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# Polypeptide-Based Nanoscale Materials

Daniel Aili



**Linköping University**  
**INSTITUTE OF TECHNOLOGY**

Division of Sensor Science and Molecular Physics  
Department of Physics, Chemistry and Biology  
Linköping University, Sweden

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Cover, front: (left) electron micrograph showing fibres composed of hetero-associated polypeptide fibre-forming units, (middle) a folded four-helix bundle (PDB entry 1u7j), and (right) polypeptide-decorated gold nanoparticles. Back: (left) ring structure composed of peptide fibres, (middle) polypeptide-decorated gold nanoparticles, and (right) polypeptide fibre.

During the course of the research underlying this thesis, Daniel Aili was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.

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*To Siri*



## Abstract

Self-assembly has emerged as a promising technique for fabrication of novel hybrid materials and nanostructures. The work presented in this thesis has been focused on developing nanoscale materials based on synthetic *de novo* designed polypeptides. The polypeptides have been utilized for the assembly of gold nanoparticles, fibrous nanostructures, and for sensing applications.

The 42-residue polypeptides are designed to fold into helix-loop-helix motifs and dimerize to form four-helix bundles. Folding is primarily driven by the formation of a hydrophobic core made up by the hydrophobic faces of the amphiphilic helices. The peptides have either a negative or positive net charge at neutral pH, depending on the relative abundance of Glu and Lys. Charge repulsion thus prevents homodimerization at pH 7 while promoting heterodimerization through the formation of stabilising salt bridges. A Cys incorporated in position 22, located in the loop region, allowed for directed, thiol-dependent, immobilization on planar gold surfaces and gold nanoparticles. The negatively charged (Glu-rich) peptide formed homodimers and folded in solution at pH < 6 or in the presence of certain metal ions, such as Zn<sup>2+</sup>. The folding properties of this peptide were retained when immobilized directly on gold, which enabled reversible assembly of gold nanoparticles resulting in aggregates with well-defined interparticle separations. Particle aggregation was found to induce folding of the immobilized peptides but folding could also be utilized to induce aggregation of the particles by exploiting the highly specific interactions involved in both homodimerization and hetero-association. The possibility to control the assembly of polypeptide-functionalized gold nanoparticles was utilized in a colorimetric protein assay. Analyte binding to immobilized ligands prevented the formation of dense particle aggregates when subjecting the particles to conditions normally causing extensive aggregation. Analyte binding could hence easily be distinguished by the naked eye. Moreover, the peptides were utilized to assemble gold nanoparticles on planar gold and silica substrates.

Fibrous nanostructures were realized by linking monomers through a disulphide-bridge. The disulphide-linked peptides were found to spontaneously assemble into long and extremely thin peptide fibres as a result of a propagating association mediated by folding into four-helix bundles.



## Populärvetenskaplig sammanfattning

Ingenjörer och vetenskapsmän har ofta inspirerats av naturen i sökandet efter lösningar på tekniska problem. Allt ifrån byggnadskonstruktioner, flygplansvingar, kompositmaterial till kardborrebandet har skapats med utgångspunkt från förebilder i naturen. Många av de material och konstruktioner som återfinns i naturen har åtråvärda egenskaper som är svåra att erhålla i syntetiska material med traditionell teknik. Även om vi i flera fall kan härma sammansättningen och formen blir resultatet inte nödvändigtvis det samma. Den största skillnaden mellan syntetiska material och material producerade av levande organismer är hur deras komponenter sinsemellan är organiserade och sammansatta. I syntetiska material är komponenterna ofta inbördes mer eller mindre slumpvis ordnade medan de i biologiska material är organiserade med en oerhörd precision som sträcker sig ända ned på molekyl- och atomnivå. Naturens byggstenar har genom evolutionens gång förfinats för att spontant kunna organisera sig och bilda komplexa material och strukturer. Denna process, som styrs genom att många svaga krafter inom och mellan byggstenarna samverkar, kallas ofta för självorganisering och är en förutsättning för allt liv. Självorganisering har också blivit en allt viktigare metod inom nanotekniken för att konstruera material och strukturer med nanometerprecision.

I den här avhandlingen beskrivs en typ av självorganiserande material där byggstenarna utgörs av nanometerstora guldpartiklar och syntetiska proteiner. De syntetiska proteinerna är designade för att efterlikna naturliga biomolekyler och antar en välbestämd tredimensionell struktur när två av dem interagerar med varandra. Denna interaktion är mycket specifik men kan styras genom att variera kemiska parametrar som surhet och jonstyrka vilket ger en möjlighet att påverka och kontrollera proteinernas struktur. Proteinerna har vidare modifierats för att spontant organisera sig till fibrer som är flera mikrometer långa men endast några nanometer tjocka. Proteinfibrer utgör en mycket viktig typ av strukturer i biologiska system och finns i alltifrån spindelväv till muskler. Syntetiska proteinfibrer är därför både ett intressant modellsystem och ett material med många potentiellt intressanta användningsområden.

Genom att fästa de syntetiska proteinerna på ytan av guldnanopartiklar går interaktionerna mellan partiklarna att kontrollera på samma sätt som interaktionerna mellan proteinerna. Krafterna mellan proteinerna och interaktionerna

involverade i proteinernas veckning har använts för att reversibelt aggregera och organisera nanopartiklarna. Ett antal olika byggstenar har studerats och utvecklats till något som liknar ett mycket enkelt nano-Lego, som på en given signal spontant bygger ihop sig eller trillar isär.

Guldnanopartiklar är intressanta eftersom de är stabila och lätta att modifiera kemiskt men också på grund av deras optiska egenskaper som ger dem en ovanligt vacker vinröd färg. Färgen uppstår på grund av partiklarnas ringa storlek och varierar naturligt med egenskaperna hos den omgivande miljön. Detta gör det enkelt att studera hur partiklarna interagerar eftersom de byter färg när de närmar sig varandra, men gör dem också intressanta för sensortillämpningar. En enkel och robust sensor beskrivs i avhandlingen där syntetiska proteiner, speciellt utformade för att upptäcka och binda andra molekyler, har fästs på nanopartiklarna. Med partiklarnas hjälp går det att med blotta ögat detektera ett mänskligt protein i koncentrationer under ett tusendels gram per liter. En tidig diagnos av sjukdomstillstånd kan i de flesta fall avsevärt underlätta behandlingen och behovet av enkla sensorer för att bestämma närvaro och koncentration av medicinskt intressanta molekyler är därför mycket stort.

## List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-VII).

- I** K. Enander, D. Aili, L. Baltzer, I. Lundström, B. Liedberg, "Alpha-Helix-Inducing Dimerization of Synthetic Polypeptide Scaffolds on Gold", *Langmuir*, **2005**, 21, 2480-2487.
- II** D. Aili, K. Enander, J. Rydberg, I. Lundström, L. Baltzer, B. Liedberg, "Aggregation-Induced Folding of a De Novo Designed Polypeptide Immobilized on Gold Nanoparticles", *J. Am. Chem. Soc.*, **2006**, 128, 2194-2195.
- III** D. Aili, K. Enander, I. Nesterenko, F. Björefors, L. Baltzer, B. Liedberg, "Folding Induced Assembly of Polypeptide Decorated Gold Nanoparticles", *J. Am. Chem. Soc.*, **2008**, 130, 5780-5788.
- IV** D. Aili, H. S. Han, K. Enander, L. Baltzer, B. Liedberg, "Controlled Assembly of Gold Nanoparticles using De Novo Designed Polypeptide Scaffolds", *Proc. SPIE*, **2008**, 6885, 688506-1-688506-8.
- V** D. Aili, F.-I. Tai, K. Enander, L. Baltzer, B. Liedberg, "Self-assembling Fibers and Nano-Rings from Disulphide-Linked Helix-Loop-Helix Polypeptides", *Angew. Chem., Int. Ed.*, **2008**, 47, 5554-5556.
- VI** D. Aili, K. Enander, L. Baltzer, B. Liedberg, "Assembly of Polypeptide Decorated Gold Nanoparticles through a Hetero-Association and Folding Dependent Bridging", *Nano Lett.*, **2008**, 8, 2473-2478.
- VII** D. Aili, R. Selegård, L. Baltzer, K. Enander, B. Liedberg, "Colorimetric Protein Sensing by Controlled Assembly of Gold Nanoparticles Functionalized with Synthetic Receptors", *In manuscript*.

## Contribution report

Paper I: D.A. and K.E. worked together with planning, performing and evaluating CD and SPR experiments. K.E. did all peptide synthesis. D.A. was responsible for all IR and ellipsometry measurements. K.E. did a major part of the writing.

Paper II: D.A. was responsible for planning, performance, and evaluation of experiments; except for peptide synthesis which was conducted by K.E. D.A. was responsible for a major part of the writing.

Paper III: D.A. was responsible for planning, and evaluation of experiments. Evaluation of impedance data was performed in collaboration with F.B. D.A. did most of the experimental work and a major part of the writing. Initial experiments on cation-induced folding were performed by J.R. Experiments on aggregation reversibility were performed by I.N.

Paper IV: D.A. and S.H. worked together with performing the experiments. D.A. did most of the planning, evaluation and most of the writing.

Paper V: D.A. was responsible for planning, performance, and evaluation of experiments. AFM was performed by F.-I.T. D.A. did most of the writing.

Paper VI: D.A. was responsible for planning, performance, and evaluation of experiments. D.A. did most of the writing.

Paper VII: D.A. was responsible for planning, and evaluation of the experiments. R.S. did the measurements on C-pTMVP, and K.E. expressed and purified the antibody fragment. D.A. did the experiments on HCAII and most of the writing.

The peptides utilized throughout this thesis were originally designed by Johan Rydberg, Sarojini Vijayalekshmi, and Karin Enander and are the result of many years of research in the group of Professor Lars Baltzer.

## Papers not included in the thesis

- VIII** D. Aili, K. Enander, L. Baltzer, B. Liedberg, "Synthetic de novo designed polypeptides for control of nanoparticle assembly and biosensing", *Biochem. Soc. Trans.*, **2007**, 35, 532-534.
- IX** F. Nayeri , J. Xu , A. Abdiu , T. Nayeri , D. Aili , B. Liedberg , U. Carlsson, "Autocrine production of biologically active hepatocyte growth factor (HGF) by injured human skin", *J. Derm. Sci.*, **2006**, 43, 49-56.
- X** C. Andréßen, S. Jalal, D. Aili, Y. Wang, S. Islam, A. Jarl, B. Liedberg, L.-G. Mårtensson, B. Wretlind, M. Sunnerhagen, "Biochemical and biophysical analysis of multidrug resistant mutations in the *Pseudomonas aeruginosa* efflux gene regulator MexR suggest that the intrinsic dynamics of MexR subdomain interactions is critical for specific gene regulation", *Submitted to Biochemistry*.
- XI** F. Nayeri, D. Aili, T. Nayeri, J. Xu, S. Almer, I. Lundström, B. Åkerlind, B. Liedberg, "Hepatocyte growth factor (HGF) in fecal samples: rapid detection by surface plasmon resonance", *BMC Gastroenterology*, **2005**, 5, 13.
- XII** F. Nayeri, T. Nayeri, D. Aili, L. Brudin, B. Liedberg, "The clinical impact of real-time evaluation of biological activity and degradation of hepatocyte growth factor", *Growth Factors*, **2008**, 26, 163-171.
- XIII** J. Wetterö, T. Hellerstedt, P. Nygren, K. Broo, D. Aili, B. Liedberg, K.-E. Magnusson, "Immobilized chemoattractant peptides mediate adhesion and distinct calcium-dependent cell signaling in human neutrophils", *Langmuir*, **2008**, 24, 6803-6811.

## Other contributions

D. Aili, A. Larsson, B. Liedberg, "Attana Technical note - Sensor Surface for Immobilization of Biotinylated Ligands", [www.attana.com](http://www.attana.com), **2004**.

## Conference contributions

D. Aili, K. Enander, J. Rydberg, I. Lundström, L. Baltzer, B. Liedberg, POSTER: "Immobilization and heterodimerization of helix-loop-helix polypeptides on gold surfaces – a model system for peptide-surface interactions" *First World Congress on Synthetic Receptors*, **2003**, Lisbon, Portugal.

D. Aili, K. Enander, J. Rydberg, I. Lundström, L. Baltzer, B. Liedberg, POSTER: "Alpha helix-inducing dimerization of synthetic polypeptide scaffolds on gold - a model system for receptor mimicking and biosensing" *The Eight World Congress on Biosensors*, **2004**, Granada, Spain.

D. Aili, K. Enander, J. Rydberg, I. Lundström, L. Baltzer, B. Liedberg, POSTER: "Folding-induced aggregation of polypeptide-decorated gold nanoparticles – a nano-scale Lego for the construction of complex hybrid materials" *The 5th International Conference on Biological Physics – ICBP*, **2004**, Gothenburg, Sweden.

D. Aili, K. Enander, I. Lundström, L. Baltzer, B. Liedberg, POSTER: "Towards novel functional materials and sensors using de novo designed polypeptides on gold nanoparticles" *Europt(r)ode VIII*, **2006**, Tübingen, Germany.

D. Aili, K. Enander, L. Baltzer, B. Liedberg, ORAL COMMUNICATION: "Synthetic de novo designed polypeptides for control of nanoparticle assembly and biosensing" *Bionanotechnology: from self-assembly to cell biology*, **2007**, Cambridge, UK.

D. Aili, K. Enander, L. Baltzer, B. Liedberg, POSTER: "Complex gold nanoparticle- and polypeptide based supramolecular hybrid materials", *Supramolecules & Assemblies, Chemistry of Functional Materials Through Bottom-Up Self-Assembly*, **2007**, Lucca, Italy.

## Abbreviations

AFM	Atomic Force Microscopy
CA	Carbonic Anhydrase
CD	Circular Dichroism
EDC	EthylDimethylaminopropylCarbodiimide
Fcc	face centered cubic
Fmoc	Flourenylmethyloxycarbonyl
FT-IR	Fourier Transform Infrared Spectroscopy
HCAII	Human Carbonic Anhydrase II
HPLC	High Performance Liquid Chromatography
LSPR	Localized Surface Plasmon Resonance
MPC	Monolayer Protected Cluster
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
mrw	mean residue weight
NHS	N-HydroxySuccinimide
NMR	Nuclear Magnetic Resonance
IRAS	Infrared Reflection Absorption Spectroscopy
PDEA	2-(2-PyridinylDithio)-EthylAmine
SAM	Self-Assembled Monolayer
SEM	Scanning Electron Microscopy
SPR	Surface Plasmon Resonance
STEM	Scanning Transmission Electron Microscopy
TEM	Transmission Electron Microscopy
UV-vis	Ultra Violet - visible

## Amino Acids

The three- and one-letter code of the twenty common amino acids.

<b>Amino Acid</b>	<b>Three-letter abbreviation</b>	<b>One-letter abbreviation</b>
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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## Preface

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The world is so much larger than we can comprehend, especially without a good microscope! Beyond the limits of our perception, there exists a micro-cosmos so different from our macroscopic world, yet so integrated into our reality. In the nano-regime, the true wonders of life take place and it is here where atoms assemble into molecules and molecules into living organisms. This thesis, on polypeptide-based nanoscale materials, intends to give a tiny glimpse into this fascinating world, and is an attempt to understand and to explain some of the underlying mechanisms and phenomena taking place there.

The work presented in this thesis has been carried out during approximately five years of studies at the Department of Physics, Chemistry and Biology (IFM) at Linköping University, from August 2003 to October 2008, and would not have been possible if it was not for the creative and friendly environment at IFM and at the division of Sensor Science and Molecular Physics. I am thus indebted to a great number of people for a great number of reasons: I would like to acknowledge and extend my heartfelt gratitude to my supervisors Professor Bo Liedberg and Dr Karin Enander who have kept me on track throughout the whole of this journey. Although they have given me the freedom to sometimes find my own way and get lost in the landscape of Science they have always been around for guidance and for sharing their great knowledge and brilliant ideas. I would also like to acknowledge Professor Lars Baltzer and Dr Johan Rydberg for giving me the opportunity to work with the JR-polypeptides, to Hsu Shu Han for being

patient enough to let me supervise her during her diploma work, Irina Nesterenko for all the hard work on peptide structures and gold nanoparticles, Robert Selegård for helping me with Paper VII, Feng-I Tai for the great work on peptide fibres, Daniel Kanmert for all the fruitful discussions on peptides and peptide fibres, Per Persson for helping me with the STEM, Per Björk, Jens Wiggenius, and Maihar Hamedi for the interesting work on conjugated polymers. I am also very grateful for the collaborations with Fariba and Tayeb Nayeri, Maria Sunnerhagen and Cecilia Andréson, and Henrik Andersson, which have given me the opportunity to widen my perspectives in many ways.

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Finally, I would like to thank my parents, my sister and brother for supporting, encouraging and believing in me, my grandfather for sharing his wisdom and reminding me about the privilege of receiving a good education and the importance of the progress of science (and for all the good fishing tips), and Siri for all the patience and love.

Linköping, August 2008

Daniel Aili

*“There’s plenty of room at the bottom”* Feynman, 1959.<sup>[1]</sup>

## 1. Introduction

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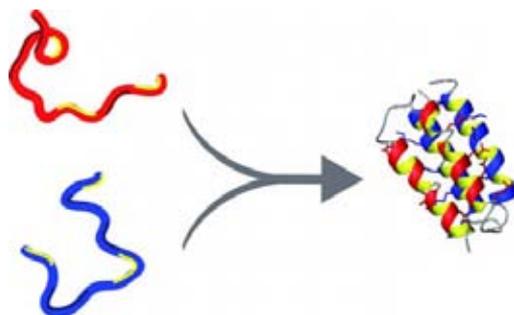
“There’s plenty of room at the bottom” was the title of a seminar talk given by Richard Feynman in 1959 where he first proposed the general ideas of nanotechnology – to manipulate individual atoms and molecules in order to construct materials and devices with nanoscale dimensions.<sup>[1]</sup> In this after dinner talk, Feynman proposed work in a new field of science “in which little has been done but an enormous amount can be done in principle”.<sup>[1]</sup> Several new avenues for research was pointed out that later came to define nanotechnology, such as making computers smaller and therefore faster and making “mechanical surgeons” that could travel to trouble spots in the body. Although the concepts of modern nanoscience was first described by Feynman, the term *nanotechnology* was not introduced until 15 years later, when Norio Taniguchi of the Tokyo University of Science suggested it to describe a technology that strives for precision at the level of about one nanometre.<sup>[2]</sup> About one decade later, in 1986, the implications and ideas of nanotechnology was brought to the general public by Eric Drexler in his controversial novel “Engines of Creation”.<sup>[3]</sup> Nanotechnology is nowadays an established field of science that is truly interdisciplinary and encompasses areas within chemistry, medicine, physics, biology, electronics and material science.

By definition, nanotechnology refers to the rational design of materials and devices with dimensions at the nano-scale. One nanometre is a millionth of a millimetre, which corresponds to the distance a standard beard in a standard face grows in 0.1 seconds.<sup>[4]</sup> By the time the razor has passed over the cheek, the

beard has in other words already grown ten nanometres. On this unimaginably small length scale the properties of materials are very different from what is seen in the macroscopic world. Nanomaterials can be stronger, lighter, conduct electricity and heat in a different way, and possess very different optical properties as compared to the corresponding bulk materials. Nanomaterials can also be assembled in completely new ways, exploiting the same principles for fabrication as is utilized by living organisms. These assembly strategies allow ordering of building blocks into complex functional architectures and devices with sub-nanometre precision. As the most complex nanostructures can be found in Nature, it is not strange that life itself, and all the amazing processes going on at the molecular and cellular levels of living organisms, has been a source of inspiration for many scientists working within this field.

