{Eq. 4}$$

Stokes diameter, D_{st}, of a structure can be calculated by equation 4. In a disc centrifuge, the liquid viscosity, η, the densities of the structure, ρ_p, and liquid, ρ_l, and the centrifuge speed, ω, are defined by the user. The sedimentation distance, from R_0 to R_f, is measured by calibration standard before each run, and the time, t, until a signal is

detected is measured by the instrument. Equation 4 gives the Stokes diameter of a spherical particle. Deviations from a spherical shape will cause the particles to move slower through the density gradient, causing an underestimation of their actual diameter. The density difference between the structure and the gradient depends on the density of the nanostructure and the density and thickness of any adsorbed biomolecular corona. When the density of a nanostructure is significantly higher than the density of biomolecules, sedimentation will be slower upon adsorption of biomolecules compared to the naked nanostructure. In case of aggregation of the nanostructures, sedimentation will be significantly faster. When the density of the nanostructure is close to the density of biomolecules, e.g. polystyrene with density of 1.05 g cm^{-3}, the effect will be much more difficult to interpret without applying complementary methods.

Even if the experimental procedure itself is quite straightforward, interpretation and presentation of the results needs careful consideration. As will be discussed in 5.1.2 on dynamic light scattering, larger particles scatter more light than smaller ones. The disc centrifuge software offers the possibility to account for the difference in scattering intensity and to convert the data to weight, surface area, or number of particles. This is very useful for the characterization of a pure NP sample since relatively few large particles might dominate the absorbance profile, even if they only account for a small portion of the number of particles. Since these calculations are based on the parameter settings provided by the user, deviations from the parameters in the actual properties of the particle will result in wrong presentation of the data. Let us take gold for an example. Gold NPs have a density of 19.3 g $cm^{-3.}$ Entering the appropriate values for gold into the program and measuring the particles diluted in water will give a diameter very similar to the diameter provided by the manufacturer and measured by other methods, e.g. dynamic light scattering or electron microscopy. However, proteins have a density of 1.37 g cm^{-3}.[50] Upon protein adsorption, the average density of the new complex will be much lower compared to the naked NP. This results in slower sedimentation of the complex compared to the naked NP, even though the actual diameter has increased. By conversion of the data to weight or number, the slower sedimenting, large complex will scatter light as efficiently as before but the program will process it as a smaller particle and overestimate the amount of small species. For NPs with an adsorbed biomolecular corona, it is therefore more appropriate to use absorbance than weight.

Using equation 4, the Stokes diameter is calculated and shown on the x-axis. For a sample of spherical, pure NPs, this gives a fair representation of the sample. As the volume inside the disc increases with each run, a calibration standard is run before each measurement. This increases the sedimentation distance, i.e. the difference between R_f and R_0 in equation 4. Therefore, it is not appropriate to use the sedimentation time to compare two runs. As described in the previous paragraph, delayed sedimentation time due to adsorbed biomolecular corona will appear as smaller diameter. It is better to refer

to the diameter as apparent diameter, i.e. our particle sediments like a spherical NP of a certain density and diameter.

5.1.2 Preparative centrifugation

Based on the same principle as DCS, preparative centrifugation separates structures according to their sedimentation behavior. In preparative centrifugation, a sample of interest is loaded on top of a density gradient. The density gradient can be made of increasing concentration of e.g. salts or sucrose. The gradient is prepared in a centrifugation tube and the sample loaded on top of the gradient. A centrifugal force is applied, causing different species of the sample to sediment and separate in the gradient. When we stop the centrifuge, the species remain suspended in different layers of the gradient, the largest ones preferably close to the bottom to increase separation efficiency. We can then elute the gradient in fractions to further study each species. In classical biochemistry, the method was used in the forties and fifties to estimate the size and shape of proteins. As described above, mathematical approaches used to relate the sedimentation rate and size assume proteins to be spherical. However, it became clear early on that discrepancies are between the sedimentation behavior and the actual size of the proteins as discussed in 3.2.3 on the work of Svedberg. As the field progressed, it became clear that the somewhat rough surface of proteins causes significantly more friction to the fluid as it sediments through combined with their elongated shape which reduces the sedimentation rate.[50]

5.1.3 Binding sedimentation assay

When studying proteins' interaction with nanostructures, the first step is to determine if there is an interaction. Different methods exist to demonstrate this binding. However, many of them have limitations and prove difficult to give a definite answer. Dynamic light scattering, which will be discussed later, will only give information about the diffusion rate and slower diffusion rate can either be due to aggregation of the nanostructure or the proteins, or due to adsorption. For nanostructures of high density, slower sedimentation by DCS is a strong indicator of adsorption, while for low density NPs like polystyrene, data can be difficult to interpret.

Finding a universal approach can be difficult due to the diversity of materials, both nanomaterials and biomolecules. The solution usually lies in the materials properties. Inspired by the work presented in paper I, where complexes of different sizes and composition were separated by preparative centrifugation, we were looking for a way to confirm that the enzyme myeloperoxidase (MPO) binds to nanowires. The nanowires were made from gallium arsenide. GaAs has a density of 5.3 g cm^{-3} and the NWs were approximately 100 nm in diameter and 2 μm long. The high density and large size cause poorly dispersed NW samples to sediment quite readily within minutes

when left undisturbed. Exploiting this difference between the protein, which moves by diffusion, and the NWs which move by sedimentation, the following assay was developed. In an Eppendorf tube, a small amount of high concentration sucrose (40-65%) in PBS was mixed with TMB, the enzyme's substrate. On top of the high density bottom layer, an intermediate density (10-40%) sucrose buffer layer was added to separate the bottom, substrate layer from the top layer. The top layer was a mixture of NWs and MPO. For poorly dispersed NWs no centrifugation was needed. After approximately 5 minutes, a blue color was formed in the bottom layer, proving that the enzyme binds to the NWs and remains active once it is bound. The system was applied in papers IV, on GaAs NWs, and paper V, on polystyrene (PS) NPs, establishing that it works on both high and low density nanostructures. In the case of PS NPs, centrifugation was needed. Figure 2 shows an illustration of the assay setup. In case of color formation, binding in an active state is proven. However, if no color develops, it either means that there is no binding or the enzyme loses its activity upon binding. In parallel, control samples were run to rule out color formation due to diffusing or sedimenting enzymes or enzyme aggregates.

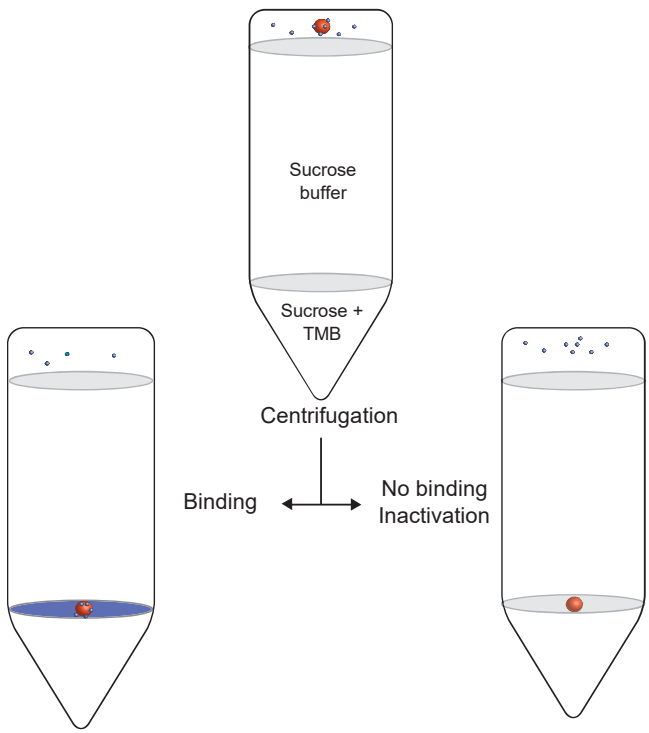

Figure 2. Illustration of the binding sedimentation assay.