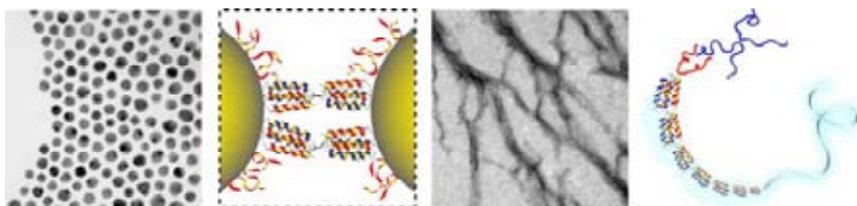
The work presented in this thesis has been focused on self-assembly processes of nanoscale objects exploiting specific biomolecular interactions, such as polypeptide folding. The constituents are however entirely synthetic although partly inspired by naturally occurring nanostructures. The aim has been to develop a kit of functional building blocks that allow flexible, controlled, and reversible assembly of nanoscale architectures, and to study their properties in order to elucidate more about the design rules for self-assembling systems. The term *functional building blocks*, refers to their ability to respond in a predictable way to changes in the physiochemical environment which can be utilized both as a trigger for assembly and disassembly as well as for sensor applications.

The common denominator of the included papers is a set of amphiphilic *de novo* designed synthetic helix-loop-helix polypeptides. The so called JR-polypeptides are 42-mer helix-loop-helix polypeptides that were designed to fold into four-helix bundles upon dimerization.<sup>[5]</sup> The JR-polypeptides are rich in either Glu (JR2E) or Lys (JR2K), which render them a high negative or positive net charge, respectively. Charge repulsion prevents homodimerization at neutral pH while promoting heterodimerization through the formation of stabilizing salt bridges. As monomers the polypeptides are random coil but they fold into four-helix bundles upon dimerization (Figure 1.1). Homodimerization can be induced at low and high pH, respectively, as well as at high salt concentrations or in the presence of certain metal ions.



**Figure 1.1** JR2E (red) and JR2K (blue) are random coil monomers at neutral pH but fold into four-helix bundles upon dimerization.

The JR-polypeptides thus offer a certain control over the state of folding and comprise a robust model system and a versatile instrument for the design of novel self-assembling supramolecular constructs and nanoscale materials. The polypeptides have been utilized for assembly of gold nanoparticles, fibrous nanostructures, and for sensing applications (Figure 1.2).



**Figure 1.2** The polypeptides have been utilized for controlled and reversible assembly of gold nanoparticles and fibrous nanostructures.

The thesis is divided into two parts, where the aim of the first part is to give an introduction and a brief overview of the subjects relating to the thesis work. The second part of the thesis is devoted to the obtained results, which are presented and discussed in seven papers. The papers are briefly summarized in chapter 9.

Molecular self-assembly is very likely to become an important methodology for nanofabrication. Building with molecules, however, requires a deep understanding of their individual structure, assembly properties and dynamic behaviour; questions that are addressed in this thesis.



*“The objectives of self-assembly are to make structures that cannot be made by other means and to understand one aspect of life.” Whitesides, 2002.<sup>[6]</sup>*

## **2. Self-Assembly and Self-Organization**

---

The terminology of “Self-Assembly” and “Self-Organization” are not really clear-cut and the two words are often used interchangeably and sometimes in association. Whitesides defines self-assembly as “the autonomous organization of components into patterns or structures without human intervention”.<sup>[6]</sup> He further divides the process of self-assembly into two groups; static and dynamic self-assembly. Static self-assembly occurs as a result of free energy minimization in a closed system and leads to an equilibrium state, whereas dynamic self-assembly occurs far from equilibrium and as a result of energy dissipation. This definition embraces a wide variety of processes covering the whole length scale from nano to parsec but is limited to such interactions that involve pre-existing components (separate or distinct parts of a disordered structure), are reversible, and can be controlled by proper design of the components.<sup>[6, 7]</sup> Lehn, on the other hand, regards self-assembly “as simple collection and aggregation of components into a confined entity”<sup>[8]</sup> and distinguish it from self-organization which he proposes is “the spontaneous but information-directed generation of organized functional structures in equilibrium conditions”<sup>[8]</sup>. The information that directs the molecular species into ordered structures is encoded in the covalent framework of the involved molecules and the organization occurs as a result of specific supramolecular interactions. Kirschner et al. consider self-organization from a biological perspective, as an extension of self-assembly<sup>[9]</sup> where self-organization in contrast to self-assembly “gives structures under a wider set of conditions; the

rules tend to be more general and the structures more variable”.<sup>[10]</sup> Kirschner et al. further concludes that self-organizing systems are characterized by reaching a steady state, where there is continuous energy consumption and gain and loss of material.<sup>[10]</sup> This view on self-organization is similar to Whitesides definition of dynamic self-assembly and both definitions also allude to the processes responsible for the assembly, and survival, of all living organisms.

Despite some discrepancies in definitions and terminology, there is a consensus in that self-assembly and self-organization are the perhaps only, or at least the most practical, strategies for making ensembles of nanostructures.<sup>[11, 12]</sup>

## 2.1 Supramolecular chemistry

Assembly of organic nanostructures and hybrid materials most often rely on non-covalent molecular interactions. This area of chemistry, focused on the association of molecular species that are held together solely by intermolecular forces, is called *supramolecular chemistry* and is often referred to as “the chemistry beyond the molecule”.<sup>[8]</sup> Whereas traditional chemistry focuses on the covalent bond, supramolecular chemistry exploits the weaker and reversible interactions between molecules such as:

- hydrogen bonding
- metal coordination
- hydrophobic effects
- van der Waals forces
- $\pi$ - $\pi$  interactions
- electrostatic interactions

These forces are utilized in order to form organized, complex entities from the association of two or more species. The assembly can, as in host-guest chemistry, be driven by association of smaller molecules and ions with larger molecules and complexes, or involve macromolecules of the same size range that interact through multiple interactions in a concerted fashion.<sup>[13]</sup> Examples of host-guest chemistry include binding of a substrate by an enzyme or the coordination of metal ions by a chelating ligand.

Molecular recognition (selectivity and specificity) is crucial in order to obtain predictable structures and is the basis for programmable assembly. Another key component in supramolecular chemistry is *cooperativity*. As the individual interactions in a supramolecule may be weak, the association of the components requires multiple interactions that act in concert. In this way the resulting binding strength can become synergistically very much stronger than the mere sum of the parts. It is also very common within self-assembling systems to have more than one type of interaction present. In such cases, the system acts to optimize the product by forming those structures that have the lowest overall free energy. The assembly then tends to occur stepwise where the most stabilizing interactions are formed first and then proceed in a descending hierarchical order.<sup>[13]</sup>

Supramolecular chemistry has provided new insights in how to design synthetic molecular compounds that self-assemble in a programmable fashion but has also put new light on the chemistry of life, which heavily relies on non-covalent interactions.

## 2.2 Bionanotechnology

Bionanotechnology is the area of science where biology and nanotechnology meet and are combined in order to create synthetic self-assembling nanostructures. Nature gives the most striking examples on the strength of self-assembly when it comes to producing complex functional nano- and micro-architectures.<sup>[12]</sup> And based on the definitions mentioned above, one can claim that the creation of all living organisms, from bacteria to humans, is a result of self-assembly.

Feynman considered biology as a source of inspiration for the new generation of physicists in their pioneering efforts within the then newly invented field of nanotechnology: “A biological system can be exceedingly small. Many of the cells are very tiny, but they are very active; they manufacture various substances; they walk around; they wiggle; and they do all kinds of marvellous things - all on a very small scale. Also, they store information. Consider the possibility that we too can make a thing very small which does what we want - that we can manufacture an object that manoeuvres at that level!”<sup>[1]</sup>

Nature is truly full of amazing examples of supramolecular constructs that spontaneously self-assemble into functional nanostructures far more complex than any man-made nanodevice. Just to mention a few; the ribosome which carry out DNA translation and synthesis of proteins, ATP-synthase which is a proton driven molecular machine that produces the intracellular energy carrier ATP, and not to forget the protein assemblies constituting the photosystems in photosynthetic cells that collects photons and converts their energy into chemical energy.<sup>[14]</sup>

It is therefore not surprising that much of the work on nanosized self-assembling systems has been based on biologically derived or biomimetic molecules and biomolecular interactions. DNA has for example been widely studied and employed as a building block for nanostructures as it combines self-assembly with programmability and a plethora of chemical techniques for its manipulation.<sup>[15]</sup> This has resulted in the development of DNA based molecular motors,<sup>[16, 17]</sup> and computational devices,<sup>[18]</sup> as well as astonishing two- and three-dimensional constructs of DNA and the development of the field called “DNA origami”.<sup>[19, 20]</sup> Micrometre long DNA chains can also be stretched on surfaces and used as wire templates for bioorganic electronic applications.<sup>[21, 22]</sup> In addition, DNA has also been demonstrated as an effective and specific means to control the assembly of nanoparticles.<sup>[23, 24]</sup>

Despite the many interesting features of DNA, it is chemically and structurally inferior to proteins. Proteins are far more complex and versatile when considering their three dimensional structure as well as their chemical and biological functionality. The complexity stems from the huge number of degrees of freedom owing to their chemical composition. Proteins have thus become flexible and powerful molecular tools for various technological applications. Of particular interest is the ability of some proteins to specifically recognize other molecular species, which has been extensively employed in bioassays. Proteins have also been extensively employed for creating supramolecular nano-structures, such as synthetic spider silk,<sup>[25]</sup> molecular wires,<sup>[26]</sup> and for assembly of nanoparticles.<sup>[27, 28]</sup>

Most proteins are, however, very sensitive to changes in their physiochemical environment and can easily and irreversible lose their native conformation. In this perspective, designed polypeptides, that spontaneously adopts an ordered secondary and tertiary structure has emerged as an interesting alternative for

proteins. Polypeptides do have the same chemical diversity as proteins but are typically more robust and can also be manufactured at a large scale to a relatively low cost. As the design rules for obtaining functional and folded polypeptides are gradually being elucidated, novel polypeptides with new shapes and new functions are being realized.<sup>[29, 30]</sup> Polypeptides have in recent years also been exploited in the design of self-assembling nanostructures,<sup>[31]</sup> and hybrid materials.<sup>[32, 33]</sup>

### **2.3 Self-Assembly of hybrid materials and nanocomposites**

Numerous well-established man-made materials, known as composites, are made as mixtures between organic and inorganic compounds which are combined in such a way that the resulting materials attain properties that the individual components by them self cannot attain.<sup>[34]</sup> The inorganic building blocks in such materials are often of macroscopic dimensions and dispersed in an organic matrix, e.g. inorganic fibre-reinforced plastics. A large number of composite materials can also be found in nature, such as bone and teeth, which consists of hard inorganic crystals of hydroxyapatite, that are reinforced with collagen fibres.<sup>[35]</sup> Many natural composites have outstanding mechanical properties, far beyond those that can be achieved using similar synthetic materials. They typically consist of both inorganic and organic materials that are hierarchically and spatially organized at the nano-, micro-, and meso-levels. In addition, the dimensions of the inorganic components in natural composites are of approximately the same size as the organic species (< 50 nm), and consequently the properties of the materials will be very different showing characteristics in between the pure compounds or even display entirely new properties.<sup>[36, 37]</sup> Large efforts have therefore been made in order to develop composite materials with spatial and chemical control on the nanoscale which not only can provide improved mechanical properties but also afford so called functional or smart materials, that are switchable and able to react to changes in their physiochemical environment.<sup>[38]</sup>

These types of materials are usually divided into two subgroups, hybrid materials and nanocomposites, based on the size of their constituents.<sup>[38]</sup> Hybrid materials are typically considered as homogenous materials that includes two moieties, one inorganic and one organic, blended at the molecular level. The term nanocomposite is used if one of the structural units, either the inorganic or organic, is

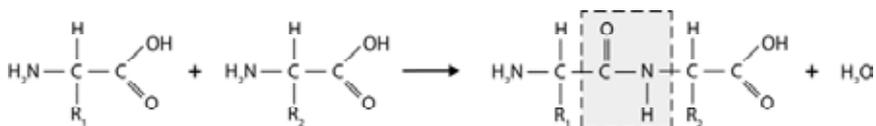
in a defined size of 1-100 nm. There is however no clear boundary between the two types of materials and the terms are often used interchangeably.<sup>[38]</sup>

Apart from conventional soft chemistry routes, self-assembly has emerged as a natural method for fabrication of both hybrid materials and nanocomposites. The large variation in possible building blocks, both when considering the inorganic part as well as the organic components, allows fabrication of an amazing amount of architectures using very different assembling strategies. Nanocomposites containing for example noble metal particles with plasmonic properties have in recent years found many interesting applications and are a subject of intense research. A large part of the work in this thesis has been devoted to the study and development of such self-assembling materials.

*“After taking into consideration the surrounding water, we actually have no idea of the magnitude of the interactions within folded proteins, of the real entropy balance, and there is no hope of evaluating them solely by the logical analysis of folding an abstract polypeptide chain into an abstract ordered conformation” Privalov, 1992.<sup>[49]</sup>*

### 3. Peptides, proteins and protein structures

A peptide is a linear chain of covalently linked amino acids with a defined length and sequence. The amino acids are joined end-to-end through an amide bond that is formed when the carboxyl group of one amino acid condenses with the amino group of another (Figure 3.1). When the number of amino acids in the peptide exceeds about 20, it is usually referred to as a polypeptide. The number of amino acids in naturally occurring polypeptides, or proteins, range from approximately 50 up to as many as 27,000, and they generally have a defined three-dimensional structure under physiological conditions.<sup>[39, 40]</sup>



**Figure 3.1** A peptide is formed when two or more amino acids are joined covalently through an amide bond (shaded area).

The peptide backbone has a distinct double bond character due to the electron delocalization over the  $\pi$ -orbital system involving the carbonyl oxygen, carbonyl carbon and the amide nitrogen. The atoms involved in the peptide bond and the connected  $\alpha$ -carbons are positioned in a common plane. The only degrees of

freedom are thus rotations around the  $\alpha$ -carbon, carboxyl carbon and amide nitrogen atoms.<sup>[41]</sup> The relative rotational positions of the backbone are defined by its dihedral angles.

There are 20 different naturally occurring amino acids, which are distinguished by their side-chain groups ( $R_1$  and  $R_2$  in Figure 3.1).<sup>[42]</sup> The different nature of the side chains give the amino acids a variety of chemical properties that roughly can be divided into three groups; nonpolar, polar, and charged. When connected into a single molecule, the sequence and number of amino acids can be varied in an infinite number of ways, giving rise to polypeptides and proteins with chemical properties far more complex than just the sum of its constituents.

### 3.1 Structure and folding of proteins

Proteins assemble into their native fold, or three-dimensional structure, as a result of a large number of concerted inter- and intra-molecular interactions. The outcome is heavily dependent on the sequence of the amino acids. Many proteins fold spontaneously after biosynthesis whilst others require some assistance from e.g. chaperones or enzymes to adopt their native conformation.<sup>[43, 44]</sup> Because of the very large number of degrees of freedom in an unfolded polypeptide chain, the molecule has an astronomical number of potential conformations. Hence, the time for a protein to find its native fold would be enormous if the protein was to attain the correct fold by sequentially sampling through all possible conformations.<sup>[45, 46]</sup> This is obviously not the case as proteins typically fold on a timescale of seconds or less. Proteins rather seem to fold as a result of an energy gradient or rugged “funnel” that guides the random tangle to its native conformation.<sup>[47]</sup>

The interactions involved in protein folding are largely the same as those mentioned in chapter 2.1 on supramolecular chemistry (vide supra). In particular, the hydrophobic effect, which can be regarded as the tendency for nonpolar substances to aggregate in aqueous media, is considered to play a central role.<sup>[48]</sup> This is manifested by the burying and clustering of hydrophobic side chains in order to minimize their contact with water as the hydrophobic residues induce an ordering of the water molecules.<sup>[41, 48]</sup> The folding of a polypeptide chain thus involves a gain in entropy due to the burial of nonpolar residues. This entropy gain is however reduced by the loss in configurational entropy of the polypeptide,

which means that the overall entropy change of protein folding and unfolding is close to zero at physiological temperatures.<sup>[49]</sup> The net stabilization of proteins is therefore mainly a result of the cooperative action of a large number of intramolecular interactions, such as salt-bridge formation and hydrogen bonds. The stability of a protein hardly ever exceeds  $50 \text{ kJ mol}^{-1}$  in free energy, which corresponds to the energy of about three hydrogen bonds.<sup>[50]</sup>

The peptide backbone is very hydrophilic, with one hydrogen bond donor, NH, and one acceptor, C=O, in each peptide unit. As the interior of proteins typically is very hydrophobic the free energy is minimized through formation of hydrogen bonds between the polar atoms of the backbone. These hydrogen bonds result in a regular secondary structure, which is usually one of two types: *alpha-helix* or *beta-sheet*.<sup>[51]</sup> The secondary structure elements then pack into often rather dense structures. The interior residues of proteins are actually packed as tightly as crystalline amino acids.<sup>[41]</sup>

Lack of ordered secondary structure is referred to as *random coil*, while a protein that is not fully folded neither fully unfolded is in a *molten globule* state.<sup>[40]</sup> Although many proteins adopt a regular and close-packed three dimensional structure they are not static, but constitute flexible molecules that can go through significant structural fluctuations.<sup>[41, 52]</sup>

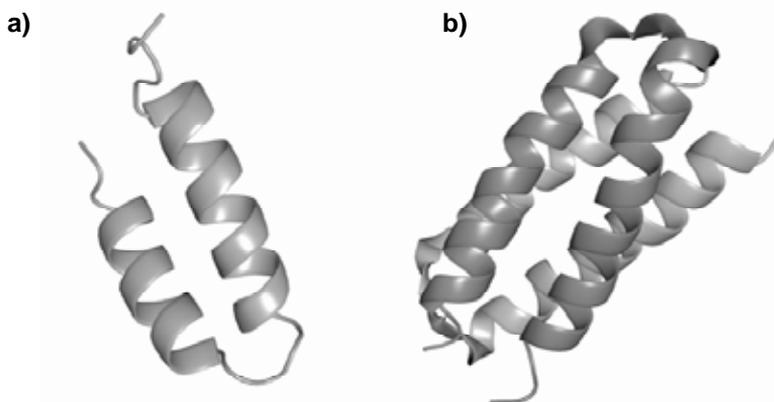
### 3.2 The alpha-helix

When a stretch of consecutive amino acid residues all have the dihedral angles for rotation around the  $\alpha$ -carbon ( $\Phi$ ,  $\Psi$ ) close to  $-60^\circ$  and  $-50^\circ$ , the polypeptide chain will adopt an  $\alpha$ -helical conformation. Each completed turn incorporates 3.6 amino acid residues and raises  $5.6 \text{ \AA}$  along the helix axis direction.<sup>[14]</sup> This conformation places the hydrogen bonding atoms in near perfect alignment. In addition, the radius of the helix allows for favourable van der Waals interactions across the helix axis and the side chains are well staggered which minimize steric interferences. Hydrogen bonds occur in the ordinary  $\alpha$ -helix between the carbonyl carbon of residue  $n$  and the amide nitrogen of residue  $n+4$ . Since all the dipole moments of each peptide unit are directed in the same direction along the helix axis, the  $\alpha$ -helix has a significant net dipole moment that gives rise to a partial

positive charge at the amino terminal and a partial negative charge at the carboxy-terminal.<sup>[51]</sup>

### 3.3 The four-helix bundle

A simple combination of a few secondary structures in a specific geometric arrangement is often referred to as supersecondary structure, or motif.<sup>[42]</sup> The simplest supersecondary structure with a specific function is the helix-loop-helix motif consisting of two  $\alpha$ -helices joined by a loop region (Figure 3.2 a). An isolated  $\alpha$ -helix is only marginally stable in solution due to the lack of stabilization of adjacent hydrophobic side chains. Stabilization can be achieved by the formation of domains. A domain is a combination of secondary structure elements and motifs, also commonly referred to as tertiary structure. A common naturally occurring domain is the four-helix bundle where four amphiphilic  $\alpha$ -helices are arranged in a bundle with the helical axis almost parallel to each other (Figure 3.2 b). In this way the hydrophobic side chains are effectively buried between the helices and the hydrophilic side chains are exposed to the solvent.