5.2 Dynamic light scattering

Dynamic light scattering (DLS) instruments can be found in most nanoscience laboratories. Their popularity undoubtedly stems from how rapidly they produce results with high throughput and ease of operation. In general, DLS instruments are equipped with a laser of a specific wavelength, a temperature controlled sample chamber, and a photon detector positioned at a certain angle that detects the scattered light. DLS measures the Brownian motion of particles suspended in a sample. Brownian motion, the random movement of particles due to collisions with the molecules of their environment, depends on temperature and viscosity of the solvent they are suspended in. These parameters must therefore be well defined in order to estimate the size of the particles. Furthermore, Brownian motion depends on the particles' size as small particles move faster than larger ones. The relationship between these factors is described by the Stokes-Einstein equation.

$$X_h = \frac{k_B T}{3\pi\eta D_t} \qquad \text{Eq. 5}$$

X_h is the particles' hydrodynamic diameter, k_B is the Boltzmann's constant, T is the temperature, η is the viscosity of the solution, and D_t is the translational diffusion coefficient. In a DLS experiment, all parameters on the right side of the equation are known, except the diffusion coefficient. To measure the diffusion coefficient, the laser illuminates the sample and as light travels through the solution, some of it will be scattered by the suspended particles. Some of this scattered light will reach the photon detector. Due to random Brownian motion of the particles, the intensity of the scattered light will fluctuate over time. This fluctuation is monitored for a time interval chosen by the user, typically between 1 and 10 seconds. Over this defined interval, the scattering intensity is measured every 0.1 μs, and the correlation within the scattering profile calculated using equation 6.

$$g^{(2)}(\tau) = \frac{\langle I(t)I(t+\tau)\rangle}{\langle I(t)\rangle^2} \qquad \text{Eq. 6}$$

The diffusion coefficient in equation 5 is calculated from the auto-correlation function according to the following relations. Equation 6 describes how the correlation function, $g^{(2)}(\tau)$, is obtained from I(t), the measured intensity of scattered light at time t, and at a delay of time τ. Larger particles have better correlation at larger τ since the scattering intensity changes less at the delay time. Equation 7 is fitted to the autocorrelation function to obtain the decay rate.

$$g^{(2)}(\tau) = B + \beta e^{-2\Gamma\tau} \qquad \text{Eq. 7}$$

Where B is the baseline of the function at infinite delay, β is the coherence factor and depends on detector area, optical alignment, and the scattering of the sample,[4] and Γ is the decay rate of the correlation function, obtained from the fitting assuming monomodal size distribution. The relationship between the diffusion coefficient and the rate of decay is described by equation 8, where q is the magnitude of the scattering vector, calculated according to equation 9.

$$\Gamma = -D_t q^2 \qquad \text{Eq. 8}$$

$$q = \frac{4\pi n}{\lambda} sin\frac{\theta}{2} \qquad \text{Eq. 9}$$

Where n is the refractive index of the solvent, λ the wavelength of the laser, and θ the scattering angle. The scattering angle and wavelength of the laser are constant for the instrument, while the refractive index is defined by the user.

So how do we use DLS? DLS is a measure of the randomness often considered noise.[128] The auto-correlation function examines the rate of fluctuations in the scattering intensity. All the parameters in equation 5 are kept constant during a measurement and the diffusion coefficient is measured to calculate the hydrodynamic diameter. A large particle has a lower diffusion coefficient, i.e. it diffuses slower, than a smaller particle. This property is directly measured by slower fluctuations in the intensity spectra for larger particles measured by the detector.

Figure 3 shows the auto-correlation functions for 20 and 80 nm gold NPs. The smaller gold NPs diffuse faster and less correlation is between measurements at time t and t+τ as indicated in equation 6. Since the decay is faster, the value of Γ is larger, and the value of D_t is higher, obtained from equation 8. Using cumulant analysis of the experimental data, the average diffusion coefficient of the sample is calculated and equation 7 contains only one decay rate, Γ. In a mixture of biomolecules, sizes can range from less than 1 nm up to micrometers. For a system of such broad distribution of sizes, an average size does not describe the sample very well. To obtain a size distribution, regularization of the data is possible where a fitting of the data with more than one diffusion coefficient can be extracted from the intensity auto-correlation function.[4]

Another important aspect of light scattering is the size dependence of scattering intensity. A particle's scattering intensity increases as the sixth power of its diameter, i.e. a four-fold increase in diameter from 20 to 80 nm will increase the intensity by 4^6 or 4,096 times.[129] Finally, it should be pointed out that the conversion from diffusion coefficient to hydrodynamic diameter, according to equation 5 assumes that the particle is spherical. When measuring a non-spherical structure, the reported hydrodynamic diameter is therefore the diameter of a spherical, solid particle with the same diffusion coefficient as the measured particle.[130]

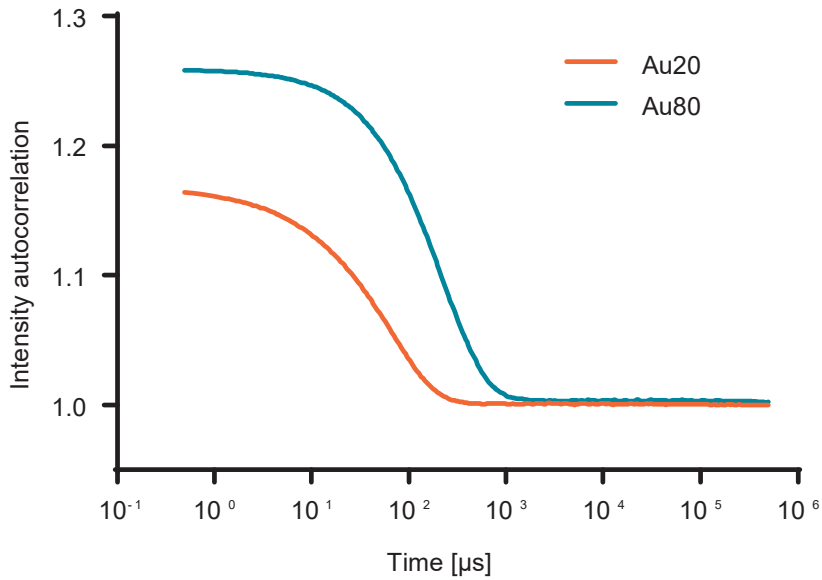

Figure 3. Dynamic light scattering of 20 and 80 nm gold nanoparticles.

5.3 Ultraviolet-visible spectroscopy

Ultraviolet-visible spectrophotometers are a standard instrument in most research labs. The spectrophotometer measures the difference in light intensity emitted from a light source and the light that reaches a detector after passing through a sample. When a chemical compound absorbs light, an electron moves from its ground state to an excited state. The energy difference between those two states is equal to the energy of the absorbed light. The energy difference depends on the compound and different compounds absorb light at different wavelengths. The spectrophotometer can usually emit light of any chosen wavelength in the mid- and near-UV from around 200 nm to the visible spectrum around 900 nm. Absorbance is concentration dependent, according to Beer-Lambert law.

$$A = \varepsilon * b * c \qquad \text{Eq. 10}$$

Where A is the absorbance, ε is the extinction coefficient, which depends on the chemical compound and the wavelength, b is the path length of the sample, and c is the concentration of the compound.

UV-Vis spectroscopy is useful both in protein and nanostructure studies. For both gold and silver NPs, a shift in the maximum absorbance wavelength is observed when the surface of the NPs is disturbed, either by aggregation or adsorption of other

molecules as described by the localized surface plasmon resonance in 2.2.2. Absorbance at 280 nm is also a common method to quantify proteins since aromatic side chains of amino acids absorb in this wavelength.

5.4 Attenuated total reflectance Fourier-transform infrared spectroscopy

One of the most common tools for chemical analysis is infrared spectroscopy (IR). IR spectroscopy measures the vibration frequencies of chemical bonds. For a chemical bond to absorb IR radiation, it has to have a changing dipole moment during its vibration. The mid-IR range, from 4,000 to 400 cm^{-1}, covers vibrations of common biological compounds, consisting of carbon, oxygen, hydrogen, and nitrogen. The modes of vibrations depend on the number of atoms in the compound and its shape. For large biomolecules, an IR spectrum is very complex and many vibrations overlap. Furthermore, measuring a biomolecule in water can be challenging due to the overlap of O-H vibrations with the analyte and can be solved by using heavy water, D_2O. As deuterium is heavier than hydrogen, the vibration peaks are shifted to lower wavenumbers.[131,132]

Although IR spectroscopy is often performed in solution in a sample cell, a useful modification of the technique is ATR-FTIR. By applying IR radiation at a certain angle to a horizontal crystal of a specific thickness, the infrared light is reflected within the crystal and every time it is reflected, it produces an evanescent wave. The wave penetrates the sample at a depth similar to the wavelength, i.e. on the order of micrometers. Therefore, it probes the sample very close to the crystal surface. This can be useful when studying sedimenting nanostructures.

5.5 Transmission electron microscopy

Due to the small wavelength of electrons compared to photons, electron microscopy enables higher resolution than light microscopy. Electrons are fired at the sample by an electron gun. Depending on the acceleration voltage of the gun, the resolution can be down to single atoms. The electrons pass through various lenses on their way to and from the sample. The electrons finally reach a screen or a camera that produces an image of the sample. The image is a representation of the atoms the electrons encounter on their way. Heavier atoms appear as black spots in the image, due to their high electron scattering and absorbance, while lighter atoms produce less contrast. In regular TEM, the sample is dried in order to withstand the high vacuum of the sample chamber.

Drying a protein sample can cause some changes in its structure, as most proteins function in a hydrated state. In 2017, Jacques Dubochet, Joachim Frank, and Richard Henderson were awarded the Nobel prize in chemistry for their development and advance of cryo-electron microscopy. By freezing the sample in liquid ethane and storing it in liquid nitrogen, the temperature is low enough for the sample to remain frozen in the high vacuum of the sample chamber. This means that the protein is kept in it hydrated state during images and eliminates many of the possible artefacts that might appear during the drying process. Furthermore, by taking multiple images of the sample while rotating the sample holder, a three dimensional image of the sample can be obtained. Cryo-TEM is therefore an extremely useful method for analyzing samples at the nanoscale, both proteins, nanostructures, and their complexes.

5.6 SDS-PAGE and mass spectrometry

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most common methods in any biochemistry lab. A protein sample is first denatured by boiling in the presence of SDS and a reducing agent. This step is very useful when extracting proteins from the surface of a nanostructure. The denatured sample is then loaded onto a polyacrylamide gel and a voltage applied through it. Proteins of different sizes will then migrate towards the anode due to their negative charge. Small proteins will migrate further than large ones. The gel is then stained, e.g. by Coomassie blue or silver, depending on the amount of protein in the sample. Silver staining can detect proteins down to the ng range while Coomassie is less sensitive. This relatively rapid and high throughput method gives a picture of the protein profile of the sample, e.g. the protein corona. In order to get relative quantities of proteins on a gel, they can be analyzed by image processing software like ImageJ.

An SDS-PAGE gel does not give information about a protein's identity, even if it shows the molecular weight of the protein. In order to identify proteins from a gel, a

stained band is excised and destained. The protein is then digested by trypsin or other proteolytic enzymes with well-defined cleaving sites. The resulting peptide pool is then analyzed by mass spectrometry, where the peptides are identified by their mass-to-charge ratio and the identified peptide pattern compared to databases of known proteins.

5.7 X-ray based detection methods

X-ray photoelectron spectroscopy (XPS) is a surface sensitive technique that irradiates a sample with X-rays, removing electrons from the surface of a sample. By controlling the voltage, electrons of specific elements are removed. The method is very useful to characterize the surfaces of nanostructures, both before and after exposure to biological fluids. The method can be used to detect an organic layer on the surface of an inorganic material as well as ensure that no unexpected molecules are on the surface, e.g. stabilizers.

Similarly, X-ray based photoemission electron microscopy (XPEEM) uses X-rays to remove photoelectrons from the surface of samples with well-defined energies and gives an image of the elemental and chemical nature of the sample.

6. Results

In this chapter, I will describe the results of the five papers presented in the thesis. I will first give a short summary of the motivation and main results of each individual paper. Following the summary, I will put the papers in context to one another using selected discussion points. Table 1 shows a summary of each paper's aim and design.

Table 1. Overview of papers.

Paper	Aim	Biomolecule	Nanostructure	Conc.	Methods
I	Aggregation Corona	Serum	TiO_2 NPs	High	DCS Prep. centr. SDS-PAGE Mass spec. XPS
II	Aggregation Corona	CCM Serum	Au NPs	Low	DLS DCS UV-Vis ATR-FTIR Cryo-TEM SDS-PAGE Mass spec.
III	Single nanostructures Corona	Laminin Albumin Plasma	GaP NWs	Very low	Cryo-TEM XPEEM XPS DLS
IV	Activity Structure Corona	MPO Laminin Albumin IgG Serum	GaAs NWs	Low	Cryo-TEM Activity Sedim. assay DCS Intr. fluoresc.
V	Activity Size Surface Corona	Secr. MPO Pur. MPO Albumin CCM Serum	PS NPs TiO_2 NPs	Various	Activity Sedim. assay DCS DLS

6.1 Summary of papers

6.1.1 Paper I

In paper I, our focus was on the biomolecular corona of complexes formed when TiO_2 NPs aggregate in blood serum. Under the experimental conditions, complexes with a broad size distribution were formed. The complexes had apparent sizes between 30 to 500 nm. Traditionally, the protein corona is portrayed as a fixed entity for each nanostructure and protein concentration ratio. We wanted to see if this was true, i.e. upon formation of a broad size distribution of NP-biomolecule complexes in biological fluids, do the complexes only differ in size but are homogenous in biomolecular content? After separating these complexes by their sedimentation rate, using preparative centrifugation, we studied the biomolecular content of each size population. We found that two main size populations were formed. One fast sedimenting population of large aggregates with a biomolecular composition independent of the NP to serum concentration ratio. A second population was also formed; a slow sedimenting population that was very dependent on the surface to serum concentration ratio. By increasing the serum to NP concentration ratio, more IgG and lipid binding proteins were found in the slow sedimenting population. These findings show that the conventional description of one protein corona for a specific NP to serum ratio is an oversimplification. It also showed that when the serum concentration is increased, less large aggregates are formed and more lipids are bound to the NPs.