**Figure 3.2** a) A helix-loop-helix motif (PDB entry 2gp8). b) A four-helix bundle formed by the association of two helix-loop-helix motifs (PDB entry 1u7j). The structures were obtained in the Protein Data Bank (PDB)<sup>[53]</sup> and rendered using the Polyview-3D visualization server.<sup>[54]</sup>

### 3.4 Protein adsorption on surfaces

Proteins have a tendency to accumulate on surfaces as a result of the high standard free energy at the interface. When proteins adsorb on a surface, the free energy of the system decreases and the surface is thermodynamically stabilized.<sup>[55]</sup> The mechanisms of protein adsorption are very complex and there seems to be a large number of dynamic constraints affecting the process. The adsorption is most often irreversible, but exchange reaction may take place in the presence of other proteins. The adsorption can also result in two-dimensional phase transitions and crystalline packing of the proteins on the surface.<sup>[56]</sup> The amount of protein adsorbed is determined by several factors including properties of the proteins, the type of surface and the solvent. The properties of the proteins affecting adsorption are such as the charge, size, stability of the protein, amino acid composition, and conformation.<sup>[55]</sup> Proteins with low internal stability (e.g. human serum albumin and immunoglobulin G) tend to adsorb on all types of surfaces irrespective of electrostatic interactions whereas proteins with high internal stability (e.g. ribonucleases and lysozymes), generally adsorb to a very low extent on hydrophilic surfaces, unless there is an electrostatic attraction.<sup>[55]</sup> Upon adsorption bonds are formed between the protein and the solid surface and the protein may unfold during the process of optimizing the contact with the surface. The unfolding is in general not complete and the degree of remaining secondary structure is often significant. Protein structure is, however, not necessarily disrupted upon adsorption and induction of secondary structure of proteins upon adsorption has also been observed.<sup>[57, 58]</sup> Possibilities to induce folding of synthetic *de novo* designed polypeptides by adsorption to monolayer protected gold clusters,<sup>[59]</sup> and silica nanoparticles<sup>[60, 61]</sup> have recently been demonstrated.

### 3.5 Determination of protein structure

To fully characterize the three-dimensional structure of proteins and polypeptides is tedious and time consuming work. Such an undertaking involves either X-ray crystallography or Nuclear Magnetic Resonance (NMR) spectroscopy. Fourier transform infrared (FT-IR) spectroscopy and circular dichroism (CD) spectroscopy are two frequently used techniques for structural characterization of proteins and polypeptides that are less laborious, but that still provide both qualitative and quantitative information.

### 3.5.1 Infrared spectroscopy of polypeptides

The infrared (IR) spectra of polypeptides show nine separate bands that originate from vibrational modes of the polypeptide backbone; amide A, amide B and amide I-VII. For structural characterization of polypeptides most information can be obtained from the amide I band. This vibrational mode is due to the C=O stretching vibration of the amide group coupled to the in plane bending of the N-H and stretching of the C-N bonds.<sup>[62]</sup> Amide II is also often studied when evaluating secondary structure. This vibrational mode is more complex and originates from an out of phase combination of NH in plane bend and CN stretch with minor contributions from CO in plane bending, CC stretch and NC stretch.<sup>[63]</sup> Unfortunately, many of the side chains of polypeptides have characteristic absorption bands in the same frequency range as the amide I and II bands and may account for as much as 15-20% of the total integrated intensity in this region.<sup>[62]</sup>

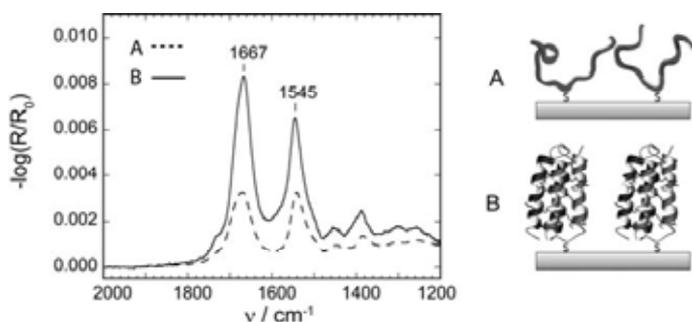
The amide bands, amide I in particular, are very sensitive to hydrogen bonding patterns, dipole-dipole interactions and the geometry of the polypeptide backbone. Different secondary structures therefore give rise to characteristic absorption frequencies. The formation of hydrogen bonds between the amide C=O and N-H groups in polypeptides leads to a redistribution of electrons among the atoms involved in the peptide bond. The original loss of negative charge at the donor atom due to the formation of the hydrogen bond tends to be overcompensated by the molecule, which actually leads to an increased electron density at the donor atom. The acceptor atom passes negative charge to the rest of the molecule to compensate for the loss of fractional positive charge. This results in weaker NH and CO bonds and stronger CN bonds.<sup>[64]</sup> The weakening of the CO bond makes the amide I band appear at lower absorption frequencies while the stronger CN bond will make the amide II band appear at higher frequencies. The stronger the hydrogen bonds the lower the wavenumber of the amide I band. The strongest hydrogen bonds are formed in extended and aggregated polypeptide chains due to the possibility for the chains to come in close proximity to each other and form very strong intermolecular hydrogen bonds. The amide I band for proteins known to be predominantly  $\alpha$ -helical is commonly positioned between 1648 and 1660  $\text{cm}^{-1}$  and for denaturated and aggregated proteins between 1610 and 1628  $\text{cm}^{-1}$ ,<sup>[65]</sup> which is in good agreement with calculations made by for example Krimm and Bandekar.<sup>[66]</sup> Typical amide I frequencies for a number of common protein structures are presented in table 3.1. The amide II peak is not as sensitive to

differences in secondary structure as the amide I but is commonly seen in the spectral range 1545 to 1551  $\text{cm}^{-1}$  for  $\alpha$ -helical proteins.<sup>[65]</sup> In contrast to the amide I, the amide II mode is rather sensitive to dehydration and typically shifts by 5-8  $\text{cm}^{-1}$  when dried as a result of the reduction of hydrogen bond interactions with water.<sup>[67]</sup>

**Table 3.1** Common protein structures and corresponding amide I frequencies<sup>[65]</sup>

Structure	Amide I frequency ( $\text{cm}^{-1}$ )
antiparallel $\beta$ -sheet/aggregated strands	1675-1695
$3_{10}$ -helix	1660-1670
$\alpha$ -helix	1648-1660
random coil	1640-1648
$\beta$ -sheet	1625-1640
aggregated strands	1610-1628

Data from studies of globular proteins known to be predominantly helical, such as albumin, also suggest that there exists a correlation between the intensity ratio of the amide II band to the amide I band and the  $\alpha$ -helix content.<sup>[68]</sup> Typically the amide I to amide II intensity ratio adopts values of 1.5-2 in isotropic (non-polarized) transmission-absorption spectra of native proteins.<sup>[67]</sup> For unfolded proteins this ratio may decrease to about 1.<sup>[68]</sup> This has also been observed for polypeptides utilized in this thesis (Figure 3.3) and is discussed in Paper I.



**Figure 3.3** Infrared reflection absorption spectra (IRAS) of a polypeptide (JR2EC) immobilized on gold surfaces, before (---) and after (—) heterodimerization with the complementary polypeptide JR2K. Heterodimerization induces folding into four-helix bundles.

### 3.5.2 Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy is a widespread technique for studies of protein secondary structure.<sup>[69-71]</sup> Different secondary structure elements give rise to characteristic spectra that enables characterization and quantification of secondary structure elements. CD is observed for molecules that are optically active, or chiral, which means that they absorb left and right circularly polarized light to a different extent. For proteins the CD arises in the far-UV region ( $\lambda < 240$  nm) as a result of the chiral properties of the polypeptide backbone. The difference in extinction coefficients ( $\Delta\varepsilon$ ) for right-handed (R) and left-handed (L) circularly polarized light results in a difference in absorption ( $\Delta A$ ) of the two components according to the Lambert-Beer relation (eq 3.1).

$$\Delta A = l \cdot c \cdot \Delta\varepsilon \quad \text{eq. 3.1}$$

where  $l$  is the optical path length and  $c$  the sample concentration. CD is usually expressed in terms of the ellipticity  $\Theta$  since the difference in amplitude between the two polarization components caused by the difference in absorption results in an elliptical polarization of the transmitted light. The ellipticity is defined by the angle between the major and minor axis of the ellipse described by the sum vector of the left and right polarized components and is measured as a function of wavelength and related to  $\Delta A$  by the expression (eq. 3.2):

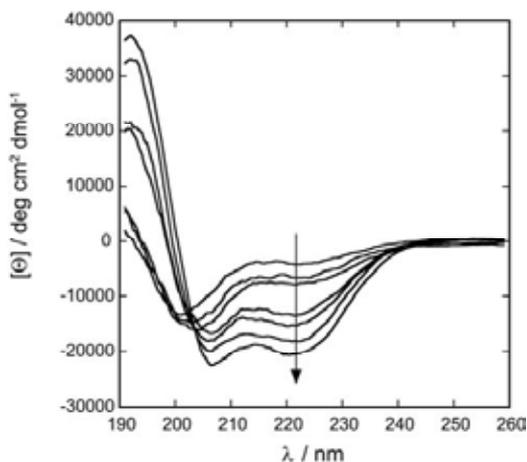
$$\Theta = \frac{\ln 10 \cdot 180 \cdot \Delta A}{4\pi} \approx 33 \cdot \Delta A \quad \text{eq. 3.2}$$

For proteins the result is commonly presented as the mean residue ellipticity  $[\Theta]$  which is related to the ellipticity ( $\Theta$ ) by the following expression (eq. 3.3).

$$[\Theta] = \frac{\Theta \cdot mrw}{10 \cdot l \cdot c} \quad \text{eq. 3.3}$$

where  $mrw$  is the mean residue weight.

The CD spectra of helical proteins typically display pronounced minima at 208 and 222 nm and a maximum around 190 nm (Figure 3.4). The mean residue ellipticity at 222 nm is often used as a measure of the helical content of a protein.



**Figure 3.4** CD-spectra in the far UV-region of a polypeptide (JR2E) that goes from random coil to a helical conformation. The helicity increases in the direction of the arrow.

To obtain quantitative data from CD spectra, access to spectra of proteins with known structure is necessary. Given a data set of reference spectra, various algorithms implemented in software packages such as the CONTIN/LL,<sup>[72]</sup> SELCON3,<sup>[73]</sup> and CDSSTR,<sup>[74]</sup> can be employed to extract the amount of the various secondary structure elements that are present in the peptide or protein.



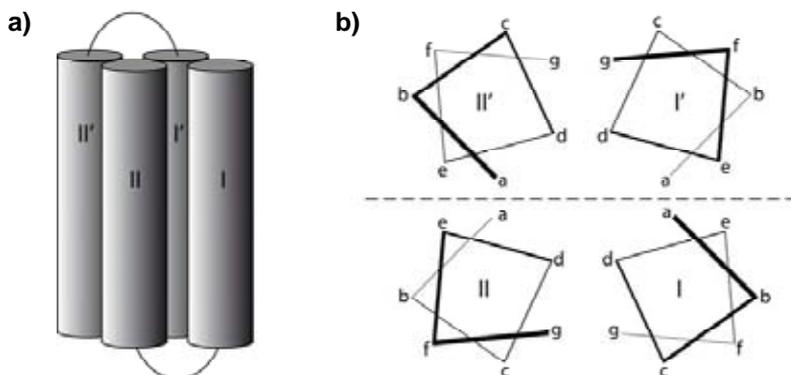
*"Where Nature finishes producing its own species, man begins, using natural things and with the help of this nature, to create an infinity of species"* Leonardo da Vinci (1452-1519).

## 4. Polypeptide design and synthesis

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An increasing knowledge on how the sequence of amino acids determines the three-dimensional structure of a polypeptide chain has made it possible to design polypeptides that adopt a regular and ordered conformation. *De novo* design of proteins not only extend our knowledge about the rules governing protein folding but enables realization of new molecular entities similar to native proteins but with entirely new properties and functions. One of the most common design motifs is the four-helix-bundle formed from amphiphilic helices (Figure 4.1 a).<sup>[29]</sup> Typically, shape complementarities and hydrophobic interactions drive the formation of the supersecondary structure. Since one turn of an  $\alpha$ -helix involves about four residues, the introduction of hydrophobic amino acids in approximately every fourth position results in an helix with a hydrophobic face. This approach can be described in terms of a heptad repeat (abcdefg)<sub>n</sub> pattern (Figure 4.1 b) where the positions *a* and *d* usually contains hydrophobic residues that form the hydrophobic core upon folding. The residues in *g* and *c* positions are part of the solvent exposed face and the residues in *b* and *e* positions are at the dimer interface and control dimerization.<sup>[51]</sup> Two designed helix-loop-helix motifs can under the right conditions dimerize to form a four-helix bundle. The dimerization process can lead to a parallel, an anti-parallel<sup>[75]</sup> or a bisecting U motif<sup>[76]</sup>.

The design of the polypeptides used throughout this thesis is based on the SA-42 polypeptide motif.<sup>[77, 78]</sup> SA-42 is a helix-loop-helix polypeptide that was *de novo*



**Figure 4.1** a) The polypeptides utilized throughout this thesis dimerize in an antiparallel fashion to form four-helix bundles.<sup>[77, 78]</sup> b) The heptad repeat pattern is utilized to illustrate the relative position of the amino acids in the antiparallel helix-loop-helix dimers. The dashed line represents the dimerization interface. Figures redrawn from <sup>[79]</sup>.

designed to dimerize and fold into a four-helix bundle. The polypeptide was also designed to catalyze ester hydrolysis, but its reactivity was found to be very low. SA-42 is largely helical with a mean residue ellipticity at 222 nm of  $-25\ 000\ \text{deg}\ \text{cm}^2\ \text{dmol}^{-1}$ , and from NMR-spectroscopy data it was concluded that it predominantly forms a hairpin helix-loop-helix that dimerize in an antiparallel mode. Dimerization was further confirmed using analytical ultracentrifugation.<sup>[77]</sup>

#### 4.1 The JR-polypeptides

The JR-polypeptides, JR2E and JR2K, were designed by Johan Rydberg and Sarojini Vijayalekshmi to form heterodimeric four-helix bundles.<sup>[80]</sup> The design of the 42-residue helix-loop-helix polypeptides was based on SA-42, but they were modified with a large abundance of either Glu (JR2E) or Lys (JR2K), giving them controllable dimerization properties. The amino acid sequences are presented in Figure 4.2. The net charge of JR2E and JR2K at pH 7 is -5 and +11, respectively. The charged residues are mainly incorporated in the *b* and *e* positions in the heptad repeat pattern, which are located on the faces of the helices that control dimerization.

**JR2E**

1	19
H <sub>3</sub> N <sup>+</sup> -N-A-A-D-L-E-K-A-I-E-A-L-E-K-H-L-E-A-K	
	20 23
	-G-P-V-D
42	24
<sup>-</sup> OOC-G-A-R-E-F-A-E-F-A-Q-E-L-Q-K-E-L-Q-A-A	

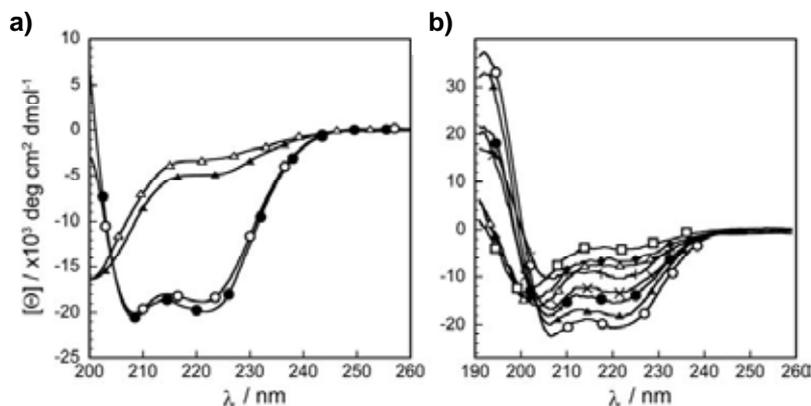
**JR2K**

1	19
H <sub>3</sub> N <sup>+</sup> -N-A-A-D-L-K-K-A-I-K-A-L-K-K-H-L-K-A-K	
	20 23
	-G-P-V-D
42	24
<sup>-</sup> OOC-G-A-R-K-F-A-K-F-A-Q-K-L-Q-K-K-L-Q-A-A	

**Figure 4.2** The amino acid sequences of JR2E and JR2K.

Due to the charge repulsion, the peptides exist as random coil monomers at neutral pH and at moderate salt concentrations (< 0.5 mM). When the two peptides are mixed at equimolar concentrations they heterodimerize and fold, resulting in a large increase in helicity (Figure 4.3 a). The driving force for folding is the formation of the hydrophobic core made up by hydrophobic residues in *a* and *d* position. Dimerization has been confirmed using analytical ultracentrifugation.<sup>[80]</sup>

Homodimerization is induced when lowering the charge repulsion between the monomers by protonating or deprotonating acidic and basic residues, respectively. For JR2K this occurs at pH > 10,<sup>[80]</sup> whereas JR2E homodimerizes below pH 6 (Figure 4.3 a). The pH dependent homodimerization of JR2E is described in Paper I and Paper II. As is reported in Paper III, homodimerization of JR2E could also be induced at neutral pH in the presence of a number of metal ions, such as Ni<sup>2+</sup>, La<sup>3+</sup>, Co<sup>2+</sup> and Zn<sup>2+</sup> (Figure 4.3 b). The largest folding tendency was seen for Zn<sup>2+</sup>.



**Figure 4.3** a) CD-spectra of ( $\Delta$ ) JR2K, ( $\blacktriangle$ ) JR2E, and ( $\circ$ ) JR2K+JR2E at pH 7 and ( $\bullet$ ) JR2E at pH 4.5. b) CD-spectra of JR2E at pH 7 in the presence of 10 mM of metal salts: ( $\circ$ )  $\text{Zn}^{2+}$ , ( $\Delta$ )  $\text{Ca}^{2+}$ , ( $\blacklozenge$ )  $\text{Mg}^{2+}$ , ( $\times$ )  $\text{Ni}^{2+}$ , ( $+$ )  $\text{Fe}^{3+}$ , ( $\bullet$ )  $\text{Co}^{2+}$ , ( $\blacktriangle$ )  $\text{La}^{3+}$ , and ( $\square$ ) buffer only.

In order to allow for directed, thiol-dependent immobilization of the JR-polypeptides on gold substrates, Val22 was exchanged for Cys, affording the peptides JR2EC and JR2KC. Position 22 is located in the loop region and was chosen in order to minimize the influence of immobilization on dimerization. No effects of the Cys on the secondary structure were observed for either the homodimer or the heterodimer when in solution, irrespectively if homodimerization was induced by  $\text{Zn}^{2+}$ . The Cys residue was also utilized to covalently join two peptide monomers via a disulphide bridge. The disulphide-linked peptides were found to spontaneously and rapidly assemble into micrometre long fibres as a result of a propagating folding mediated association (Paper V). Both hetero- and homo-associating fibres were obtained.

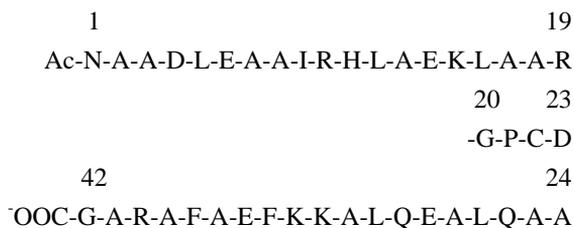
An additional peptide that was unable to dimerize and fold was designed and utilized as a reference polypeptide when investigating the influence of dimerization and folding on the assembly of peptide-modified gold nanoparticles and peptide fibres. This peptide, JR2ECref, has the same amino acid sequence as JR2EC with the exception that all L-Ala were replaced by D-Ala, while the other residues were kept in the L-state. Peptides with mixed D- and L-amino acids cannot generally adopt an ordered secondary structure.