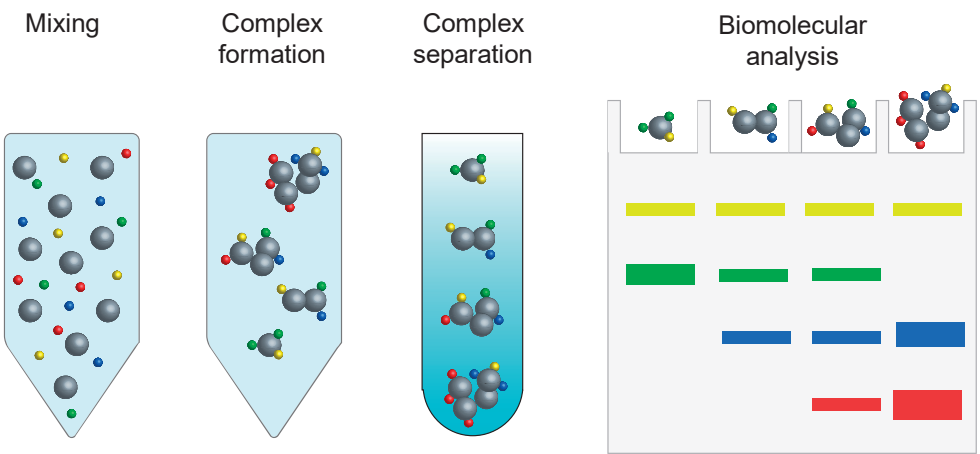

Figure 4. Graphical summary of paper I. TiO_2 NPs (gray spheres) were mixed with a mixture of biomolecules (colored spheres), separated by preparative centrifugation and the biomolecular content analyzed.

6.1.2 Paper II

In paper II we studied the behavior of 20 nm gold NPs in low concentration in cell culture medium (CCM). CCM is a mixture of amino acids and other nutrients, often supplemented with fetal calf serum, applied to cells grown *in vitro*. Using a combination of complementary analytical methods, we were able to study interactions of NPs in the pM concentration range in an environment rich in biomolecules. The concentration used is very low compared to biomolecular corona studies and on a similar level to those used in cell assays. Two different CCM were used, one protein rich and one protein poor. Both CCM are rich in amino acids, electrolytes, carbohydrates, and other molecules. The Au NPs behave very differently in the two media. In protein rich CCM the NPs are fully stable as the protein corona forms rapidly and prevents them from aggregating. In protein poor CCM the NPs aggregated slowly. By studying the NPs' diffusion, sedimentation, and optical properties, we distinguished between aggregation and biomolecule adsorption. We also described the aggregates' morphology and confirmed our conclusion by cryo-TEM. In protein poor CCM, we detected amino acids in the biomolecular corona that, in isolation, have been shown to induce the aggregate morphology we described.

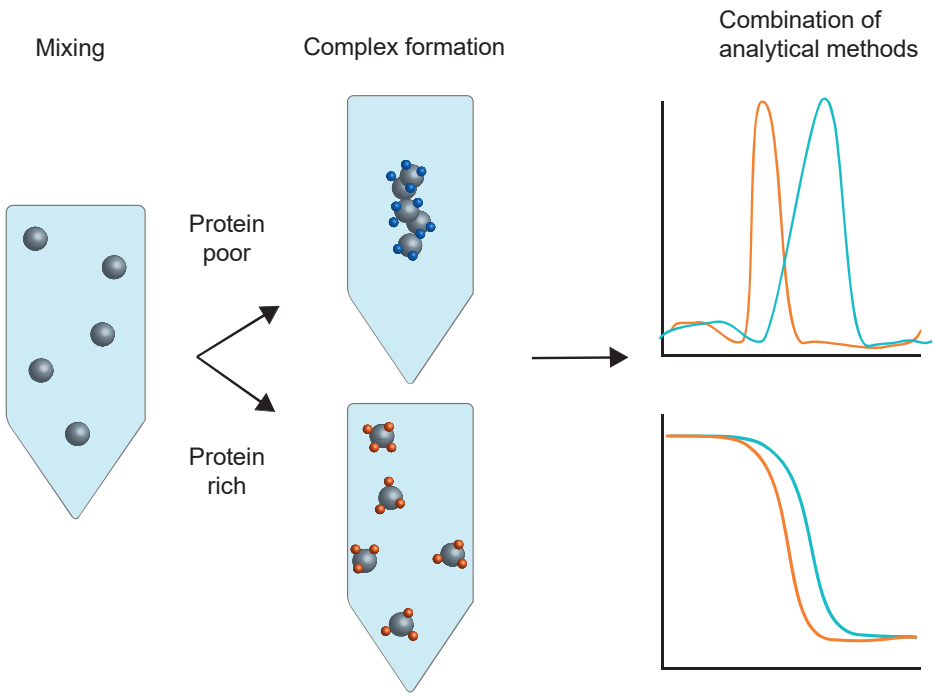

Figure 5. Graphical summary of paper II. Gold NPs in low concentration (gray spheres) were mixed with protein poor or rich cell culture media and the complexes formed were analyzed by a combination of techniques with regard to biomolecular corona and aggregate morphology.

6.1.3 Paper III

In paper III we studied protein adsorption to gallium phosphide nanowires (NWs). New methods for NW fabrication are constantly developed in NanoLund. Their potential applications range from medical implants and drug delivery systems to solar cells. During the development of novel nanomaterials, it is important to study their interactions with biological systems. The risk of overlooking a potential hazard is reduced and can even lead to novel application ideas. Studying materials under development requires a lot of flexibility in the experimental approach due to the limited material availability before scaling up of their production. Using a combination of cryo-TEM and X-ray based analytical methods, we could image the adsorbed protein layer formed by different unlabeled proteins on single NWs. The very large protein laminin, the smaller albumin, and a protein mixture from blood plasma were imaged and the observed protein corona matched quite well the measured hydrodynamic diameters of the proteins. Due to the low electron scattering of organic materials, we used the X-ray based techniques to confirm the presence of organic materials in very low concentrations.

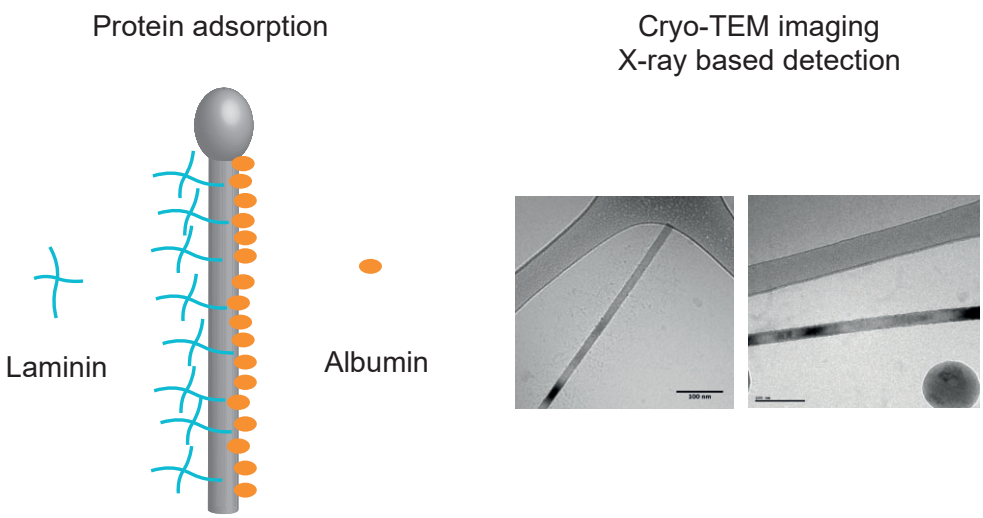

Figure 6. Graphical summary of paper III. NWs were coated with laminin or albumin and imaged by cryo-TEM and X-ray based method for confirmation of the presence of organic material.