## 4.2 The KE-polypeptides

The 42-residue helix-loop-helix polypeptide KE2 was designed by Karin Enander as a scaffold for biosensor applications.<sup>[81, 82]</sup> The design was based on LA-42b, which is closely related to SA-42, but modified in order to catalyze site-selective self-functionalization.<sup>[83]</sup> KE2 was modified with an affinity ligand and fluorescent probes to enable specific binding of target proteins and reporting on this event combined in the same molecular entity. A number of different derivatives of benzenesulphonamide were investigated as ligands and were attached to the polypeptide scaffold using an orthogonal protection group strategy. Benzenesulphonamide ( $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_5$ ) is an inhibitor of the 29 kDa, enzyme human carbonic anhydrase II (HCAII) and binds with a  $K_d$  of 1.5  $\mu\text{M}$ .<sup>[84]</sup> In order to detect binding, a number of different environmentally sensitive fluorescent probes were incorporated in various positions. In addition, the affinity for HCAII could be tailored by varying the length and hydrophobicity of the spacer between the benzenesulphonamide moiety and the polypeptide, which was utilized in order to create an affinity array.<sup>[85]</sup>

In this thesis, KE2 was modified to allow for thiol dependent immobilization on gold surfaces by replacing Val in position 22 by Cys, affording the polypeptide KE2C (Figure 4.4). KE2C was further functionalized with a benzenesulphonamide derivate with an aliphatic spacer having five methylene units in order to obtain the sensor peptide KE2C-C6. The corresponding fluorophore modified sensor peptide KE2-D(15)-5 binds HCAII with a  $K_d$  of 0.02  $\mu\text{M}$ .<sup>[82]</sup>

### KE2C

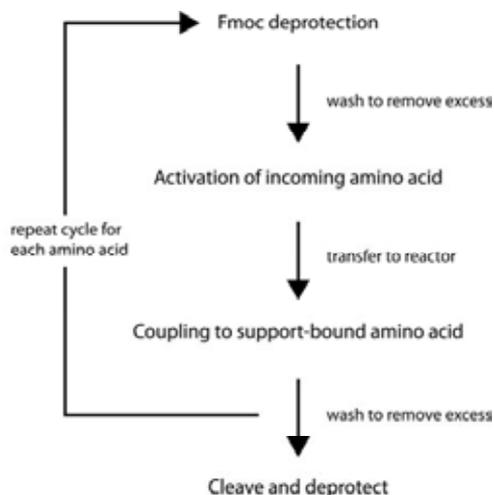


**Figure 4.4** The amino acid sequence of KE2C.

### 4.3 Peptide Synthesis

The peptides utilized throughout this thesis were prepared using an automated continuous flow solid-phase peptide synthesizer. The procedure is schematically summarized in Figure 4.5, below. The insoluble solid support allows soluble by-products and excess reagents to be conveniently removed. The C-terminal amino acid was covalently bound to the solid support (typically polyethylene glycol grafted polystyrene) and the following amino acids were added sequentially one at the time to the growing chain. The  $\alpha$ -amino group of the amino acids was protected with a fluorenylmethoxycarbonyl (Fmoc) group that was removed by treatment with a mild base before addition of the following activated amino acid. Amino acids with reactive side chains were protected with groups that could be removed under the conditions used when cleaving the peptide from the solid support.

After synthesis and cleavage from the support material the peptides were purified by reversed phase high-performance liquid chromatography (HPLC) and identified from their matrix-assisted laser desorption ionization time of flight (MALDI-TOF) spectra.



**Figure 4.5** Overview of the steps involved in solid-phase synthesis of polypeptides.

*“The state of division of these particles must be extreme; they have not as yet been seen by any power of the microscope.”* Faraday, 1857.<sup>[92]</sup>

## 5. Colloidal Gold

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In the bulk form, gold is a soft, yellow metal, with the face centred cubic crystal structure, a melting point of 1064° C and excellent electrical conductivity. Interestingly, not one of these properties necessary applies to gold colloids on the nanometre scale,<sup>[86]</sup> and the smaller they are the more do their properties deviate from the bulk material.<sup>[87, 88]</sup>

The many interesting properties of colloidal gold have fascinated humanity since the 5<sup>th</sup> or 4<sup>th</sup> century B.C. The technique of preparing “soluble gold” was first discovered and used in Egypt and China and adopted by the Romans who utilized it for staining of glass.<sup>[89]</sup> The Lycurgus Cup, is the perhaps most famous preserved piece from that time and is one of a class of Roman Vessels known as *cage cups* or *diatreta*.<sup>[90]</sup> The Lycurgus Cup has a very remarkable feature; it appears ruby red in transmitted light while it has an opaque greenish-yellow tone in reflected light. The origin of this dichroic property has been confirmed to the presence of gold and silver particles, 50-100 nm in diameter, embedded in the glass.<sup>[91]</sup> The Roman glass makers, however, probably had a very vague idea about the cause of the beautiful colours and it was not until the mid 19<sup>th</sup> century that the properties of colloidal gold was examined and described in more detail by Michael Faraday. Faraday presented his results on the interaction of light with metal particles that were “*very minute in their dimensions*” in a ground breaking lecture to the Royal Society of London in 1857.<sup>[92]</sup> Although the term “colloid”



**Figure 5.1** (Left) Electron micrograph of a 13 nm gold nanoparticle (scale bar 5 nm), that are the cause of the beautiful colours of the dispersions in the photo in the middle. (Right) UV-visible spectrum of a dispersion of gold nanoparticles showing the characteristic extinction peak that gives rise to these colours. (Photo courtesy of Prof. M. Cortie, Institute for Nanoscale Technology, Sydney, Australia.)

was not coined until a few years later by Graham<sup>[93]</sup> from the Greek word for glue, Faradays work laid the foundation to modern colloid science.<sup>[94]</sup>

In addition to the interesting optical properties of colloidal gold that gives rise to their beautiful colours (Figure 5.1), monodisperse particles in the size range from ~2 to 100 nm can straightforwardly be prepared using standard protocols. Moreover, they do not easily oxidize and can be functionalized with a wide variety of different molecules, which are properties that make gold nanoparticles very attractive as components in various technological applications.

Except as a colourant in stained glass, the first practical use of gold nanoparticles was as immunohistochemical contrast agent for electron microscopy of tissue samples, a method still widely used.<sup>[95]</sup> When used for staining, antibodies raised against an antigen of interest are adsorbed on the surface of gold nanoparticles which are subsequently exposed to the sample. If the antigen is present the antibody coated particles might bind, which can easily be visualized using Transmission Electron Microscopy (TEM). Gold nanoparticles functionalized with a range of other biomacromolecules, such as lectins and polysaccharides, have also been employed for cytochemical applications.<sup>[96, 97]</sup> In addition, gold nanoparticles have been extensively used as a label in immunoassays,<sup>[98, 99]</sup> and in a number of commercially available products for self-testing, such as the home pregnancy test “First Response”,<sup>[100]</sup> and more recently as a component in a large number of nanocomposite materials.

## 5.1 Synthesis of gold nanoparticles

Synthesis of gold nanoparticles are commonly performed by reduction of hydrogen tetrachloroaurate ( $\text{HAuCl}_4$ ) dissolved in water.<sup>[89, 101]</sup> Since the lowest surface energy shape of a particle is that of a sphere, solution phase synthesis of gold nanoparticles tends to yield approximately spherical particles.<sup>[102]</sup> Like bulk gold, nanoparticles larger than 10 nm adopts the face centred cubic (fcc) crystal structure and the particles exhibit the cubic, octahedral or rhombohedral crystal forms associated with the fcc structure.<sup>[86]</sup> The number of defects in the crystal structure is however usually high. Non-spherical particles can be generated by e.g. addition of surfactants with preferential binding to certain crystal facets,<sup>[103]</sup> or by photo conversion of spherical particles.<sup>[104]</sup> Two different and widely used procedures to synthesize gold nanoparticles are the methods developed by Brust and Schiffrin<sup>[105]</sup> and Turkevich et al.<sup>[106]</sup> Both methods allow a certain degree of size control by varying the reaction temperature and relative concentrations of the reagents.<sup>[107, 108]</sup>

The Brust-Schiffrin method results in mono disperse gold nanoparticles, ~1.5-6 nm in diameter, dissolved in an organic solvent. The synthesis strategy is inspired by Faradays two-phase system.<sup>[92]</sup>  $\text{AuCl}_4^-$  is typically transferred to toluene from an aqueous solution using tetraoctylammonium bromide as a phase-transfer reagent, and reduced with aqueous sodium borhydride ( $\text{NaBH}_4$ ) in the presence of dodecanethiol, resulting in monolayer protected clusters (MPC). This method has further been improved to involve other functional thiol ligands.<sup>[109, 110]</sup> By utilizing a thiol exchange reaction, MPCs with a wide variety of surface functionalities has been produced and characterized.<sup>[111-114]</sup>

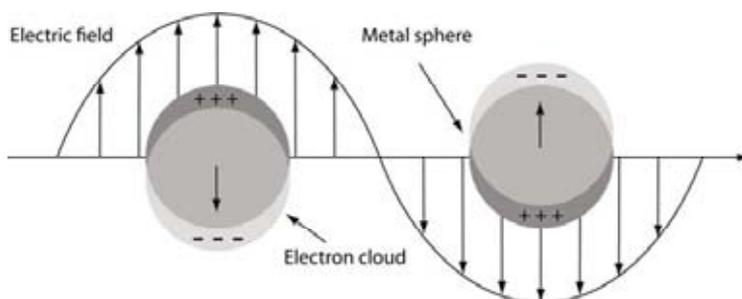
The procedure for reduction of gold salt by citrate anions was introduced by Turkevich et al.<sup>[106]</sup> in 1951, and was further improved by Frens.<sup>[108]</sup> The method utilizes citrate both as a reducing agent and an electrostatic stabilizer, yielding fairly monodisperse gold nanoparticles in the range 7-100 nm in diameter.<sup>[108]</sup> The reaction is performed entirely in aqueous solution and the excess citrate stabilizes the particles by forming a complex multilayered assembly of anions, with different oxidation states lending an overall negative surface charge. The size of the particles can rather easily be controlled by alternating the gold salt/citrate ratio. In contrast to MPCs, citrate stabilized nanoparticles cannot withstand dehydration without aggregating irreversibly. The stability is also sensitive to the

presence of organic compounds, changes in pH and the ionic strength of the medium.<sup>[115]</sup> Citrate stabilized particles are very suitable for applications involving biomolecules as they are dispersed in water from start.<sup>[23, 95, 116, 117]</sup> This method was also chosen for the preparation of the gold nanoparticles used in this thesis, using a protocol first described by Storhoff et al.<sup>[118]</sup>

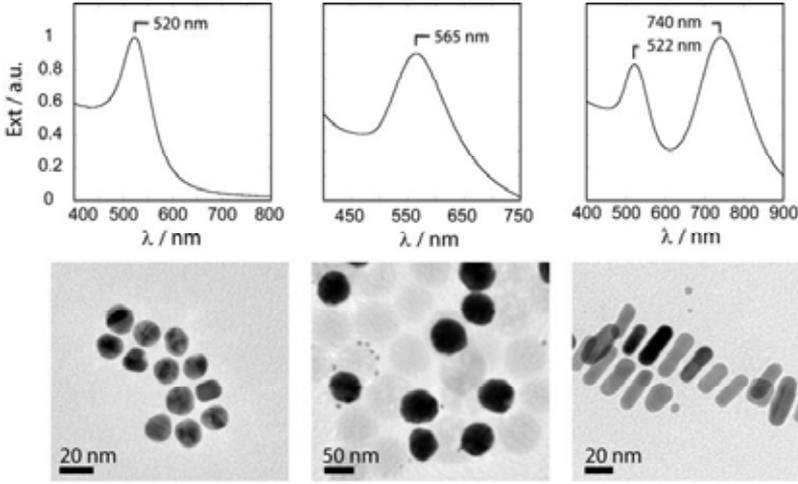
## 5.2 Optical properties of gold nanoparticles

The colours of the Lycurgus Cup are the result of a coherent motion of the conduction electrons in the metal particles embedded in the glass as they are exposed to visible light.<sup>[91]</sup> These so called localized surface plasmons are a consequence of the high electronic polarizability of noble metal nanoparticles. When irradiated with light, the conduction electrons in the particles experience a force that displaces them from their equilibrium position, which in turn induces a restoring force that in combination with the oscillatory nature of the radiation, give rise to an oscillatory motion of the electrons (Figure 5.2).<sup>[119]</sup>

A number of factors influence the optical response of the particles, such as particle size, geometry and type of metal (Figure 5.3). The influence of the dielectric constant of the embedding medium is also significant as the polarization of the particles induces a polarization in the opposite direction in the surrounding medium that reduces the restoring force.



**Figure 5.2** Schematic illustration of a localized surface plasmon of a metal sphere showing the displacement of the electron cloud relative to the nuclei. Redrawn from <sup>[120]</sup>.



**Figure 5.3** UV-visible spectra and corresponding electron micrographs of (from left to right) gold nanoparticles approximately 15 and 60 nm in diameter and gold nanorods.

The optical properties of metal nanoparticles were described in detail by Mie,<sup>[121]</sup> who developed analytical solutions to Maxwell's equations for the interaction of electromagnetic radiation with small spheres. For particles much smaller than the wavelength of light (roughly  $2r < \lambda_{max}/10$ ) the incident field can be treated as a plane wave and only the dipole oscillations contribute significantly to the extinction.<sup>[122]</sup> In the dipole approximation the extinction spectra of spherical particles can be described using the following expression (eq 5.1):

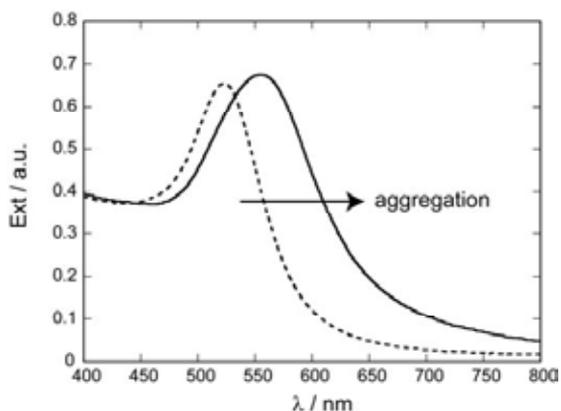
$$E(\lambda) = \frac{24\pi}{\lambda \ln(10)} r^3 \epsilon_m^{3/2} N_A \frac{\epsilon_i(\lambda)}{[\epsilon_r(\lambda) + 2\epsilon_m]^2 + \epsilon_i(\lambda)^2} \quad \text{eq. 5.1}$$

where  $E(\lambda)$  is the extinction, defined as the sum of the absorption and Rayleigh scattering contributions,  $N_A$  the areal density of nanoparticles,  $r$  the particle radius, and  $\epsilon_m$  the wavelength independent dielectric constant of the medium in the vicinity of the nanoparticles. The dielectric constant of metal nanoparticles is wavelength dependent and has one imaginary part  $\epsilon_i(\lambda)$ , and one real part  $\epsilon_r(\lambda)$ .

The conditions for localized surface plasmon resonance (LSPR) is met when the resonance term in the denominator ( $\epsilon_r(\lambda) - 2\epsilon_m$ ) approaches zero. The extinction is

thus highly dependent on the wavelength ( $\lambda$ ) of the incident radiation and the refractive index of the surrounding medium ( $n_m = \epsilon_m^{0.5}$ ). At resonance, nanoparticles display a maximum in extinction (Figure 5.4). A change in refractive index close to the particle surface, for example as a result of adsorption of molecular species on the particle surface, will accordingly result in a shift of the extinction maximum ( $\lambda_{Extmax}$ ).<sup>[120]</sup> The magnitude of this shift varies with the thickness and refractive index of the adsorbed layer but is also heavily dependent on the properties of the nanoparticles. A number of models based on Mie theory that describes the dependence of the LSPR shift on the adsorption of molecular species on the particle surface have been proposed.<sup>[123, 124]</sup>

Mie theory is only valid under the assumption that the particles are well separated from one another and do not interact, which means that the resulting electric field that is created as a result of the resonating surface plasmons is not felt by any surrounding particles. For small inter-particle distances the plasmon resonance conditions are highly affected by near-field coupling,<sup>[125]</sup> which gives rise to a dramatic redshift of the LSPR extinction maximum upon particle aggregation (Figure 5.4). The coupling coefficient can be approximated as an exponential function of the interparticle distance, and the smaller the distance and the larger the aggregates, the larger the resulting shift will be.<sup>[125-128]</sup>



**Figure 5.4** At resonance, the UV-visible spectrum of dispersed gold nanoparticles (---) displays a pronounced maximum. Upon aggregation (—), the extinction maximum experiences a dramatic redshift as well as a change in intensity.

The large influence of the interparticle separation on the position of the LSPR peak can thus be utilized to tune the colorimetric response of aggregating gold nanoparticles. The LSPR peak shift can also give valuable information about the extent of aggregation, which was utilized in paper II to distinguish between a folding and a non-folding peptide and in Paper VII to monitor the binding of a protein to the particle surface.



*“The colloid, although often dissolved in a large proportion by its solvent, is held in solution by a singularly feeble force. Hence colloids are generally displaced and precipitated by the addition to their solution of any substance from the other class.”* Graham, 1861.<sup>[93]</sup>

## 6. Nanoparticle stability

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In colloidal science, the term *stability* is often used to describe how well particles are dispersed in the continuous phase and can resist aggregation. Whether a colloidal system is stable or not will depend upon the balance of repulsive and attractive forces acting between the particles. Colloidal particles are always subject to Brownian motion and in many cases also hydrodynamic forces, and will frequently undergo collisions. If the repulsion is stronger than the attraction they will separate again after contact whereas aggregation is the outcome if the opposite is true. There are a number of forces that play a role in the interaction between colloids where the four most important are considered to be van der Waals forces, electrostatic interactions, solvation (hydration), and osmotic and entropic interactions related to macromolecular adsorption.<sup>[129]</sup> At long range (typically < 100 nm) the interactions between particles in liquid are dominated by electrostatic, steric-polymer and van der Waals forces, whereas at shorter range (<1-3 nm) solvation and other types of steric forces tend to be most important.<sup>[130]</sup>

### 6.1 van der Waals forces

van der Waals forces are always present at varying degree and are always attractive between similar particles. The origin of the van der Waals forces is the presence of permanent or induced dipoles and multipole moments and is usually subdivided into three groups; orientation, induction, and dispersion forces,

depending on the nature of the interactions.<sup>[131]</sup> Orientation forces are a result of dipole-dipole (or higher multipole) interactions whereas induction forces arise due to dipole-induced dipole interactions. Dispersion forces, or London forces, are quantum mechanical in origin and are caused by interactions due to fluctuating dipoles that can induce polarity in neighbouring objects.<sup>[130]</sup> Even though all the van der Waals forces have the same distance dependence (assuming that the interactions are non-retarded), it is generally the dispersion forces that are dominating, except for highly polar molecules such as water.<sup>[130]</sup>

## 6.2 Electrostatic double-layer forces

Particles suspended in water are usually charged as a result of either the dissociation or ionization of surface groups, or by the adsorption of ions from the solution on the surface of the particles.<sup>[132]</sup> Proteins and peptides acquire their charge mainly through ionization of acidic and basic amino acids, such as Glu and Lys, respectively. This means that the charge of these types of molecules is highly dependent on the molecular isoelectric point (pI) and the pH of the solution. At low pH, most proteins and peptides will be positively charged, while they are negatively charged at high pH. A surface charge can also result from the unequal adsorption of ions with different charge. As cations in general are more hydrated than anions they are less prone to adsorb on surfaces whereas the smaller, less hydrated and more polarizing anions, tend to adsorb more easily. Surfaces that are in contact with aqueous media therefore often have a net negative charge. Independently of the mechanism of charging, the surface charge will have an influence on the distribution of ions in the solution in the vicinity of the interface.

In order to balance the surface charge, counter-ions are attracted to the surface whereas co-ions are repelled. The layer of adsorbed ions and the loosely associated counter ions (the Stern layer) is only a couple of Ångström thick. Surrounding the Stern layer there is a cloud of ions that undergo thermal motion that together with the charged surface forms the *diffuse electric double layer*.<sup>[130]</sup> The overlap of the diffuse electric double layers of two similarly charged particles dispersed in aqueous media will result in a repulsive force, known as the electrostatic double layer repulsion force. The origin of the repulsive force is not the electrostatic contribution (which actually is attractive) but entropic.<sup>[130]</sup> When two charged surfaces are brought together, there is a decrease in the

configurational entropy of the ions in the diffuse double layer that leads to an osmotic repulsion.

### 6.3 Hydration forces and the hydrophobic effect

Water is a remarkable solvent that has a number of interesting features due to its small size and high polarity. Water has a profound effect on the chemistry of life and the stability and structure of dissolved compounds. At separations below a few nanometres, colloids in water experiences a force that arises from the presence and ordering of water molecules at the interface.<sup>[133]</sup> This *hydration force* is thought to account for the stability of many biologically important colloidal systems such as vesicles of phospholipid bilayers,<sup>[134]</sup> and can be monotonically repulsive and in some cases oscillatory.<sup>[130, 133]</sup>

Water molecules in contact with nonpolar (hydrophobic) solutes have to arrange in such a way that the loss of hydrogen bonds are minimized.<sup>[130]</sup> The reorganization around the inert solute is entropically unfavourable as it disrupts the existing water structure and induces a new and more ordered structure. To reduce the surface exposed to the surrounding water, nonpolar molecules tend to aggregate as this reduces the total free energy of the system. This effect, known as the *hydrophobic effect*, prevents nonpolar substances from being dissolved in aqueous media and is also, as was previously mentioned, an important contribution in protein folding where the hydrophobic residues aggregate in order to avoid contact with water.

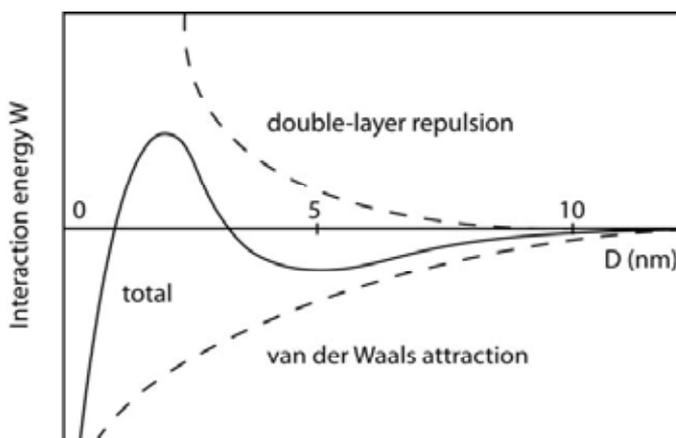
### 6.4 Steric- and bridging forces

Adsorption of macromolecules, such as polymers, on the surface of colloid particles has a significant impact on their stability. The resulting interactions can either be attractive or repulsive depending on the properties of the adsorbed molecules, the solvent, and how the molecules are grafted to the surface.<sup>[130]</sup> When the outer layers of two polymer-coated surfaces overlap they experience a repulsive osmotic force due to the decrease in configurational entropy as the polymer chains are compressed.<sup>[135]</sup> This force is commonly referred to as the *steric- or overlap repulsion* and is extensively used in various industrial processes to prevent particles from aggregating. The range of the steric force increases with

the size of the polymer and may extend as far as ten times the radius of gyration ( $R_G$ ).<sup>[136]</sup> The grafting density and the nature of the polymer solvent interactions are also of great importance.<sup>[130, 137]</sup> In a theta solvent (a solvent where the individual polymer segments do not interact with each other), the repulsive force is larger as compared to in a poor solvent where the polymer chain is less extended. In poor solvents polymer/polymer interactions can be more favourable than polymer/solvent interactions, which can result in segments bridging between the polymer-coated particles giving rise to an attractive force. Bridging can also occur if the grafting density is low, exposing available surface sites were polymers grafted to an opposing surface can bind. Attractive bridging forces can under suitable conditions be considerably stronger than any van der Waals attraction between two surfaces. The range of the force is dependent on the size of the adsorbed polymers and has a decay length that is about the same as the radius of gyration of the tails and loops protruding from the surface.

## 6.5 DLVO-theory

The most classical theoretical work on colloidal stability, DLVO-theory, was proposed by Derjaguin and Landau, and Verwey and Overbeek in 1941 and 1948, respectively.<sup>[130]</sup> In DLVO theory the total interaction energy is treated as the sum of the electrostatic and van der Waals contributions and predicts that the van der Waals attractions always exceeds the double-layer repulsion at short distances, and that particle stability is highly dependent on the electrolyte concentration and surface potential. Highly charged particles in dilute electrolytes usually experience strong repulsion and are therefore very stable. The repulsion typically peaks at a distance ( $D$ ) between 1 and 4 nm, illustrated by the energy barrier in Figure 6.1. At higher electrolyte concentrations, the diffuse double layer is compressed (shielding) and there will be a significant secondary minimum in the energy-distance profile. If the energy barrier to the primary minimum is high enough, the particles will either be in the secondary minimum or remain dispersed. In this case the particles are referred to as kinetically stable. If the surface charge density is lower also the energy barrier to the primary minimum will be lower which results in less stable particles and eventually aggregation. At low enough surface charge, the particle stability will be totally dependent on the van der Waals attraction. Unlike the double-layer interaction, the van der Waals interactions are not so sensitive to changes in the electrolyte concentration or pH.



**Figure 6.1** Schematic illustration of the energy ( $W$ ) versus distance ( $D$ ) profiles described by DLVO-theory. The solid line is the total sum of the interaction energies of the van der Waals and double-layer interactions (broken lines). Picture adopted from <sup>[130]</sup>.

## 6.6 Controlling nanoparticle stability using the JR-polypeptides

Controlling nanoparticle assembly means controlling the forces between the particles. In this thesis a number of strategies for stability control have been investigated that have exploited the versatile properties of the JR-polypeptides.

Immobilization of JR2EC on gold nanoparticles profoundly affected the stability of the particles. JR2EC-modified particles were stable under conditions where unmodified particles would rapidly and irreversibly aggregate, such as in the presence of high concentrations of salt, certain buffers and when subjected to hard centrifugations. The main reason for the observed stability is presumably the relatively high negative net charge of the peptide emanating from the large number of acidic amino acids. The importance of the electrostatic stabilization was clearly demonstrated when gradually protonating these residues by lowering the pH (Paper II). The isoelectric point (pI), which is the pH where the net charge of a polyelectrolyte is zero, was calculated to be 4.56 for JR2EC. Well above this pH the JR2EC-modified particles were stable, whereas at pH 4-4.5 the particles aggregated. The particles were redispersed again when the pH was raised and the charge repulsion was restored. At lower pH ( $\text{pH} < 3.5$ ) the peptide obtains a

positive net charge as the positively charged residues outnumber any remaining unprotonated acidic groups. Although this charge is not enough to disrupt the homodimer in solution, it was well enough to prevent the peptide-modified particles from aggregating. Some of the properties of the free polypeptides were thus transferred to the particles when immobilized, which enabled a strategy for controlling particle stability. Particle aggregation further allowed for peptide monomers immobilized on adjacent particles to dimerize and fold, which resulted in a well defined interparticle distance that corresponded to the size of the four-helix bundle. Particles functionalized with the reference peptide (JR2ECref) also aggregated in the same pH interval, but the resulting redshift of the LSPR-peak was considerably larger indicating a smaller interparticle separation, which was also confirmed using TEM. Here, the ability of the peptides to fold did clearly not influence the stability of the particles, although the morphology of the aggregates was affected.

At neutral pH, JR2EC fold in the presence of  $Zn^{2+}$ . When immobilized on gold nanoparticles, exposure to  $Zn^{2+}$  caused a rapid and extensive particle aggregation (Paper III). Interestingly,  $Zn^{2+}$  did not induce any aggregation of particles modified with the reference peptide (JR2ECref), indicating that the ability of the peptides to fold was a prerequisite for causing aggregation. The two peptide monomers formed sub-monolayers on planar gold surfaces with comparable properties regarding coverage, conformation, and ability to interact with  $Zn^{2+}$ . It is thus very likely that the coordination of  $Zn^{2+}$  by the immobilized JR2EC monomers reduced the interparticle charge repulsion enough to allow for peptides on adjacent particles to dimerize and fold. The interactions involved in folding then contributed in destabilizing the particles and to keep the particles in the aggregated state. The particles were redispersed when the  $Zn^{2+}$  was removed and the charge repulsion restored.

A second example of folding-induced particle assembly is presented in Paper VI where the hetero-association between the disulphide linked peptide JR2KC<sub>2</sub> and immobilized JR2EC was investigated. Upon association with two JR2EC monomers on separate particles, JR2KC<sub>2</sub> formed a heterotrimeric complex that bridged between the particles resulting in a bridging flocculation. Also in this case, the ability to fold was essential as no aggregation was obtained for the JR2ECref-modified particles.

*“SAMs provide the needed design flexibility, both at the individual molecular and at the material levels, and offer a vehicle for investigation of specific interactions at interfaces, and of the effect of increasing molecular complexity on the structure and stability of two-dimensional assemblies.” Ullman, 1996.<sup>[139]</sup>*

## **7. Surface functionalization of gold**

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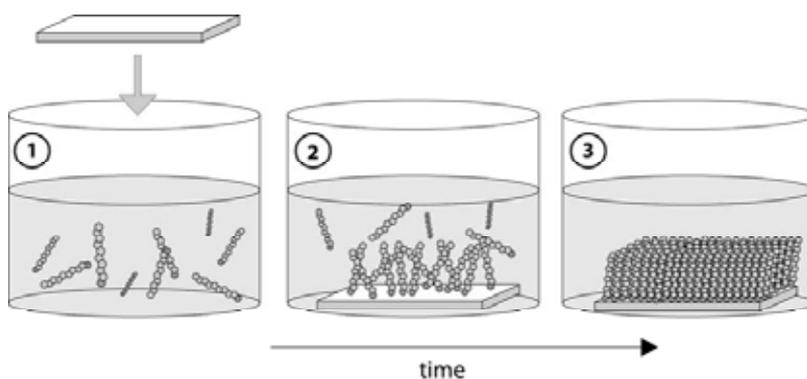
Organic material readily adsorb on metal surfaces as this lowers the free energy of the interface between the metal and the ambient environment. The adsorption of organic molecules can have a dramatic influence on the interfacial properties of the metal and by controlling the chemical composition and orientation of the adsorbed species these properties can be tailored with great precision.

### **7.1 Self-Assembled Monolayers**

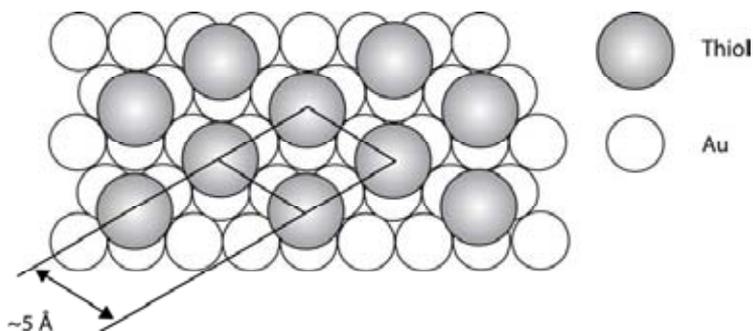
The technique for preparation of molecular self-assembled monolayers (SAMs) on semi-flat two-dimensional gold surfaces was first described by Nuzzo and Allara in 1983,<sup>[138]</sup> and have been extensively employed to achieve various types of functional surfaces.<sup>[139, 140]</sup> Gold does not only afford a surface that is stable to oxidation and corrosion but can also readily bind organosulfur compounds, such as thiols and disulphides, with high affinity. A thiol/gold bond is covalent but slightly polar with a binding strength of approximately 170 kJ/mol.<sup>[141]</sup> As a result of the strong interaction, the thiols strive to occupy every possible binding site on the surface. The assembly process can be divided into two kinetically different steps (Figure 7.1) where the first reaction, the pinning of the thiols to the surface is very rapid and

occurs within seconds after immersion of the surface in a thiol containing solution. The second step is more time consuming and generally requires more than 12 hours to complete, and involves ordering of the chemisorbed molecules. This step is driven by the weak lateral interactions between molecules on the surface, such as van der Waals interactions. Depending on the chemical properties of the molecules, the lateral interactions may result in the formation of very homogenous, well-ordered, crystalline-like monolayers.

The sulphur atoms in monolayers of alkanethiols have a hexagonal symmetry on Au(111) surfaces with an S-S spacing of 4.97 Å, forming a hexagonal ( $\sqrt{3}\times\sqrt{3}$ )R30° overlayer (Figure 7.2).<sup>[139]</sup> Since the van der Waals diameter of an alkyl chain is slightly smaller, approximately 4.5 Å, the alkyl chains are tilted about 26-28° in order to optimize the lateral interactions within the monolayer. The stabilizing van der Waals force increases with the length of the alkyl chains and molecules with longer alkyl chains tend to exhibit a larger fraction of all-trans conformers, i.e. are better ordered, than smaller molecules.



**Figure 7.1** A schematic illustration describing the process of preparing self-assembled monolayers on planar gold surfaces. A clean gold surface is immersed in a thiol or disulphide containing solution (1). The initial adsorption step (2) is very fast, but organization into a well-ordered monolayer usually takes more than 12 hours (3).



**Figure 7.2** Alkanethiols adsorbed on a (111) Au surface as a  $(\sqrt{3} \times \sqrt{3})R30^\circ$  hexagonal overlayer with a nearest neighbour distance of approximately 5 Å.

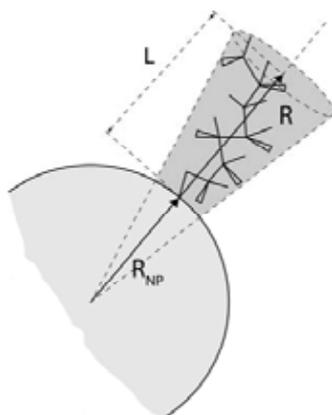
## 7.2 Surface functionalization of gold nanoparticles

The principles for assembly of monolayers on nanoparticles are about the same as on flat 2D-surfaces. There are however a few differences between a particle and a planar surface, and some practical issues that that has to be taken into consideration. One important aspect is that on the surface of dispersed particles, molecules or ions that promote particle stability are already adsorbed. Removal of these stabilizers can cause an irreversible aggregation of the particles and they must therefore be replaced by molecules that do not jeopardize stability. Furthermore, the surface of a nanoparticle is finite in size and often presents a lot of defects. It may also present variable crystal facets and not only the (111) lattice plane commonly used for two-dimensional SAMs. In particular, the number of edges and corners increases as the size of the particles decreases, whereas the number of (111) facets on gold nanoparticles increases with increasing particle diameter.<sup>[142]</sup> On smaller particles ( $r < \sim 5$  nm) the curvature of the surface can also have a substantial influence on the conformation of adsorbed species.<sup>[143, 144]</sup> On planar surfaces the adsorbate can occupy a volume in space with a cylindrical geometry, whereas on surfaces with high curvature the available space is conical. The smaller the particles the larger will the conical segment be, which decreases the stabilizing lateral interactions between adsorbed molecules and allow for a more flexible conformation. Assuming that the surface density of molecules

are identical on a planar and a spherical surface, molecules that protrude the distance  $L$  nanometres from the surface will then when immobilized on a particle with radius  $R_{NP}$  have access to a fractionally larger volume, where the volume ratio ( $f$ ) is given by (eq 7.1):

$$f = \frac{V_{sphere}}{V_{planar}} = \frac{1}{3} \left( \frac{R^3 - R_{NP}^3}{LR_{NP}^2} \right) \quad (\text{eq. 7.1})$$

where  $R$  is the radius of the sphere described by  $R = R_{NP} + L$ , as illustrated in Figure 7.3. A molecule protruding 2 nm from the surface of a particle with  $R_{NP} = 6.5$  nm, can thus occupy a ~30% larger volume than the corresponding molecule immobilized on a planar substrate. The smaller the particles the larger will the difference in accessible volume be. Furthermore, the higher curvature on spherical particles may result in a higher number of adsorbate molecules per metal surface atom, from the bulk value of one thiol per three gold surface atoms to roughly one thiol per two gold surface atoms on clusters 2 nm in diameter.<sup>[143, 145]</sup>



**Figure 7.3** A schematic illustration of the available space for a 1-octanethiol on the surface of a gold nanoparticle, where  $R_{NP}$  is the radius of the gold nanoparticle,  $R$  the radial distance, and  $L$  the protrusion length of the molecule, respectively.

On planar gold surfaces amines only form weak bonds and chemically very unstable monolayers.<sup>[146]</sup> On nanoparticles, on the other hand, the amines are reported to bind in a manner best described by a weak covalent bond. The

amine/gold surface interaction is charge neutral but much weaker than the thiol/gold bond.<sup>[147, 148]</sup> Specific and directed immobilization of molecules rich in primary amines, such as JR2KC, on gold surfaces and gold nanoparticles can turn out to be problematic as the sum of the weak amine/gold interactions presumably can be quite large, resulting in multiple attachment points between the molecule and the surface. The affinity of amine groups for gold nanoparticles can however also be useful. For example, Natan et al have utilized 2-mercaptoethylamine as a bi-functional linker to capture and assemble gold nanoparticles in 2-dimensional arrays on planar gold substrates.<sup>[149]</sup> A similar strategy was employed in Paper IV where gold nanoparticles were anchored to aminosilane functionalized silica surfaces.

### 7.3 Immobilization of the JR- and KE-peptides

The thiol group of Cys in JR2EC, JR2KC and KE2C facilitates directed immobilization on gold substrates but can also be used to covalently couple the peptides to functionalized surfaces through other types of immobilization strategies, such as the thiol-ligand coupling to carboxy-methylated dextrane hydrogel matrixes<sup>[150]</sup> utilized in Paper I or through Michael-addition to maleimide groups on maleimide-terminated SAMs<sup>[151]</sup> described in Paper IV.

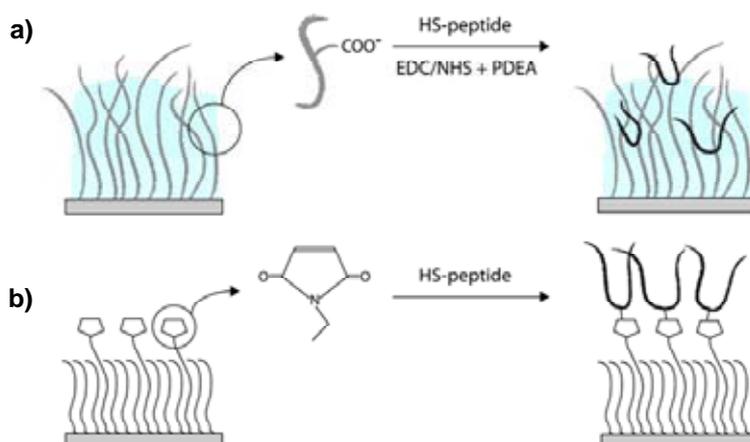
#### 7.3.1 Immobilization on planar gold

Immobilization of JR2EC and JR2KC at pH 7 on bare gold substrates rendered approximately 1-1.3 nm thick films. The film thickness was converted into a surface mass concentration,  $\Gamma$ , using the method developed by Stenberg and Nygren (eq. 7.2),<sup>[152]</sup> where the density of a dry protein layer is approximately  $\rho_0 = 1.27 \text{ g/cm}^3$ , the refractive index for a dry and hydrated protein layer is  $N_{\text{dry}} = 1.465$  and  $N_{\text{hydrated}} = 1.55$ , respectively, which gives the constant  $K$ , the value of  $K \approx 1.2 \times 10^{-6} \text{ ng/mm}^3$ .

$$\Gamma = d_{\text{dry}} \cdot \rho_0 = d_{\text{hydrated}} \cdot \frac{1 - 1/N_{\text{hydrated}}^3}{1 - 1/N_{\text{dry}}^2} \cdot \rho_0 \approx K \cdot d_{\text{dry}} \quad (\text{eq. 7.2})$$

Assuming that a single monomer of JR2EC or JR2KC occupies an area of  $2 \times 2 \text{ nm}^2$ , a 1 nm film thickness thus corresponds to approximately 60% of monolayer, or  $\sim 10^{13}$  peptides/cm<sup>2</sup>.