6.1.4 Paper IV

In paper IV we expanded on the work presented in paper III. Rapid advances in the production scale of NWs made it possible to revisit some of the remaining questions from our previous work. We imaged the protein corona formed by the enzyme myeloperoxidase (MPO) and laminin by cryo-TEM. We obtained a better resolution on the corona thickness by labelling it with gold NPs. To distinguish between optical errors due to the two dimensional projection of standard cryo-TEM images, we rotated the sample to image the it in three dimensions. By measuring the distance between the gold NPs and the NWs' surface, we identified two distinct distance populations at 58 and 85 nm, respectively. This shows irregularities in the protein corona, consistent with the cross shape of laminin. Furthermore, due to the improvements in material availability, we were able to show the non-specific binding of a series of proteins to the NWs by differential centrifugal sedimentation. We also studied the effect of NWs on MPO's activity. Upon adsorption, the enzyme lost some of its activity while no structural changes were observed by probing its intrinsic fluorescence. In a more complex environment produced by addition of blood serum to the sample, the enzyme regained its activity and in fact became more active than the control sample. During the early stages of this work, a sedimentation assay system was developed to confirm the binding of enzymes to nanostructures.

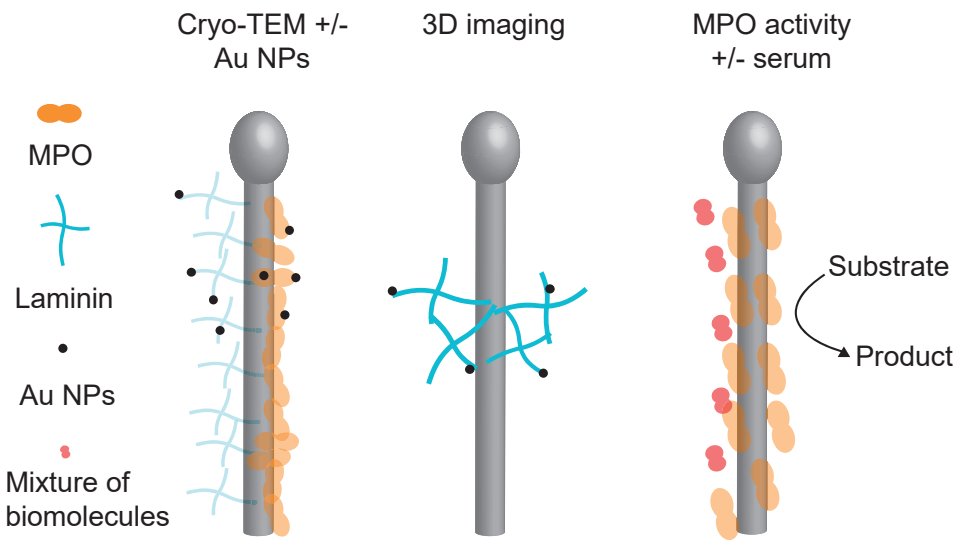

Figure 7. Graphical summary of paper IV. NWs were coated with laminin or MPO and imaged by cryo-TEM both label free and with gold NP labelled protein corona. The gold labelled laminin NWs were imaged in three dimensions. The activity of MPO on the NWs was studied, with and without the addition of mixture of biomolecules from blood serum.

6.1.5 Paper V

In paper V we studied the effect of polystyrene (PS) NPs of different sizes and surface functionalization on MPO activity. The complexity of the protein environment varied from NPs with purified enzyme to MPO secreted by neutrophils during an immune response. By doing so, we assessed the effect of the NPs on the secondary immune response, i.e. not the immunological response to the NPs but the effect of NPs on the molecules produced by the response. Neutrophil secreted MPO was studied in CCM with and without fetal calf serum. As mentioned in paper IV, when the enzyme's environment becomes more complex, the results become difficult to interpret. At physiological pH level and lower, MPO is positively charged. The experimental conditions can be divided in four levels of complexity. First and simplest was purified MPO in PBS with NPs. Under those conditions, the PS NPs in general increased MPO's activity. TiO_2 on the other hand almost completely inactivated it at low NP concentration.

Increasing the level of complexity, bovine serum albumin was added to the mixture. This caused a further increase in MPO's activity, up to almost three-fold activity of the control in presence of 1 mg mL^{-1} of BSA. At higher BSA concentration, the activity increase was less, and at very high BSA concentration there was inactivation.

Further increase in complexity, using neutrophil secreted MPO in cell culture medium without any added proteins, negatively charged PS NPs reduced the activity while the positively charged particles had no significant effect. This can be explained by the affinity of positively charged MPO for negatively charged PS. However, when the proteins were added to the cell culture medium, the positively charged NPs reduced the activity more.

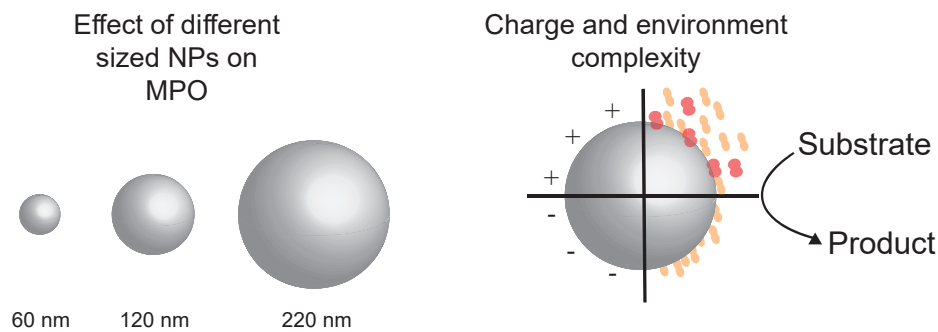

Figure 8. Graphical summary of paper V. The effect of different sizes and surface modifications of polystyrene NPs on the activity of neutrophil secreted or purified MPO was studied. The enzyme's environment ranged from a simple buffer to cell culture medium supplemented with fetal bovine serum.

6.2 Papers in context

One of the main challenges in the field of nanosafety is the difficulty in comparing results from different studies due to the differing experimental conditions. Similarly, there are many ways to compare these five papers and discuss their interrelationship. In order to structure the discussion, the papers have been numbered from one to five as indicated by figure 9. All five papers are united by the center circle, representing nanostructure interaction with biomolecules. While each paper has its own focus, there are some overlaps. These overlaps demonstrate the need for a bigger context when assessing the interactions of complex systems. Papers I and II deal with NP aggregation. In papers II and III we look at nanostructures at very low concentrations. Papers III and IV investigate nanowires at two different stages in the product development. The first experiments were done when the material was available by the numbers, rather than weight. Later, when more material was available, we could go back to remaining questions and in new directions. By flexibility in analytical methods, it is possible to make progress the fields of fabrication and nanosafety in parallel. Papers IV and V focus on protein function upon adsorption to different nanomaterials under varying environmental complexity. Finally, papers I and V show the importance and dynamic nature of the protein corona.

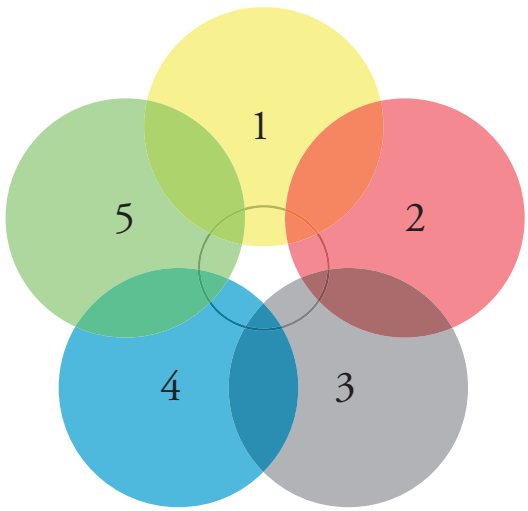

Figure 9. The interrelationship of the presented papers.

6.2.1 Aggregation

Papers I and II illustrate two different approaches when characterizing NPs' aggregation in biological environment. The nanomaterials used in these two papers are titanium dioxide and Au NPs, respectively. TiO_2 NPs are kept colloidally stable by low pH and gold NPs by citrate capping. TiO_2 NPs undergo severe aggregation in cow serum. The largest aggregates are more than ten times larger than the NPs diluted in water and the smallest complexes formed are only slightly larger than the naked NP. On the other hand, in the protein rich environment of serum, the Au NPs are perfectly stable. The difference in colloidal stability of the two NPs can to some extent be explained by their stabilization method.