When immobilized from buffered loading solutions at pH 7, the IR-spectra and film thicknesses of JR2EC and JR2KC monolayers suggested that the peptides were immobilized as random coil monomers. When the complementary peptides were introduced, only JR2EC-functionalized surfaces responded with an increase in thickness and changes in the IR-spectra that indicated conformational alterations (Paper I). JR2KC thus seemed unable to dimerize and fold when immobilized on bare gold surfaces. Although primary amines only form a weak bond to gold surfaces,<sup>[153]</sup> the cooperative effect arising from the large number of Lys residues in JR2KC presumably kept the peptide monomers tightly bound to the surface, thus reducing its ability to interact and dimerize with JR2E. In order to facilitate the dimerization of immobilized JR2KC, the peptides were immobilized via a linker layer comprised of either a maleimide-terminated EG<sub>3</sub> containing SAM or a carboxy-methylated dextran matrix (Figure 7.4).



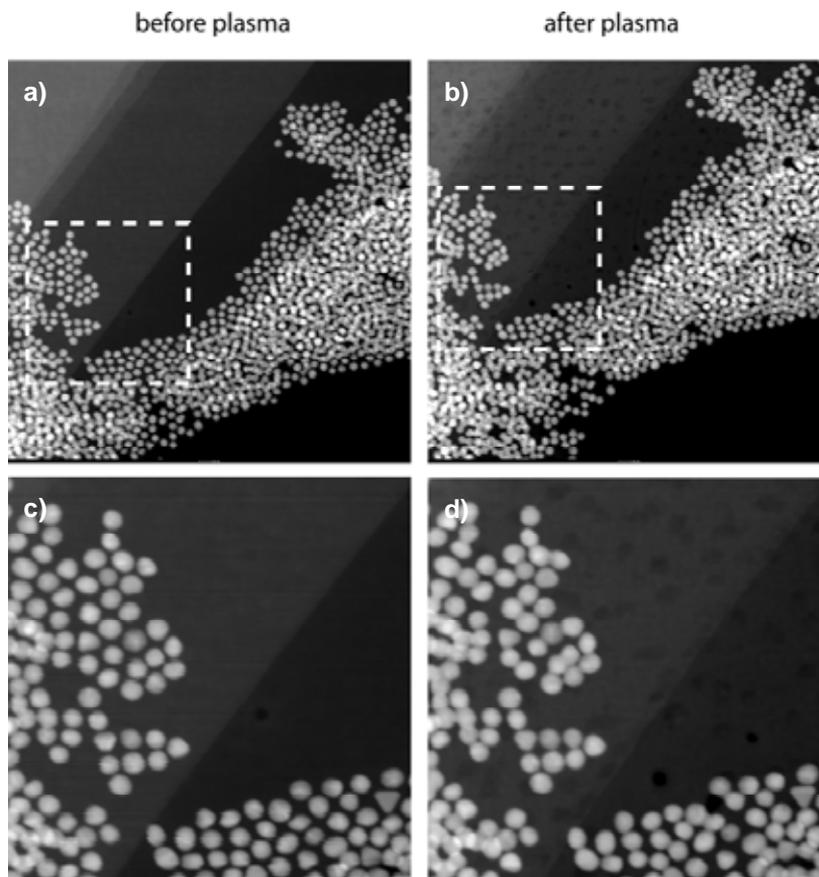
**Figure 7.4** Two strategies for covalent immobilization of JR2EC and JR2KC on modified gold substrates: a) Coupling to a carboxy-methylated dextran matrix using carbodiimide chemistry followed by derivatization with 2-(2-pyridyldithio)ethylamine. The thiol containing peptides are then coupled to the surface through a thiol-disulphide exchange reaction. b) Thiol containing peptides can also be covalently linked to monolayers displaying a maleimide group through a Michael addition.

For immobilization in the dextran matrix the carboxyl-groups were first activated using conventional carbodiimide chemistry (EDC/NHS) followed by a derivatization with 2-(2-pyridinyldithio)ethylamine (PDEA).<sup>[150]</sup> These strategies enabled JR2KC to dimerize with JR2E when immobilized. JR2EC on the other hand was able to both hetero- and homodimerize when immobilized directly on bare gold surfaces.

### 7.3.2 Immobilization on gold nanoparticles

Immobilization of JR2KC on gold nanoparticles was only possible at pH > 10. At lower pH the particles immediately and irreversibly aggregated upon addition of the peptide. This loss of stability is most probably correlated to the properties of JR2KC that prevented dimerization when immobilized on planar gold surfaces, and related to the large number of Lys residues. If a majority of the Lys residues are directed towards the gold surface the peptides exposes a large number of hydrophobic residues to the surrounding water, which severely destabilizes the particles. Also, as the citrate stabilized particles are negatively charged and JR2KC highly positively charged, the peptide might cause an unspecific bridging aggregation of the particles.

Immobilization of JR2EC on gold nanoparticles was on the other hand rather straightforward and the peptide modified particles displayed excellent stability. The JR2EC functionalized particles could be exposed to various buffers and high concentrations of salt (> 200 mM) without aggregating. The high stability of the particles enabled the use of repeated centrifugations to remove excess peptides remaining in solution after immobilization. The ability of the peptides to dimerize and fold was, as was mentioned previously, retained when immobilizing them on gold nanoparticles, which enabled a convenient approach for controlling the assembly and organization of the particles (Paper II, III, IV and VI). As estimated from electron micrographs, the distances between the aggregated particles were in general quite homogenous and in good agreement with the expected size of the folded peptides. Scanning Electron Transmission Microscopy (STEM) images (Figure 7.5) clearly illustrates how this organization can be disrupted when the peptides are removed using an Argon plasma.



**Figure 7.5** Electron micrographs obtained using Scanning Electron Transmission Microscopy (STEM), of JR2EC decorated particles, assembled at pH 4.5, before and after Ar plasma treatment. The areas within the squares in a) and b) are shown in higher magnification in c) and d), respectively.

Immobilization of KE2C and KE2C-C6 on gold nanoparticles was also possible at close to neutral pH without presenting any large difficulties. As is described in Paper VII, the ability of the sensor peptide to bind its target protein, human carbonic anhydrase II, was preserved upon immobilization.

*”The particles in these fluids are remarkable for a set of physical alterations occasioned by bodies in small quantities, which do not act chemically on the gold, or change its intrinsic nature; for through all of them it seems to remain gold in a fine state of division.”*, Faraday, 1857.<sup>[92]</sup>

## 8. Gold nanoparticles in biosensors

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A *biosensor* is a device that utilizes the interactions of biological, biologically derived or biomimetic sensing elements for detection of compounds in combination with a transducer that facilitates the readout.<sup>[154, 155]</sup> In general a biosensor exploits the excellent ability of some biomolecules to discriminate between similar compounds and to bind the target molecule (analyte) with high affinity. The binding event induces changes in physio-chemical parameters, e.g. refractive index, that can be detected by the transducer. A biosensor that is designed for single use (i.e. disposable) and that cannot continuously monitor the analyte concentration is usually referred to as a *bioprobe*.<sup>[156]</sup>

Gold nanoparticles have been extensively utilized as a component in various biosensors and bioprobes due their large surface-to-volume ratio, robustness and optical properties that enable detection of small changes in refractive index close to the particle surface.<sup>[101, 157]</sup> As was discussed in chapter 5.2, nonpropagating localized plasmon excitations (LSPR) can be resonantly excited on gold nanoparticles. A change in the dielectric environment close to the particle surface alters the resonance conditions, which consequently gives rise to a shift in both the intensity and position of the LSPR peak. Nanoparticles functionalized with biomolecules able to recognize an analyte can thus be utilized in label-free biosensors where the binding of the analyte induces a detectable change in refractive index in the vicinity of the particle surface. The sensitivity depends on

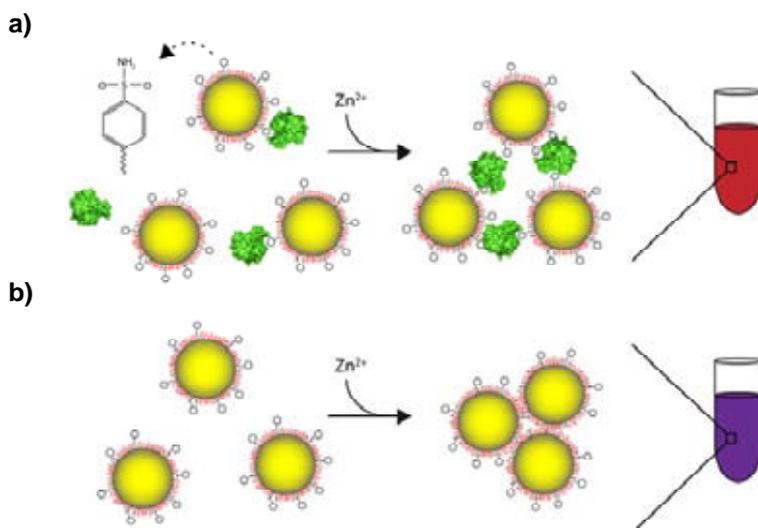
the type of metal, size and morphology of the particle. For spherical gold nanoparticles ~13 nm in diameter, a peak position sensitivity of ~76 nm/refractive index unit (RIU) has been reported.<sup>[158]</sup> Higher sensitivities, ~167 nm/RIU and ~252 nm/RIU, have been obtained for silver nanoparticles,<sup>[159]</sup> and gold nanorods,<sup>[160]</sup> respectively. These figures are significantly lower than the refractive index sensitivity obtained using flat surface SPR sensors, which is about  $10^3$ - $10^4$  nm/RIU in the wavelength range 600-1000 nm.<sup>[161]</sup> The decay length of the plasmonic fields associated with nanoparticles is however considerably shorter (~10 nm) than for flat gold (~100 nm), and adsorbed species will hence occupy a larger fraction of the total sensing volume, which makes them more surface sensitive. For biosensor applications, the particles can either be dispersed<sup>[162]</sup> or bound to a substrate,<sup>[157]</sup> where the latter strategy has the advantage of not being sensitive to particles aggregation.

Particle aggregation can, however, be advantageous if it can be controlled and e.g. occurs as a result of binding of the analyte.<sup>[101, 119]</sup> The shifts in the LSPR peak position and intensity upon aggregation are very large, which produce a colour change that can be readily detected by the naked eye. This type of colorimetric detection can be made very robust and simple but still provide extremely low limit of detection. The use of controlled nanoparticle aggregation for sensor applications was pioneered by Mirkin et al, in a paper where aggregation of gold nanoparticles modified with single stranded DNA was induced upon addition of a complementary strand.<sup>[23]</sup> The demonstrated strategy was sensitive enough to allow for detection of single base pair mismatches.<sup>[118]</sup> The principle is similar to that of conventional latex agglutination and sol particle immunoassays which have existed for decades, but relies on the large colour shift induced upon nanoparticle aggregation that facilitates a very simple readout.<sup>[163, 164]</sup> The field has experienced a rapid development and a large number of papers on oligo-nucleotide-functionalized nanoparticles for sensor applications have appeared.<sup>[119, 165-167]</sup>

Although immunoassays using antibody modified metal nanoparticles has been around for more than twenty years,<sup>[98, 99]</sup> colorimetric detection of proteins exploiting the large spectral shifts upon particle aggregation is less widely reported. Examples of such assays involve particles modified with antibodies and other proteins,<sup>[163, 168]</sup> and carbohydrates<sup>[169, 170]</sup>.

### 8.1 Colorimetric biosensing using KE2C-C6

The established procedures for colorimetric biosensing using gold nanoparticles typically rely on nanoparticle aggregation induced by binding of an analyte, which results in large and easily detectable colour shifts.<sup>[101]</sup> Nanoparticles are however very rarely stable and easily aggregate as a result of high salt concentrations or unspecific interactions, which can result in false positives. False positives may be more easily avoided in an assay where the particles are redispersed in the presence of the analyte,<sup>[171]</sup> or aggregate in the absence of an analyte. In Paper VII a sensor concept based on particles functionalized with a mixed monolayer of JR2EC and KE2C-C6, is described. The interaction of JR2EC with  $Zn^{2+}$  (Paper III) was utilized to control particle aggregation whereas analyte binding was provided by KE2C-C6. Binding of the target protein (HCAII) prevented the formation of dense aggregates upon addition of  $Zn^{2+}$  and the dispersion remained red. In the absence of the target protein the particles aggregated extensively (Figure 8.1). The presence of the analyte could thus be determined by the naked eye.



**Figure 8.1** a) Binding of HCAII to the polypeptide functionalized particles prevented the formation of dense aggregates upon addition of  $Zn^{2+}$ , and the dispersion remained red. b) In the absence of HCAII, addition of  $Zn^{2+}$  induced a rapid colour shift from red to purple.



*“There are no facts, only interpretations.”* Nietzsche (1844 - 1900).

## 9. Summary of the papers

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Seven papers are included in this thesis, of which five are published in peer review journals, one is published as a conference proceeding and one is in manuscript form.

### Paper I

K. Enander, D. Aili, L. Baltzer, I. Lundström, B. Liedberg, “Alpha-Helix Inducing Dimerization of Synthetic Polypeptide Scaffolds on Gold”, *Langmuir*, **2005**, 21, 2480-2487.

The aim of the paper was to investigate the properties of JR2E and JR2K when immobilized on gold substrates, primarily using ellipsometry and infrared absorption-reflection spectroscopy. The Val in position 22, located in the loop region, was replaced by a Cys to facilitate directed, thiol-dependent immobilization. The Cys containing peptides were designated JR2EC and JR2KC, respectively.

Both JR2EC and JR2KC formed monolayers with film thicknesses of about 1 nm when immobilized from buffered loading solutions. Immobilized JR2KC was unable to heterodimerize with the complementary peptide JR2E. JR2EC on the other hand was able to heterodimerize and fold when exposed to JR2K.

When immobilized from unbuffered loading solutions (pH ~5) JR2EC was immobilized as a homodimer. Utilizing CD-spectroscopy and surface plasmon resonance (SPR) the dissociation constants for heterodimerization in solution ( $K_{d,sol} = 0.02$  mM) and when immobilized in a dextran matrix ( $K_{d,immob} = 0.2$  mM) were determined.

## Paper II

D. Aili, K. Enander, J. Rydberg, I. Lundstrom, L. Baltzer, B. Liedberg, "Aggregation-Induced Folding of a De Novo Designed Polypeptide Immobilized on Gold Nanoparticles", *J. Am. Chem. Soc.*, **2006**, 128, 2194-2195.

This paper describes how dimerization and folding of JR2EC, immobilized on gold nanoparticles, can be induced upon particle aggregation. The influence of folding was assessed using a reference polypeptide (JR2ECref) with the same primary sequence as JR2EC but without folding ability. In this peptide all L-Ala residues were exchanged by D-Ala, while keeping all other amino acids in the L-state. By mixing D- and L-amino acids in the sequence the polypeptide is prevented from folding into four-helix bundles under any circumstances.

When the pH was lowered close to the isoelectric point ( $pI_{calc} \sim 4.6$ ) of the immobilized peptides, the loss of charge repulsion caused extensive particle aggregation. Aggregation occurred irrespectively of the ability to fold. The redshift of the plasmon peak was however significantly smaller for particles functionalized with JR2EC, indicating a larger interparticle distance. Electron micrographs revealed that the interparticle distance for JR2EC-functionalized particles ( $d = 2.3 \pm 0.1$  nm) were considerably larger than for JR2ECref ( $d = 1.4 \pm 0.1$  nm), and the distance corresponded well to the expected size of the folded four-helix bundle. No differences in aggregate size were observed for the two peptides.

**Paper III**

D. Aili, K. Enander, I. Nesterenko, F. Björefors, L. Baltzer, B. Liedberg, "Folding Induced Assembly of Polypeptide Decorated Gold Nanoparticles", *J. Am. Chem. Soc.*, **2008**, 130, 5780-5788.

The aim of the paper was to describe how dimerization and folding of JR2EC immobilized on gold nanoparticles could be utilized to induce particle aggregation. JR2E in solution was demonstrated to fold in upon coordination of a number of metal ions,  $Zn^{2+}$  in particular. In the presence of  $Zn^{2+}$ , JR2EC decorated gold nanoparticles aggregated whereas particles with JR2ECref remained dispersed. Both peptides formed monolayers on gold with similar properties regarding coverage, organization and ability to coordinate  $Zn^{2+}$ . Particle aggregation could hence be attributed to the ability of the immobilized JR2EC to dimerize and fold. The particles could repeatedly be aggregated and redispersed by alternately adding  $Zn^{2+}$  and EDTA.

**Paper IV**

D. Aili, H. S. Han, K. Enander, L. Baltzer, B. Liedberg, "Controlled Assembly of Gold Nanoparticles using De Novo Designed Polypeptide Scaffolds", *Proc. SPIE*, **2008**, 6885, 688506-1-688506-8.

The possibility to assemble gold nanoparticles on planar surfaces using the JR-polypeptides was investigated. Thin gold films were modified with a self-assembled monolayer (SAM) of maleimide-terminated disulphides on which JR2KC was covalently immobilized. Introducing JR2EC-functionalized gold nanoparticles over the surface resulted in rapid particle assembly. Multilayers of particles could be formed by subsequently introducing JR2KC and JR2EC-functionalized particles over a surface with citrate stabilized gold nanoparticles adsorbed to (3-aminopropyl)triethoxysilane modified silicon surfaces.

## Paper V

D. Aili, F.-I. Tai, K. Enander, L. Baltzer, B. Liedberg, "Self-assembling Fibers and Nano-Rings from Disulphide-Linked Helix-Loop-Helix Polypeptides", *Angew. Chem., Int. Ed.*, **2008**, 47, 5554-5556.

The properties of oxidized JR-polypeptides were investigated. The thiol group of the Cys was utilized to link the monomers through a disulphide bridge into ~9 kDa fibre forming units, JR2KC<sub>2</sub> and JR2EC<sub>2</sub>. The disulphide-linked monomers were found to spontaneously assemble into long and extremely thin peptide fibres as a result of a propagating association mediated by folding into four-helix bundles. Fibres were obtained as a result of both hetero-association (hetero-fibres) or homo-association (homo-fibres), depending on pH. In addition to extended fibres, hetero-fibres occasionally assembled into closed loops, or nano-rings with diameters ranging from ~400 nm to 5 µm.

## Paper VI

D. Aili, K. Enander, L. Baltzer, B. Liedberg, "Assembly of Polypeptide-Decorated Gold Nanoparticles through a Hetero-Association and Folding-Dependent Bridging", *Nano Lett.*, **2008**, 8, 2473-2478.

The disulphide-linked polypeptide JR2KC<sub>2</sub> was utilized to induce a reversible aggregation of JR2EC-functionalized gold nanoparticles exploiting the ability of JR2KC<sub>2</sub> to associate with two JR2EC molecules. Upon association, the polypeptides formed a heterotrimeric complex that folded into two disulphide-linked four-helix bundles. Extensive particle aggregation was induced upon addition of the linker polypeptide to the JR2EC-decorated particles. The interparticle distance, as measured from electron micrographs, was 4.6±0.2 nm, which is in good agreement with the expected size of the heterotrimeric complex. No aggregation was obtained for particles decorated with JR2EC<sub>ref</sub>, which demonstrated the importance of folding. Nor was any aggregation obtained upon addition of JR2K which indicated that JR2KC<sub>2</sub> must bridge between peptides immobilized on two separate particles. The particles were redispersed when the disulphide bridge in JR2KC<sub>2</sub> was reduced.

**Paper VII**

D. Aili, R. Selegård, L. Baltzer, K. Enander, B. Liedberg, “Colorimetric Protein Sensing by Controlled Assembly of Gold Nanoparticles Functionalized with Synthetic Receptors”, *In manuscript*.

This paper describes a strategy for colorimetric protein detection, based on induced assembly of polypeptide functionalized gold nanoparticles. Recognition of the target protein was carried out using a polypeptide (KE2C-C6) designed to specifically bind the enzyme human carbonic anhydrase II (HCAII). The extent of particle aggregation induced by the coordination of  $Zn^{2+}$  to a second polypeptide (JR2EC) also present on the nanoparticle surface gave a readily detectable colorimetric shift depending on the concentration of the target protein. In the absence of HCAII, particle aggregation resulted in a major redshift of the plasmon peak whereas binding of the target protein prevented formation of dense aggregates and the magnitude of the redshift was hence significantly lower. Detection of carbonic anhydrase down to ~15 nM was possible. The versatility of the technique was further illustrated using a second model system based on the recognition of a short peptide from the tobacco mosaic virus coat protein by an antibody fragment.



*"What is it with humans and size anyways? Just because something is very, very small doesn't mean that it can't be important!" Men in Black (Movie, 1998).*

## 10. Future outlook

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Nanotechnology as science may still be embryonic and few practical applications have yet been made commercially available. There is however a strong belief in that the rapid progress within this field will cause an industrial revolution in the 21<sup>st</sup> century. Besides the conventional top-down approaches, biomolecular self-assembly has emerged as an appealing and promising route for fabrication of materials and devices with feature sizes at the nanoscale. Nanobiotechnology provides the tools for designing novel synthetic versions of many of the interesting materials found in nature, such as spider silk, bone and enamel, etc, that in many aspects are far more advanced than most manmade materials and that possess many attractive mechanical and chemical properties.

As was mentioned in the introduction, biomolecular assisted assembly of nanomaterials is not an entirely new idea. The polypeptides studied in this thesis, however, provide a new and interesting set of building blocks for this purpose and with a great potential to be further developed into a more complex nano-Lego-like toolbox. The most attractive and perhaps unique properties of these peptides are the numerous ways to control their association and folding and also that this ability is preserved when the peptides are immobilized or linked into fibre-forming units. The peptides can most likely, with only minor modifications, be utilized in a large number of potentially interesting

supramolecular materials by for example making units where three or more monomers are covalently linked to a common scaffold. Depending on the geometry and nature of this scaffold a variety of interesting assemblies can be created.

The polypeptides can also be utilized as a template for assembly of other molecules. It has previously been demonstrated that the JR-polypeptides can form a supramolecular complex with certain types of conjugated polyelectrolytes (CPEs).<sup>[5, 172]</sup> Initial experiments have shown that also fibres of JR-polypeptides can be decorated with the polythiophene-based polyelectrolyte PTAA. Although the conductivity of the utilized molecules is very low, they demonstrate a possibility to extend the applications of polypeptide based fibres into the area of organic electronics.

As the materials studied in this thesis are environmentally responsive, i.e. changes properties as a result of changes in the physiochemical environment, they have many potentially interesting applications in e.g. biosensors and as biomaterials. The ability to combine the JR- and KE-polypeptides to simultaneously control nanoparticle assembly and provide molecular recognition has been demonstrated as a simple and relatively robust procedure for colorimetric detection of proteins. Novel synthetic receptors with more biologically relevant targets are currently being developed which will add more relevance to the procedure for biosensing described in paper VII.

The immobilization of the peptides does not have to be restricted to gold surfaces and gold nanoparticles. For example, membrane bound polypeptides can be utilized as a model system for studies of protein/membrane interactions and to control the assembly of vesicles. The incorporation of biomolecules into model lipid membranes is interesting from many perspectives and has been utilized among others for self-sorting of vesicles, and fundamental studies of membrane biophysics. Anchoring of the JR-polypeptides to membranes can be facilitated by the incorporation of reactive groups in the membranes to which the peptides can be bound or by the modification of the peptides with cholesterol groups or fatty acid chains.

The dimerization and folding of JR-polypeptides might then be utilized to control the assembly of vesicles into larger aggregates or for fusion of vesicles. The polypeptides can also be utilized to study lateral mobility and assembly of molecules in supported lipid bilayer membranes in order to simulate raft formation. Initial studies of making “fibre crossings” where three polypeptides are connected via a tri-maleimide have been performed. Such architectures, in combination with the peptide fibres can be utilized to create peptide hydrogels or to connect the membrane bound peptides to artificial cytoskeleton-like structures.



## References

- [1] Feynman, R. P., There's Plenty of Room at the Bottom - An Invitation to Enter a New Field of Physics, *Engineering and Science*, **1960**, 23, 22-36.
- [2] Taniguchi, N., On the Basic Concept of Nano-Technology, In: *Proc. Intl. Conf. Prod. Eng. Tokyo, Part II*, Japan Society of Precision Engineering, Tokyo, **1974**.
- [3] Drexler, K. E., *Engines of Creation. The Coming Era of Nanotechnology*, Doubleday, New York, **1986**.
- [4] Nordling, C., Österman, J., *Physics handbook for science and engineering*, Studentlitteratur ;Chartwell-Bratt, Lund, **1996**.
- [5] Nilsson, K. P. R., Rydberg, J., Baltzer, L., Inganäs, O., Twisting macromolecular chains: Self-assembly of a chiral supermolecule from nonchiral polythiophene polyanions and random-coil synthetic peptides, *P. Natl. Acad. Sci. U.S.A.*, **2004**, 101, 11197-11202.
- [6] Whitesides, G. M., Grzybowski, B., Self-assembly at all scales, *Science*, **2002**, 295, 2418-2421.
- [7] Whitesides, G. M., Mila, B., Beyond molecules: Self-assembly of mesoscopic and macroscopic components, *P. Natl. Acad. Sci. U.S.A.*, **2002**, 99, 4769-4774.
- [8] Lehn, J.-M., Supramolecular Chemistry And Self-assembly Special Feature: Toward complex matter: Supramolecular chemistry and self-organization, *P. Natl. Acad. Sci. U.S.A.*, **2002**, 99, 4763-4768.
- [9] Kirschner, M., Mitchison, T., Beyond Self-Assembly - from Microtubules to Morphogenesis, *Cell*, **1986**, 45, 329-342.
- [10] Kirschner, M., Gerhart, J., Mitchison, T., Molecular "vitalism", *Cell*, **2000**, 100, 79-88.
- [11] Lehn, J. M., Toward self-organization and complex matter, *Science*, **2002**, 295, 2400-2403.
- [12] Whitesides, G. M., Mathias, J. P., Seto, C. T., Molecular Self-Assembly and Nanochemistry: A Chemical Strategy for the Synthesis of Nanostructures, *Science*, **1991**, 254, 1312-1319.
- [13] Ariga, K., Kunitake, T., *Supramolecular Chemistry - Fundamentals and Applications: Advanced Textbook*, Springer Verlag, Heidelberg, **2006**.
- [14] Zubay, G. L., *Biochemistry 4:th ed.*, The McGraw-Hill Companies, Inc., **1998**.
- [15] Seeman, N. C., DNA in a material world, *Nature*, **2003**, 421, 427-431.
- [16] Mao, C., Sun, W., Shen, Z., Seeman, N. C., A nanomechanical device based on the B-Z transition of DNA, **1999**, 397, 144-146.
- [17] Chen, Y., Wang, M., Mao, C., An Autonomous DNA Nanomotor Powered by a DNA Enzyme, *Ang. Chem., Int. Ed.*, **2004**, 43, 3554-3557.
- [18] Adleman, L. M., Molecular Computation of Solutions to Combinatorial Problems, *Science*, **1994**, 266, 1021-1024.
- [19] Rothemund, P. W. K., Folding DNA to create nanoscale shapes and patterns, *Nature*, **2006**, 440, 297-302.

- [20] He, Y., Ye, T., Su, M., Zhang, C., Ribbe, A. E., Jiang, W., Mao, C., Hierarchical self-assembly of DNA into symmetric supramolecular polyhedra, *Nature*, **2008**, 452, 198-201.
- [21] Björk, P., Herland, A., Scheblykin, I. G., Inganäs, O., Single molecular imaging and spectroscopy of conjugated polyelectrolytes decorated on stretched aligned DNA, *Nano Lett.*, **2005**, 5, 1948-1953.
- [22] Björk, P., Holmström, S., Inganäs, O., Soft lithographic printing of patterns of stretched DNA and DNA/electronic polymer wires by surface-energy modification and transfer, *Small*, **2006**, 2, 1068-1074.
- [23] Mirkin, C. A., Letsinger, R. L., Mucic, R. C., Storhoff, J. J., A DNA-based method for rationally assembling nanoparticles into macroscopic materials, *Nature*, **1996**, 382, 607-609.
- [24] Alivisatos, A. P., Johnsson, K. P., Peng, X., Wilson, T. E., Loweth, C. J., Bruchez, M. P., Schultz, P. G., Organization of 'nanocrystal molecules' using DNA, *Nature*, **1996**, 382, 609-611.
- [25] Hinman, M. B., Jones, J. A., Lewis, R. V., Synthetic spider silk: a modular fiber, *Trends Biotechnol.*, **2000**, 18, 374-379.
- [26] Herland, A., Björk, P., Nilsson, K. P. R., Olsson, J. D. M., Asberg, P., Konradsson, P., Hammarström, P., Inganäs, O., Electroactive luminescent self-assembled bio-organic nanowires: Integration of semiconducting oligoelectrolytes within amyloidogenic proteins, *Adv. Mater.*, **2005**, 17, 1466-1471.
- [27] S. Mann, W. S., M. Li, S. Connolly, D. Fitzmaurice., Biologically Programmed Nanoparticle Assembly, *Adv. Mater.*, **2000**, 12, 147-150.
- [28] Niemeyer, C. M., Functional Hybrid Devices of Proteins and Inorganic Nanoparticles, *Ang. Chem., Int. Ed.*, **2003**, 42, 5796-5800.
- [29] Baltzer, L., Functionalization and Properties of Designed Folded Polypeptides, *Top. Curr. Chem.*, **1999**, 202, 39-76.
- [30] Baltzer, L., Nilsson, H., Nilsson, J., De novo design of proteins - What are the rules?, *Chem. Rev.*, **2001**, 101, 3153-3163.
- [31] Pandya, M. J., Spooner, G. M., Sunde, M., Thorpe, J. R., Rodger, A., Woolfson, D. N., Sticky-End Assembly of a Designed Peptide Fiber Provides Insight into Protein Fibrillogenesis, *Biochemistry*, **2000**, 39, 8728-8734.
- [32] Ryadnov, M. G., Ceyhan, B., Niemeyer, C. M., Woolfson, D. N., "Belt and braces": A peptide-based linker system of de novo design, *J. Am. Chem. Soc.*, **2003**, 125, 9388-9394.
- [33] Stevens, M. M., Flynn, N. T., Wang, C., Tirrell, D. A., Langer, R., Coiled-coil peptide-based assembly of gold nanoparticles, *Adv. Mater.*, **2004**, 16, 915-918.
- [34] Hull, D., Clyne, T. W., An Introduction to Composite Materials, Cambridge Solid State Science Series, **1996**.
- [35] Meyers, M. A., Lin, A. Y. M., Seki, Y., Chen, P.-Y., Kad, B. K., Bodde, S., Structural Biological Composites: An Overview, *JOM*, **2006**, 58, 35-41.
- [36] Wagner, H. D., Nanocomposites: Paving the way to stronger materials, *Nature*, **2007**, 2, 742-744.
- [37] Sanchez, C., Julian, B., Belleville, P., Popall, M., Applications of hybrid organic-inorganic nanocomposites, *J. Mater. Chem.*, **2005**, 15, 3559-3592.

- [38] KICKELBICK, G., Hybrid materials synthesis, characterization, and applications, Wiley - VCH, Weinheim, **2007**.
- [39] FURST, D., OSBORN, M., NAVE, R., WEBER, K., The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy: a map of ten nonrepetitive epitopes starting at the Z line extends close to the M line, *J. Cell Biol.*, **1988**, 106, 1563-1572.
- [40] CREIGHTON, T. E., Proteins : structures and molecular properties, W.H. Freeman, New York, **1993**.
- [41] FERSHT, A., Structure and mechanism in protein science : a guide to enzyme catalysis and protein folding, W.H. Freeman, New York, **1999**.
- [42] BRANDEN, C.-I., TOOZE, J., Introduction to protein structure, Garland Pub., New York, **1999**.
- [43] ROTHMAN, J. E., Polypeptide Chain Binding Proteins: Catalysts of Protein Folding and Related Processes in Cells, *Cell*, **1989**, 59, 591-601.
- [44] BARDWELL, J. C. A., BECKWITH, J., The Bonds That Tie: Catalyzed Disulfide Bond Formation, *Cell*, **1993**, 74, 769-771.
- [45] LEVINTHAL, C., How to fold graciously, In: "Mössbaun Spectroscopy in Biological Systems" *Proceedings of a meeting held at Allerton house, Monticello, Illinois.* (eds) P. De-Brunner, J. Tsibris, E. Munck, University of Illinois Press, **1969**, 67, 22-24.
- [46] KARPLUS, M., The Levinthal paradox: yesterday and today, *Fold. Des.*, **1997**, 2, 69-75.
- [47] ONUCHIC, J. N., WOLYNES, P. G., Theory of protein folding, *Curr. Opin. Struc. Biol.*, **2004**, 14, 70-75.
- [48] DILL, K. A., Dominant forces in protein folding, *Biochemistry*, **1990**, 29, 7133-7155.
- [49] PRIVALOV, P. L., Physical basis of the stability of the folded conformations of proteins, In: *Protein folding*, Freeman and Co., New York, **1992**.
- [50] FRANKS, F., Water : a matrix of life, RSC paperbacks, Royal Society of Chemistry, Cambridge, **2000**.
- [51] BRANDEN, C., TOOZE, J., Introduction to proteins structure 2:nd ed, Garland Publishing, Inc., New York, **1999**.
- [52] FRAUENFELDER, H., SLIGAR, S. G., WOLYNES, P. G., The Energy Landscapes and Motions of Proteins, *Science*, **Dec. 13, 1991**, 254, 1598-1603.
- [53] BERMAN, H. M., WESTBROOK, J., FENG, Z., GILLILAND, G., BHAT, T. N., WEISSIG, H., SHINDYALOV, I. N., BOURNE, P. E., The Protein Data Bank, *Nucl. Acids Res.*, **2000**, 28, 235-242.
- [54] POROLLO, A. A., ADAMCZAK, R., MELLER, J., POLYVIEW: a flexible visualization tool for structural and functional annotations of proteins, *Bioinformatics*, **2004**, 20, 2460-2462.
- [55] NAKANISHI, K., SAKIYAMA, T., IMAMURA, K., On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon, *J. Biosci. Bioeng.*, **2001**, 91, 233-244.
- [56] LUNDSTRÖM, I., ELWING, H., Simple Kinetic-Models for Protein Exchange-Reactions on Solid-Surfaces, *J. Colloid. Interf. Sci.*, **1990**, 136, 68-84.
- [57] CAESSENS, P. W. J. R., DE JONGH, H. H. J., NORDE, W., GRUPPEN, H., The adsorption-induced secondary structure of beta-casein and of distinct parts of its sequence in

- relation to foam and emulsion properties, *B.B.A.-Protein Struct. M.*, **1999**, 1430, 73-83.
- [58] Vermeer, A. W. P., Conformation of adsorbed proteins, In, *Encyclopedia of surface and colloid science*, Marcel Dekker, New York, **2002**.
- [59] Verma, A., Nakade, H., Simard, J. M., Rotello, V. M., Recognition and Stabilization of Peptide  $\alpha$ -Helices Using Templatable Nanoparticle Receptors, *J. Am. Chem. Soc.*, **2004**, 126, 10806-10807.
- [60] Martin Lundqvist, P. N., Bengt-Harald Jonsson, Klas Broo., Induction of Structure and Function in a Designed Peptide upon Adsorption on a Silica Nanoparticle13, *Ang. Chem., Int. Ed.*, **2006**, 45, 8169-8173.
- [61] Nygren, P., Lundqvist, M., Broo, K., Jonsson, B.-H., Fundamental Design Principles That Guide Induction of Helix upon Formation of Stable Peptide-Nanoparticle Complexes, *Nano Lett.*, **2008**, 8, 1844-1852.
- [62] Fabian, H., Mäntele, W., Infrared spectroscopy of proteins, In: *Handbook of Vibrational Spectroscopy*, J. Wiley, New York, **2002**.
- [63] Bandekar, J., Amide Modes and Protein Conformation, *Biochim. Biophys. Acta.*, **1992**, 1120, 123-143.
- [64] Herrebout, W., Clou, K., Desseyn, H. O., Blaton, N., Vibrational characterization of the peptide bond, *Spectrochim Acta A*, **2003**, 59, 47-59.
- [65] Jackson, M., Mantsch, H. H., The Use and Misuse of FT-IR Spectroscopy in the Determination of Protein-Structure, *Crit. Rev. Biochem. Mol.*, **1995**, 30, 95-120.
- [66] Krimm, S., Bandekar, J., Vibrational Spectroscopy and Conformation of Peptides, Polypeptides, and Proteins, *Adv. Protein. Chem.*, **1986**, 38, 181-364.
- [67] Koenig, J. L., Tabb, D. L., Infrared spectra of globular proteins in aqueous solution, In: *Analytical Applications of FT-IR to Molecular and Biological Systems*, (eds) D. Reidel Publishing Co., Dordrecht, **1980**.
- [68] Kato, K., Matsui, T., Tanaka, S., Quantitative Estimation of Alpha-Helix Coil Content in Bovine Serum-Albumin by Fourier Transform-Infrared Spectroscopy, *Appl. Spectrosc.*, **1987**, 41, 861-865.
- [69] Greenfield, N. J., Fasman, G. D., Computed circular dichroism spectra for the evaluation of protein conformation, *Biochemistry*, **1969**, 8, 4108-4116.
- [70] Johnson Jr., W. C., Protein secondary structure and circular dichroism: A practical guide, *Proteins*, **1990**, 7, 205-214.
- [71] Kelly, S. M., Price, N. C., The Use of Circular Dichroism in the Investigation of Protein Structure and Function, *Curr. Prot. Pep. Sci.*, **2000**, 1, 349-384.
- [72] Provencher, S. W., Gloeckner, J., Estimation of globular protein secondary structure from circular dichroism, *Biochemistry*, **1981**, 20, 33-37.
- [73] Sreerama, N., Woody, R. W., A Self-Consistent Method for the Analysis of Protein Secondary Structure from Circular-Dichroism, *Anal. Biochem.*, **1993**, 209, 32-44.
- [74] W. Curtis Johnson, Analyzing protein circular dichroism spectra for accurate secondary structures, *Proteins*, **1999**, 35, 307-312.
- [75] Betz, S. F., DeGrado, W. F., Controlling topology and native like behaviour of de novo-designed peptides: design and characterisation of antiparallel four-stranded coiled coils, *Biochemistry*, **1996**, 35, 6955-6962.