When the TiO_2 NPs enter the physiological pH and salinity of blood serum, the repulsion effect is diminished. Simultaneously, proteins and other biomolecules collide with the TiO_2 NPs' surface. This results in competition between aggregation and adsorption and explains the formation of two main populations. The first population is larger, sediments faster and is similar in biomolecular content regardless of the concentration ratio of the serum and TiO_2 NPs. This population is therefore likely to be formed quickly with proteins adsorbed to the surface, single protein molecules binding two NPs, and proteins trapped within aggregated NPs. By increasing the serum concentration, less of the large population is formed since the probability of NP-protein interaction is higher due to increased availability of proteins. The second population sediments slower and its protein content is highly dependent on the NP surface to serum concentration ratio. Relative to the serum albumin content, there are large variations in IgG content depending on the concentration ratios, as well as differences in lipid, and lipid binding protein content. This small population would therefore be a better comparison to the Au NPs with regard to their protein corona development and their interaction and effect on cells and organisms.

In comparison, the colloidal stability of the 20 nm Au NPs in protein rich CCM can be attributed to two important factors. First, the method of stabilization. The Au NPs are capped with citrate, which is added during the Au NP production process. Citrate contains three negatively charged carboxyl groups at physiological pH. For two Au NPs to collide and form an aggregate, the citrate needs to be displaced or its charges shielded by high ionic strength. Displacement happens when proteins and other biomolecules adsorb to the Au NP surface. Unless the protein molecule serves as a bridge between two Au NPs, proteins induce stabilization of the Au NPs. Smaller biomolecules, amino acids for example, can also displace the citrate and induce the formation of fractal aggregates.

The second important factor to keep in mind when considering NP aggregation is the NP concentration. In paper I, the TiO_2 concentration was 500 μg mL^{-1}. This provides plenty of surface for NP-NP interaction when there is competition between adsorption and aggregation. In comparison, the Au NP concentration was 2.8 μg mL^{-1}. Taking into account their size and density difference, the surface area of the TiO_2

NPs is roughly 900 times larger than the Au NPs. Paper I was to a large extent based on analysis of SDS-PAGE gels where we detected the relative abundance of each protein. This required quite a lot of serum and NP starting material. In order to decrease the concentration of TiO_2, protein detection by SDS-PAGE required silver staining. When the concentration of TiO_2 was reduced to 50 µg mL^{-1}, or tenfold lower than presented in paper I, the same behavior was observed. Figure 10 shows the difference between the fast and slow sedimenting populations in 10% serum. On the left, the fast sedimenting population has similar amount of IgG, marked by a circle, and albumin, marked by a star. However, on the right, the slow sedimenting fraction contains more IgG than albumin. These results show exactly the same trend as the higher NP concentration samples. However, due to the sensitive nature of silver staining, where the signal intensity is dependent on the development time, it proved difficult to get fair comparisons between experiments.

Figure 10. Cut-outs of silver stained SDS-PAGE gels of the fast (left) and slow (right) sedimenting populations formed when 50 µg mL-1 TiO2 NPs are mixed with 10% cow serum.

Aggregation is an often overlooked aspect in studies of NPs in biological fluids. During aggregation, complexes of different sizes and biomolecular compositions are formed. Since cellular uptake depends on both the composition of the corona and the NP size, this might affect the cells' response. Furthermore, when concentration is low, aggregation is slowed down. Even in the absence of a protein corona, a biomolecular corona is still formed. The presence of amino acids, e.g. in a cell culture medium, can both affect the aggregation rate and morphology, as well as the surface chemistry of the NPs.

6.2.2 Concentration

When designing a chemistry experiment, one of the most important factors is concentration of reacting or interacting components. This is also very true for nanostructure and biomolecule studies. Concentration will affect the rate of reactions and in a multi-component system, where a number of interacting molecules are present, the results are difficult to predict. Experiments involving NPs with therapeutic applications, high localized concentration might be necessary, e.g. if injected into a tumor. Long term environmental exposure, on the other hand, requires assessment at much lower concentrations. Often, there needs to be a compromise between available analytical methods and the relevance of the concentration. Furthermore, during the formation of hypotheses and method development, concentrations might be chosen out of convenience rather than their expected exposure levels to an organism.

Compared to paper I, the conditions in papers II and III deal with interactions of nanostructures at the lowest end of the concentration scale. In paper II, the Au NP concentration of 2.8 μg mL^{-1} equals 58 pM which is anywhere between 2-200 fold lower than the concentrations found when studying the protein corona. Working in this low concentration comes with major challenges. The protein concentration in serum is between 40-70 mg mL^{-1}. After dilution of serum to 10% in CCM, the protein concentration is roughly 2,000 fold higher than the Au NP concentration.

Rather than choosing a larger NP or higher concentration, we chose a different approach. Compared to the biomolecules in their environment, two major physicochemical properties of Au NPs are different. First, their density. Gold has a density of 19.3 g cm^{-3} while proteins have a density of 1.37 g cm^{-3} and lipids and lipoproteins even lower. By only applying DLS, it would be very difficult to distinguish between two Au NPs in a small aggregate and a single Au NP with an adsorbed protein corona. However, due to the huge difference in the mass of a small aggregate and a NP-biomolecule complex, they sediment very differently. By combining DLS and DCS, we can tell if an increase in size is due to aggregation or adsorption. In certain materials, the unique properties of nanostructures can be exploited when studying their interactions with biological environment. One of these unique properties is the localized surface plasmon resonance of noble metal nanoparticles like gold and silver. Surface sensitive properties of the Au NPs are of course ideal for this kind of study since it is very fast and requires only a spectrophotometer and reveals the surface conditions of the NPs.

In paper III, even lower concentrations were used. These concentration limitations ruled out the possibility to use many conventional methods. By applying electron and X-ray based techniques, it is possible to obtain image resolution down to the scale of single NWs and proteins. At the single molecule scale, it is difficult to image the detailed features of proteins and to distinguish between optical artefacts and proteins. However, by increasing the resolution, important information about the adsorbed protein layer's topography can be obtained that we do not see by other methods. In

order to confirm the presence of proteins on the NW surface we used X-ray based chemical detection methods. We studied both laminin, a very large extracellular matrix protein, with dimensions up to 90 nm, as well as the smaller albumin, and a protein mixture of blood plasma. We were able to image the protein layer to establish protein binding, and to measure the protein layer thickness.

6.2.3 Studying novel nanomaterials

With rapid advances in NW synthesis, we were able to obtain much more material for the work done in paper IV compared to paper III. Close collaboration between producers and bionanoscience is very important to enable nanosafety assessments to keep up with the rapid developments in nanomaterial fabrication.

The NWs used in papers III and IV differ in two important ways. First, the NWs in paper III were grown vertically on a flat substrate which made their handling somewhat easier. Using immobilized NWs, it was possible to coat them with protein, wash away unbound protein, and break the NWs off the substrate before the sample was put on the electron microscope grid. This also eliminated the possibility of aggregation of NWs during the binding and washing steps. In paper IV, the NWs were produced by aerotaxy, a novel method where the NWs are grown from an aerosol seed particle. The seed particle can be seen at the top of the NWs in the cryo-TEM images. NWs produced by this method are free, i.e. they are not attached to any substrate, and require delicate handling. A second important difference is the material of the NWs. In paper III, the NWs were made of GaP, while in paper IV they were made of GaAs. Since GaAs is soluble in aqueous environment, the NWs were stored in ethanol until their use.