- [76] Hill, R. B., DeGrado, W. F., Solution structure of a2D, a native-like de novo designed protein, *J. Am. Chem. Soc.*, **1998**, 120, 1138-1145.
- [77] Olofsson, S., Johansson, G., Baltzer, L., Design, Synthesis and Solution Structure of a Helix-Loop-Helix Dimer - a Template for the Rational Design of Catalytically Active Polypeptides, *J. Chem. Soc. Perk. T. 2*, **1995**, 2047-2056.
- [78] Olofsson, S., Baltzer, L., Structure and dynamics of a designed helix-loop-helix dimer in dilute aqueous trifluoroethanol solution. A strategy for NMR spectroscopic structure determination of molten globules in the rational design of native-like proteins, *Fold. Des.*, **1996**, 1, 347-356.
- [79] Enander, K., *Folded Polypeptide Scaffolds for Biosensor and Biochip Applications - Design, Synthesis, Functionalization and Characterisation*, Dissertation No. 848, Linköping Studies in Science and Technology, Linköping University, Linköping, **2003**.
- [80] Rydberg, J., *Protein-protein interactions in model systems - design, control of catalytic activity and biosensor applications*, Dissertation No. 1037, Linköping Studies in Science and Technology, Linköpings universitet, Linköping, **2006**.
- [81] Enander, K., Dolphin, G. T., Baltzer, L., Designed, functionalized helix-loop-helix motifs that bind human carbonic anhydrase II: A new class of synthetic receptor molecules, *J. Am. Chem. Soc.*, **2004**, 126, 4464-4465.
- [82] Enander, K., Dolphin, G. T., Andersson, L. K., Liedberg, B., Lundström, I., Baltzer, L., Designed, folded polypeptide scaffolds that combine key biosensing events of recognition and reporting, *J. Org. Chem.*, **2002**, 67, 3120-3123.
- [83] Andersson, L., Stenhagen, G., Baltzer, L., The Site-Selective Glycosylation of a Designed Helix-Loop-Helix Polypeptide Motif, *J. Org. Chem.*, **1998**, 63, 1366-1367.
- [84] Taylor, P. W., King, R. W., Burgen, A. S. V., Kinetics of complex formation between human carbonic anhydrases and aromatic sulfonamides, *Biochemistry*, **1970**, 9, 2638-2645.
- [85] Enander, K., Dolphin, G. T., Liedberg, B., Lundström, I., Baltzer, L., A versatile polypeptide platform for integrated recognition and reporting: Affinity arrays for protein-ligand interaction analysis, *Chem.-Eur. J.*, **2004**, 10, 2375-2385.
- [86] Cortie, M., *The Weird World of the Nanoscale Gold*, *Gold Bull.*, **2004**, 37, 12-19.
- [87] Barnard, A. S., Lin, X. M., Curtiss, L. A., Equilibrium Morphology of Face-Centered Cubic Gold Nanoparticles > 3 nm and the Shape Changes Induced by Temperature, *J. Phys. Chem. B*, **2005**, 109, 24465-24472.
- [88] Petkov, V., Peng, Y., Williams, G., Huang, B., Tomalia, D., Ren, Y., Structure of gold nanoparticles suspended in water studied by x-ray diffraction and computer simulations, *Phys. Rev. B*, **2005**, 72, 195402.
- [89] Daniel, M. C., Astruc, D., Gold nanoparticles: Assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology, *Chem. Rev.*, **2004**, 104, 293-346.
- [90] Freestone, I., Meeks, N., Sax, M., Higgitt, C., The Lycurgus Cup – A Roman Nanotechnology, *Gold Bull.*, **2007**, 40, 270-277.
- [91] Barber, D. J., Freestone, I. C., An investigation of the origin of the colour of the Lycurgus Cup by analytical transmission electron microscopy, *Archaeometry*, **1990**, 32, 33.
- [92] Faraday, M., The Bakerian Lecture: Experimental Relations of Gold (and Other Metals) to Light, *Philos. T. Roy. Soc.*, **1857**, 147, 145-181.

- [93] Graham, T., Liquid Diffusion Applied to Analysis, *Philos. T. Roy. Soc.*, **1861**, 151, 183-224.
- [94] Edwards, P. P., Thomas, J. M., Gold in a Metallic Divided State - From Faraday to Present-Day Nanoscience, *Ang. Chem., Int. Ed.*, **2007**, 46, 5480-5486.
- [95] Faulk, W. P., Taylor, G. M., An Immunocolloid Method for the Electron Microscope, *Immunochem.*, **1971**, 8, 1081-1083.
- [96] Horisberger, M., Rosset, J., Colloidal gold, a useful marker for transmission and scanning electron microscopy, *J. Histochem. Cytochem.*, **1977**, 25, 295-305.
- [97] Horisberger, M., Colloidal Gold as a Cytochemical Marker in Electron Microscopy, *Gold Bull.*, **1981**, 14, 90-94.
- [98] Moeremans, M., Daneels, G., Van Dijck, A., Langanger, G., De Mey, J., Sensitive visualization of antigen-antibody reactions in dot and blot immune overlay assays with immunogold and immunogold/silver staining, *J. Immunol. Methods*, **1984**, 74, 353-360.
- [99] Hsu, Y.-H., Immunogold for detection of antigen on nitrocellulose paper, *Anal. Biochem.*, **1984**, 142, 221-225.
- [100] Bangs, L. B., New developments in particle-based immunoassays: Introduction, *Pure Appl. Chem.*, **1996**, 68, 1873-1879.
- [101] Stewart, M. E., Anderton, C. R., Thompson, L. B., Maria, J., Gray, S. K., Rogers, J. A., Nuzzo, R. G., Nanostructured Plasmonic Sensors, *Chem. Rev.*, **2008**, 108, 494-521.
- [102] Hu, M., Chen, J. Y., Li, Z. Y., Au, L., Hartland, G. V., Li, X. D., Marquez, M., Xia, Y. N., Gold nanostructures: engineering their plasmonic properties for biomedical applications, *Chem. Soc. Rev.*, **2006**, 35, 1084-1094.
- [103] Gao, J., Bender, C. M., Murphy, C. J., Dependence of the Gold Nanorod Aspect Ratio on the Nature of the Directing Surfactant in Aqueous Solution, *Langmuir*, **2003**, 19, 9065-9070.
- [104] Jin, R., Cao, Y., Mirkin, C. A., Kelly, K. L., Schatz, G. C., Zheng, J. G., Photoinduced Conversion of Silver Nanospheres to Nanoprisms, *Science*, **2001**, 294, 1901-1903.
- [105] Brust, M., Walker, M., Bethell, D., Schiffrin, D. J., Whyman, R., Synthesis of Thiol-Derivatized Gold Nanoparticles in a 2-Phase Liquid-Liquid System, *J. Chem. Soc. Chem. Comm.*, **1994**, 801-802.
- [106] Turkevich, J., Stevenson, P. C., Hillier, J., A study of the nucleation and growth processes in the synthesis of colloidal gold, *Discussions of the Faraday Society*, **1951**, 11, 55-75.
- [107] Templeton, A. C., Wuelfing, M. P., Murray, R. W., Monolayer protected cluster molecules, *Accounts. Chem. Res.*, **2000**, 33, 27-36.
- [108] Frens, G., Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Suspensions, *Nature (London) Phys. Sci.*, **1973**, 241, 20-22.
- [109] Brust, M., Fink, J., Bethell, D., Schiffrin, D. J., Kiely, C., Synthesis and Reactions of Functionalized Gold Nanoparticles, *J. Chem. Soc. Chem. Comm.*, **1995**, 1655-1656.
- [110] Chen, S., 4-Hydroxythiophenol-Protected Gold Nanoclusters in Aqueous Media, *Langmuir*, **1999**, 15, 7551-7557.

- [111] Hostetler, M. J., Green, S. J., Stokes, J. J., Murray, R. W., Monolayers in three dimensions: Synthesis and electrochemistry of omega-functionalized alkanethiolate-stabilized gold cluster compounds, *J. Am. Chem. Soc.*, **1996**, 118, 4212-4213.
- [112] Hostetler, M. J., Templeton, A. C., Murray, R. W., Dynamics of place-exchange reactions on monolayer-protected gold cluster molecules, *Langmuir*, **1999**, 15, 3782-3789.
- [113] Zheng, M., Huang, X. Y., Nanoparticles comprising a mixed monolayer for specific bindings with biomolecules, *J. Am. Chem. Soc.*, **2004**, 126, 12047-12054.
- [114] Zheng, M., Davidson, F., Huang, X. Y., Ethylene glycol monolayer protected nanoparticles for eliminating nonspecific binding with biological molecules, *J. Am. Chem. Soc.*, **2003**, 125, 7790-7791.
- [115] Dahl, J. A., Maddux, B. L. S., Hutchison, J. E., Toward Greener Nanosynthesis, *Chem. Rev.*, **2007**, 107, 2228-2269.
- [116] Chah, S., Hammond, M. R., Zare, R. N., Gold nanoparticles as a colorimetric sensor for protein conformational changes, *Chem. Biol.*, **2005**, 12, 323-328.
- [117] Verma, A., Srivastava, S., Rotello, V. M., Modulation of the interparticle spacing and optical behavior of nanoparticle ensembles using a single protein spacer, *Chem. Mater.*, **2005**, 17, 6317-6322.
- [118] Storhoff, J. J., Elghanian, R., Mucic, R. C., Mirkin, C. A., Letsinger, R. L., One-pot colorimetric differentiation of polynucleotides with single base imperfections using gold nanoparticle probes, *J. Am. Chem. Soc.*, **1998**, 120, 1959-1964.
- [119] Rosi, N. L., Mirkin, C. A., Nanostructures in biodiagnostics, *Chem. Rev.*, **2005**, 105, 1547-1562.
- [120] Willets, K. A., Van Duyne, R. P., Localized Surface Plasmon Resonance Spectroscopy and Sensing, *Annu. Rev. Phys. Chem.*, **2007**, 58, 267-297.
- [121] Mie, G., Beiträge zur Optik trüber Medien, speziell kolloidaler Metallösungen, *Ann. Phys.*, **1908**, 25, 377-445.
- [122] Link, S., El-Sayed, M. A., Shape and size dependence of radiative, non-radiative and photothermal properties of gold nanocrystals, *Int. Rev. Phys. Chem.*, **2000**, 19, 409 - 453.
- [123] Xu, H. X., Kall, M., Modeling the optical response of nanoparticle-based surface plasmon resonance sensors, *Sensor. Actuat. B-Chem.*, **2002**, 87, 244-249.
- [124] Miller, M. M., Lazarides, A. A., Sensitivity of Metal Nanoparticle Surface Plasmon Resonance to the Dielectric Environment, *J. Phys. Chem. B*, **2005**, 109, 21556-21565.
- [125] Su, K. H., Wei, Q. H., Zhang, X., Mock, J. J., Smith, D. R., Schultz, S., Interparticle coupling effects on plasmon resonances of nanogold particles, *Nano Lett.*, **2003**, 3, 1087-1090.
- [126] Chen, C.-F., Tzeng, S.-D., Chen, H.-Y., Lin, K.-J., Gwo, S., Tunable Plasmonic Response from Alkanethiolate-Stabilized Gold Nanoparticle Superlattices: Evidence of Near-Field Coupling, *J. Am. Chem. Soc.*, **2008**, 130, 824-826.
- [127] Rechberger, W., Hohenau, A., Leitner, A., Krenn, J. R., Lamprecht, B., Aussenegg, F. R., Optical properties of two interacting gold nanoparticles, *Opt. Commun.*, **2003**, 220, 137-141.

- [128] Storhoff, J. J., Lazarides, A. A., Mucic, R. C., Mirkin, C. A., Letsinger, R. L., Schatz, G. C., What controls the optical properties of DNA-linked gold nanoparticle assemblies?, *J. Am. Chem. Soc.*, **2000**, 122, 4640-4650.
- [129] Stuart, M. A. C., Fleer, G. J., Lyklema, J., Norde, W., Scheutjens, J. M. H. M., Adsorption of Ions, Polyelectrolytes and Proteins, *Adv. Colloid. Interfac.*, **1991**, 34, 477-535.
- [130] Israelachvili, J. N., Intermolecular and surface forces, Academic Press, London, **1992**.
- [131] Israelachvili, J. N., Tabor, D., The Measurement of Van Der Waals Dispersion Forces in the Range 1.5 to 130 nm, *P. Roy. Soc. Lond. A Mat.*, **1972**, 331, 19-38.
- [132] Somasundaran, P., Markovic, B., Yu, X., Krishnakumar, S., Colloid Systems and Interfaces - Stability of Dispersions through Polymer and Surfactant Adsorption, In: *Surface and Colloid Chemistry*, CRC Press LLC, Boca Roton, **2003**.
- [133] Israelachvili, J., Wennerstrom, H., Role of hydration and water structure in biological and colloidal interactions, **1996**, 379, 219-225.
- [134] Leikin, S., Parsegian, V. A., Rau, D. C., Rand, R. P., Hydration Forces, *Annu. Rev. Phys. Chem.*, **1993**, 44, 369-395.
- [135] Mackor, E. L., A theoretical approach of the colloid-chemical stability of dispersions in hydrocarbons, *J. Coll. Sci.*, **1951**, 6, 492-495.
- [136] Witten, T. A., Pincus, P. A., Colloid stabilization by long grafted polymers, *Macromolecules*, **1986**, 19, 2509-2513.
- [137] Pincus, P., Colloid stabilization with grafted polyelectrolytes, *Macromolecules*, **1991**, 24, 2912-2919.
- [138] Nuzzo, R. G., Allara, D. L., Adsorption of bifunctional organic disulfides on gold surfaces, *J. Am. Chem. Soc.*, **1983**, 105, 4481-4483.
- [139] Ulman, A., Formation and structure of self-assembled monolayers, *Chem. Rev.*, **1996**, 96, 1533-1554.
- [140] Love, J. C., Estroff, L. A., Kriebel, J. K., Nuzzo, R. G., Whitesides, G. M., Self-assembled monolayers of thiolates on metals as a form of nanotechnology, *Chem. Rev.*, **2005**, 105, 1103-1169.
- [141] Dubois, L. H., Nuzzo, R. G., Synthesis, Structure, and Properties of Model Organic-Surfaces, *Annu. Rev. Phys. Chem.*, **1992**, 43, 437-463.
- [142] van Hardefeld, R., Hartog, F., Statistics of surface atoms and surface sites on metal crystals, *Surf. Sci.*, **1969**, 15, 189-230.
- [143] Terrill, R. H., Postlethwaite, T. A., Chen, C. H., Poon, C. D., Terzis, A., Chen, A. D., Hutchison, J. E., Clark, M. R., Wignall, G., Londono, J. D., Superfine, R., Falvo, M., Johnson, C. S., Samulski, E. T., Murray, R. W., Monolayers in three dimensions: NMR, SAXS, thermal, and electron hopping studies of alkanethiol stabilized gold clusters, *J. Am. Chem. Soc.*, **1995**, 117, 12537-12548.
- [144] Weeraman, C., Yatawara, A. K., Bordenyuk, A. N., Benderskii, A. V., Effect of nanoscale geometry on molecular conformation: Vibrational sum-frequency generation of alkanethiols on gold nanoparticles, *J. Am. Chem. Soc.*, **2006**, 128, 14244-14245.
- [145] Leff, D. V., Ohara, P. C., Heath, J. R., Gelbart, W. M., Thermodynamic Control of Gold Nanocrystal Size - Experiment and Theory, *J. Phys. Chem.*, **1995**, 99, 7036-7041.

- [146] Xu, C., Sun, L., Kepley, L. J., Crooks, R. M., Ricco, A. J., Molecular Interactions between Organized, Surface-Confined Monolayers and Vapor-Phase Probe Molecules. 6. In-Situ FTIR External Reflectance Spectroscopy of Monolayer Adsorption and Reaction Chemistry, *Anal. Chem.*, **1993**, 65, 2102-2107.
- [147] Leff, D. V., Brandt, L., Heath, J. R., Synthesis and characterization of hydrophobic, organically-soluble gold nanocrystals functionalized with primary amines, *Langmuir*, **1996**, 12, 4723-4730.
- [148] Ooka, A. A., Kuhar, K. A., Cho, N. J., Garrell, R. L., Surface interactions of a homologous series of alpha.omega-amino acids on colloidal silver and gold, *Biospectroscopy*, **1999**, 5, 9-17.
- [149] Lyon, L. A., Musick, M. D., Natan, M. J., Colloidal Au-enhanced surface plasmon resonance immunosensing, *Anal. Chem.*, **1998**, 70, 5177-5183.
- [150] Biacore, *Biacore Sensor Surface Handbook*, Version AA, **2003**.
- [151] Houseman, B. T., Gawalt, E. S., Mrksich, M., Maleimide-Functionalized Self-Assembled Monolayers for the Preparation of Peptide and Carbohydrate Biochips, *Langmuir*, **2003**, 19, 1522-1531.
- [152] Stenberg, M., Nygren, H., The use of the isoscopic ellipsometer in the study of adsorbed proteins and biospecific binding reactions, *J. de Phys.*, **1983**, 44, 83-86.
- [153] Xu, C., Sun, L., Kepley, L. J., Crooks, R. M., Ricco, A. J., Molecular interactions between organized, surface-confined monolayers and vapor-phase probe molecules. 6. In-situ FT-IR external reflectance spectroscopy of monolayer adsorption and reaction chemistry, *Anal. Chem.*, **1993**, 65, 2102-2107.
- [154] Nakamura, H., Karube, I., Current research activity in biosensors, *Anal. Bioanal. Chem.*, **2003**, 377, 446-468.
- [155] Scheller, F. W., Wollenberger, U., Warsinke, A., Lisdat, F., Research and development in biosensors, *Curr. Opin. Biotech.*, **2001**, 12, 35-40.
- [156] Thevenot, D. R., Toth, K., Durst, R. A., Wilson, G. S., International union of pure and applied chemistry physical chemistry division, steering committee on biophysical chemistry analytical chemistry division, commission V.5 (electroanalytical chemistry) Electrochemical biosensors: proposed definitions and classification synopsis of the report, *Sensor. Actuat. B-Chem.*, **1996**, 30, 81.
- [157] Nath, N., Chilkoti, A., Label free colorimetric biosensing using nanoparticles, *J. Fluoresc.*, **2004**, 14, 377-389.
- [158] Nath, N., Chilkoti, A., A Colorimetric gold nanoparticle sensor to interrogate biomolecular interactions in real time on a surface, *Anal. Chem.*, **2002**, 74, 504-509.
- [159] Malynych, S., Chumanov, G., Coupled planar silver nanoparticle arrays as refractive index sensors, *J. Opt. A-Pure Appl. Op.*, **2006**, S144.
- [160] Marinakos, S. M., Chen, S., Chilkoti, A., Plasmonic Detection of a Model Analyte in Serum by a Gold Nanorod Sensor, *Anal. Chem.*, **2007**, 79, 5278-5283.
- [161] Homola, J., Piliarik, M., Surface plasmon resonance (SPR) sensors, In: *Surface plasmon resonance based sensors*, Springer-Verlag, Berlin Heidelberg, **2006**.
- [162] Englebienne, P., Use of colloidal gold surface plasmon resonance peak shift to infer affinity constants from the interactions between protein antigens and antibodies specific for single or multiple epitopes, *Analyst*, **1998**, 123, 1599-1603.
- [163] Hirsch, L. R., Jackson, J. B., Lee, A., Halas, N. J., West, J. L., A Whole Blood Immunoassay Using Gold Nanoshells, *Anal. Chem.*, **2003**, 75, 2377-2381.

- [164] Thanh, N. T. K., Rosenzweig, Z., Development of an Aggregation-Based Immunoassay for Anti-Protein A Using Gold Nanoparticles, *Anal. Chem.*, **2002**, 74, 1624-1628.
- [165] Huang, C.-C., Huang, Y.-F., Cao, Z., Tan, W., Chang, H.-T., Aptamer-Modified Gold Nanoparticles for Colorimetric Determination of Platelet-Derived Growth Factors and Their Receptors, *Anal. Chem.*, **2005**, 77, 5735-5741.
- [166] Liu, J. W., Lu, Y., Adenosine-dependent assembly of aptazyme-functionalized gold nanoparticles and its application as a colorimetric biosensor, *Anal. Chem.*, **2004**, 76, 1627-1632.
- [167] Juewen Liu, Y. L., Fast Colorimetric Sensing of Adenosine and Cocaine Based on a General Sensor Design Involving Aptamers and Nanoparticles, *Ang. Chem., Int. Ed.*, **2006**, 45, 90-94.
- [168] Thanh, N. T. K., Rosenzweig, Z., Development of an aggregation-based immunoassay for anti-protein A using gold nanoparticles, *Anal. Chem.*, **2002**, 74, 1624-1628.
- [169] Tsai, C.-S., Yu, T.-B., Chen, C.-T., Gold nanoparticle-based competitive colorimetric assay for detection of protein-protein interactions., *Chem. Commun.*, **2005**, 4273-4275.
- [170] Aslan, K., Zhang, J., Lakowicz, J. R., Geddes, C. D., Saccharide sensing using gold and silver nanoparticles - A review, *J. Fluoresc.*, **2004**, 14, 391-400.
- [171] Laromaine, A., Koh, L. L., Murugesan, M., Ulijn, R. V., Stevens, M. M., Protease-triggered dispersion of nanoparticle assemblies, *J. Am. Chem. Soc.*, **2007**, 129, 4156-4157.
- [172] Nilsson, K. P. R., Rydberg, J., Baltzer, L., Inganas, O., Self-assembly of synthetic peptides control conformation and optical properties of a zwitterionic polythiophene derivative, *P. Natl. Acad. Sci. U.S.A.*, **2003**, 100, 10170-10174.