Due to the low material availability, it was necessary to study the NWs in paper III on a single NW basis. This was done by cryo-TEM. Cryo-TEM offers the huge benefit that proteins are imaged in their hydrated state. However, the biological material does not withstand being bombarded with electrons and it does not scatter them very well. We could, however, see a layer of biological material on the NWs. The presence of biological material was confirmed by X-ray based, chemical sensitive methods. The protein layer was larger for laminin than for albumin. This fits very well with their hydrodynamic sizes. However, the sparse, outermost part of the protein layer is not visible using only cryo-TEM and unlabeled protein.

Preliminary experiments showed that by labelling the protein corona with gold NPs, we could better determine its thickness. Figure 11 shows regular transmission electron microscopy images of NWs incubated with gold NPs on the left. On the right, the NWs were incubated with laminin before adding gold NPs.

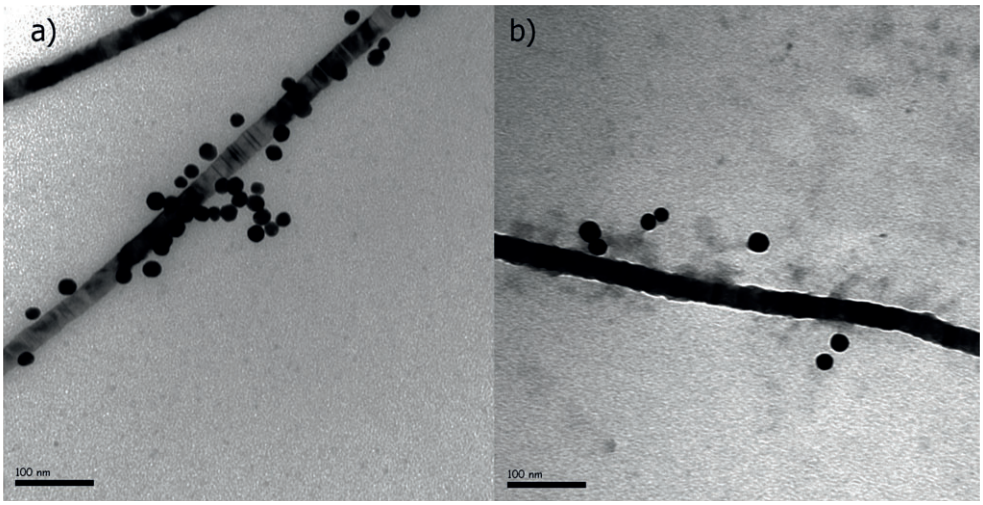

Figure 11. Transmission electron microscopy images of a) NWs incubated with 20 nm Au NPs. b) NWs incubated with laminin before exposure to Au NPs.

Comparing the distance between the NW and Au NPs in figure 11, the difference is presumably due to laminin preventing the Au NPs from binding directly to the NW surface. Two important aspects need to be considered, looking at this figure. First, the sample is dried to withstand the high vacuum in the sample chamber. During the drying process, we do not have any control over how the sample behaves. Second, we only see the picture in two dimensions and some Au NPs might be further away from the NW surface than they appear.

Given the advances in material availability and installation of a new electron microscope, we revisited some of the remaining questions from paper III in paper IV. We imaged laminin and the enzyme myeloperoxidase on nanowires, with and without gold labelling. In order to measure the corona thickness, we rotated NWs with gold labelled laminin corona by 120° and imaged the NW every 15° which greatly improved the accuracy of the measurement and made it possible to distinguish between bound and unbound Au NPs (figure 12). It also revealed some interesting information about the corona topography that is not revealed by other methods. The presence of two distinct distances between the NW and the Au NPs used to label the corona show that the corona is quite irregular.

Furthermore, we could apply more robust and less expensive methods like DCS to confirm the binding and get a relative estimate of the protein corona thickness formed by a series of proteins. According to the sedimentation behavior, blood serum forms a corona similar in thickness to IgG. The largest dimension of IgG is approximately 14 nm. This is smaller than the estimated protein corona formed in plasma in paper III, which was estimated to be between 20-30 nm. The reason for this difference can be

due to the presence of fibrinogen, a large fibrillar protein in plasma which is mostly depleted in serum, or simply due to an overestimation in paper III. Mass spectrometry analysis of the protein corona on the GaAs NWs showed the presence of lipoproteins, complement system factors, and hemoglobin, for example. The corona is therefore composed of a variety of proteins and other biomolecules of different sizes. These variations could very well be studied by a method similar to the one applied on laminin topology.

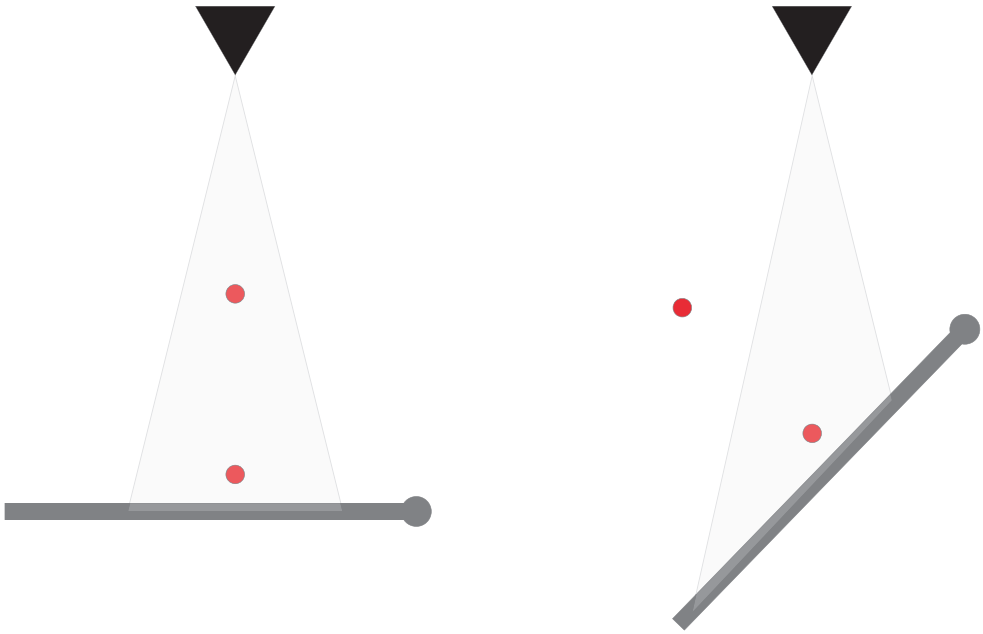

Figure 12. Illustration of transmission electron microscopy of a nanowire (gray matchstick) and two Au NPs (red). As the image stage is rotated, the Au NP further away from the NW moves out of the imaged plane while the Au NP closer to the NW remains in the image.

6.2.4 Protein function

As described in the introduction to protein function on surfaces, it is difficult to predict the effect of adsorption on the protein's function. Using enzymes, this effect can be quantified, even if it is still challenging to explain. In paper IV, one of the proteins studied was the enzyme myeloperoxidase (MPO) and the NWs' effect on its activity. First, we needed a way to establish that MPO actually binds to the NWs and if it remains active after binding. Having just finished the work on paper I, inspired by nanostructures suspended in different layers of a sucrose gradient, I made a simple system discussed in 5.1.3 (figure 2).

When MPO is mixed with NWs, there are three possibilities. The first one is that there is no binding, the second that there is binding and inactivation, and the third that the enzyme binds in an active state. If the enzyme binds in an active state, the intersect between the buffer region and the bottom layer turns blue. The system worked very well and MPO proved to be active on the NWs. An activity assay had already shown that adding MPO and NWs in 1:2 weight ratio reduced the activity by approximately 20%. It was therefore clear that MPO interacts with the NWs and the interaction affects its activity. Next, we tried to explain why.

Upon adsorption, the enzyme might undergo structural changes. Using intrinsic fluorescence, we probed for changes in the environment of the enzyme's tryptophan residues without finding any major difference. Another possibility is that the movement of the enzyme and substrate is somehow restricted, perhaps by the slower diffusion of MPO when it is bound to the large NWs. It is also possible that at least one of the two active sites of MPO is partially or completely blocked. For NWs of 100 nm diameter and 2 μm length, the available surface area for each MPO molecule in a 1:2 MPO and NW concentration ratio, would be approximately 4 nm^2. This is of course much lower than the area needed for all MPO molecules to bind. Therefore, not all enzyme molecules have lost 20% of their activity, and it is more likely that the activity of the adsorbed molecules is reduced significantly. An interesting observation during the activity measurements was that upon addition of cow serum to the NW-MPO complex, the activity was restored and was even higher than the control sample.

The effect of NWs on MPO activity was built on earlier work presented in paper V. There, we studied the effect of TiO_2 and PS NPs of different sizes and surface functionalization on the activity of MPO. The environmental conditions studied can be ranked according to their complexity. The simplest conditions consisted of purified MPO and NPs in PBS. The intermediate conditions were purified MPO and NPs in the presence of bovine serum albumin. Of higher complexity was neutrophil secreted MPO in protein poor cell culture medium (CCM), and the highest complexity was in serum supplemented CCM.

Under the simplest conditions, a concentration of 0.02 mg mL^{-1} of 220 nm negatively charged PS NPs increased the activity of MPO by 80%. At this concentration, the available surface area for each MPO molecule is 13 nm^2 which is not sufficient for all molecules to bind. However, increasing the concentration five-fold, available surface area for each molecule is 65 nm^2 which should provide enough surface for all MPO molecules. Interestingly, it did not affect the activity and it remained 80% higher than the control. The most impact PS had on MPO was observed when BSA in a concentration of 1 mg mL^{-1} was added to MPO before mixing with negatively charged 60 nm PS NPs. The activity almost tripled, while in the absence of NPs the addition of BSA did not affect the activity.

In chapter 4.4.2, the problem facing the field of enzyme immobilization on nanosurfaces was presented. Increased enzymatic activity is common following immobilization. Under the controlled conditions of a mixture of NPs, enzyme, and its

substrate, there is still no explanation, only hypotheses. In a designed NP-enzyme construct, the enzyme's orientation is somewhat controlled. In our case, the enzyme adsorbs in an uncontrolled manner, which adds one more level of complexity for interpreting the system's behavior. Adding the biomolecular cocktail of blood serum increases the complexity even further. This brings us to the protein corona.

6.2.5 The importance of the protein corona

In paper I we studied the aggregation of TiO_2 NPs in serum. The protein to NP ratio determined both the aggregation state and biomolecular content of different aggregate size populations. On the other hand, in paper V we studied the effect of TiO_2 NPs on MPO activity, both in protein poor and protein rich environment. It is therefore likely that the protein rich environment, where the TiO_2 NPs aggregate, will contribute to the NPs' effect on MPO.

To determine the impact of the corona on the total effect of TiO_2 NPs, we start by looking at the simplest system. In a system of TiO_2 NPs and purified MPO, NP concentration of 0.02 mg mL^{-1} reduced the activity of MPO by more than 90%. At a higher concentration, TiO_2 NPs reduce the activity less due to aggregation. Looking at the more complicated system, their effect on neutrophil secreted MPO in protein poor CCM was similar to polystyrene NPs. Interestingly, they had no effect on MPO activity in the protein rich CCM. This shows that not only is the protein corona important for the fate of a NP in an organism, the presence of a variety of proteins in the corona can also change its effect on biological systems.

Similarly, for PS NPs, all particles reduced the activity at 0.1 mg mL^{-1} in protein poor CCM. In the absence of proteins, negatively charged PS NPs have more effect than positively charged. Due to MPO's positive charge, this seems reasonable. However, upon addition of serum, positively charged NPs have more effect on the activity. Assessing the effect of nanostructures on biological systems is obviously a complicated matter. In order to make conditions biologically relevant, variables are added to the system that increase the probability of unexpected interactions. The experimental design and data interpretation therefore require careful consideration.

6.3 Concluding remarks and future perspectives

Working at the nanoscale is both fascinating and challenging. Implementing the huge potential of these materials in a responsible way requires a dynamic interdisciplinary approach. Previous technological advances provide warnings, e.g. the tragic use of asbestos as a building material. More recent examples include genetically modified foods and organisms, where the public discussion is sometimes lead by people with non-professional qualifications. It is therefore important for the scientific community to make sure that the application of nanotechnology is done in a responsible way and in constant harmony and dialogue with nature and society.

The impact of nanomaterials needs assessment from the molecular up to large scale, ecotoxicological studies. The tools of physics allow us to see these interactions with constantly improving resolution. Using the molecular insight of biochemistry, we can describe the behavior increased resolution shows us. When we can describe what we see, we can better explain the cell's response to it. For that we need cellular biology to quantify the toxicological impact. Finally, we need to move from the test tubes to food web models. This requires a huge effort and a constant conversation between the involved researchers.

Considering my link in this long chain, there are some answers but also many questions. First of all, due to the broadness of the field, where each study has its own interest, there appear to be gaps between sub-fields. In order to obtain sufficient material, biomolecular corona studies tend to use high concentrations of nanomaterials. The effect of varying concentration needs to be studied in order to apply those results to cell studies using much lower concentrations. An obvious first step would be to see if the different complexes formed in paper I had different toxicological response in *in vitro* assays. Of particular interest would be the smaller complexes formed. By changing the NP to serum ratio, the smaller complexes showed great variation in biomolecular content. Comparing the same fractions (i.e. populations of same size but different biomolecular content) from two different concentration ratios could then show if the size is more important than biomolecular content. On a similar note we can consider aggregation. Aggregation depends on the concentration of nanostructures and the biomolecules. Still, this seems to be largely dismissed and often reported as a single number of nanoparticle size after mixing with the biomolecular source of interest.

The third important aspect is the protein corona. Its composition is increasingly well reported. However, looking at its effect on enzyme activity indicates that this environment is very much alive. Enzymatic activity is easily quantifiable. Other protein functions are more difficult to measure but could be affected in similar ways at this poorly understood interface. Better understanding of this environment has great benefits in nanosafety, nanotherapeutics, and biotechnology. Tuning the catalytic efficiency and protein stability can also reveal physicochemical properties that can determine possible biological impact of the material. In paper V we added BSA to MPO

and PS NPs. This activity tripled. What is the role of BSA in this? Is it a general effect irrespective of the protein or do the properties of the added protein play a role? In the absence of proteins, the biomolecular corona is not very well described. In cell culture medium, amino acids adsorb on the NPs. Are there more biomolecules that are more difficult to detect?

Papers III and IV deal, in part, with imaging single nanostructures with their adsorbed coronas. Following the success of protein structures solved by tomographic cryo-TEM, the protein corona could very well be imaged directly without labelling. In order to obtain a three dimensional structure of a protein, thousands of images are averaged. This would of course cause a problem in a heterogeneous protein corona as proteins of different sizes and topologies might fail to show irregularities in the corona. Combining these images with gold labelling, both directly on the adsorbed proteins and by gold labelled antibodies against proteins of interest, will provide a more detailed picture than presented in paper IV.

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Printed by Media-Tryck, Lund 2018 NORDIC SWAN ECOLABEL 3041 0903

ISBN: 978-91-7422-592-1

Biochemistry and Structural Biology
Faculty of Science
Lund University