On the Nanoscale Interactions and the Self-Assembly of Recombinant Proteins and Hybrid Nanostructures: an AFM Study

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The study presented in this Thesis is focussed on the characterization and the design of new polymeric materials, taking inspiration from the Nature. Here, new hybrid architectures in which adhesive and elastic proteins coexist with inorganic or cellulosic surfaces, or where ligand capped metal nanoclusters self-assemble in monolayer films, are investigated. Genetic engineering is used to produce new synthetic fusion proteins having specific functionalities starting from microbes. The particle self-assembly is indeed inspired on the symmetrical and directional arrangement of natural architectures such as globular proteins and viral capsids. The study is fundamental and performed at nanoscale level. Single molecular interactions on surfaces are analysed as well as the structure and the conformation of individual fusion proteins. The self-assembly process of protein films is deeply studied as well as the stiffness and elastic modulus of self-assembled silver nanocluster composite films. The candidate proteins for making biohybrids are hydrophobins, cellulose binding modules and resilins. Hydrophobins (HFB), with their unique assembly mechanism, are well known for their hydrophobic patch, that strongly bind to hydrophobic surfaces. Cellulose binding modules (CBMs), turned out to be highly interesting domains for their binding affinity to their primary substrate, the cellulose. On the other hand, resilin, for its ability to dissipate energy upon tensile stress, could find use as a sacrificial bond in high strength materials. Atomic force microscope (AFM) is here used for detecting the binding and interaction forces between proteins and surfaces. For the resilin, this such powerful tool is also used to characterize the length of the biopolymer under different environments, upon stretching. AFM was also employed for determining the elastic modulus of the nanocluster monolayers. According to the results achieved, HFB1 ranged a quite high adhesion force value near 100 pN on the chosen hydrophobic surfaces, whereas the CBMs reported a binding affinity for different kind of cellulosic surfaces between 40-50 pN. The silver nanoclusters ligand-capped films revealed an elastic modulus value around 20 GPa. The Thesis sheds light on the importance of replacing plastic materials with new bio hybrids for a more sustainable approach, in an age where the ecosystem risks to be compromised by pollution and not biodegradable waste.
Acknowledgments

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I strongly thank Professor Karin Jacobs, the head of the Experimental Physics group in Saarbrucken to have given to me the possibility to join as visitor to the group, for the financial support and to always let me feel welcome and part of her lovely group. Your balance of strength and kindness represented for me an example of leadership.

I thank the collaborative research centre of Germany (SFB) for the financial support.
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<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
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<td>ATPS</td>
<td>Aqueous Two-Phase Separation</td>
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<td>AgNCs</td>
<td>Silver Nanoclusters</td>
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<td>ATR</td>
<td>Attenuated Total Reflection</td>
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<td>Au-NPs</td>
<td>Gold nanoparticles</td>
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<td>CBHI</td>
<td>Cellobiohydrolase I</td>
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<td>CBMs</td>
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<td>ChNCs</td>
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<td>CNCs</td>
<td>Cellulose Nanocrystals</td>
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<td>dCBM-RLP-HFBI</td>
<td>Double Cellulose Binding Modules-Resilin like Polypeptide-Hydrophobin type I class II</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>$F_A$</td>
<td>Adhesion Force</td>
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<td>$FD$</td>
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<td>Glycosylated Hydrophobin</td>
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<td>FT-IR</td>
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<td>$F_I$</td>
<td>Force – Indentation</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>HFB</td>
<td>Hydrophobin</td>
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<td>HFBI</td>
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<td>HFBI-D30N-K32Q</td>
<td>Mutant of hydrophobin type I class II</td>
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<td>HFBIII</td>
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<tr>
<td>HFBI-DCBD</td>
<td>Hydrophobin type I class II – double cellulose binding</td>
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<td>Abbreviation</td>
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<tr>
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<tr>
<td>HOPG</td>
<td>Highly Ordered Pyrolytic Graphite</td>
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<td>IMF</td>
<td>Intermolecular Forces</td>
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<td>P</td>
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<td>pMBA</td>
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<td>QCM-D</td>
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<td>QNM</td>
<td>Quantitative Nanomechanical Mapping</td>
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<td>REC</td>
<td>Resilin-elastin-collagen</td>
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<td>RLP</td>
<td>Resilin-Like Polypeptide</td>
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<tr>
<td>SMFS</td>
<td>Single Molecule Force Spectroscopy</td>
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<td>SpyCatcher</td>
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<td>ST</td>
<td>SpyTag</td>
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<td>ST-GFP-ST</td>
<td>SpyTag-Green Fluorescent Protein-SpyTag</td>
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<td>TCI</td>
<td>Crystallinity Index</td>
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<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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<td>TMSC</td>
<td>Trimethylsilyl Cellulose</td>
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<tr>
<td>Tyr</td>
<td>Tyrosine</td>
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<tr>
<td>W_a</td>
<td>Work of Adhesion</td>
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<td>WCA</td>
<td>Water Contact Angle</td>
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<td>WLC</td>
<td>Worm-like Chain model</td>
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List of Publications

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


Author’s Contribution

**Publication 1: Single-Molecule Force Spectroscopy Study on Modular Resilin Fusion Protein**

The author carried out the experiments, analysed the data and had a leading role in writing the paper. In detail, sample preparation by spin coating, dip coating and drop casting. Characterization by AFM both for Force Measurements and imaging. WCA, QCM-D and ellipsometry. Analysed and interpreted the data. Dr. H. Hähl designed the Matlab app for analyse the FD curves and helped in data analysis and into the interpretation of the results. Dr. S. Grandthyll and Dr. F. Muller carried out the XPS measurements and analysis. Dr. A. Paananen, Dr. M. Ilmén, Dr. G. R. Szilvay, Dr. C. P. Landowski and Prof. M. Penttilä expressed and purified the fusion proteins dCBM–RLP–HFBI and HFBI–dCBM. Prof. K Jacobs helped in the design of the experiments and interpretation of the results. Prof. P. Laaksonen supervised the project and guided the writing process of the paper.

**Publication 2: Binding Forces of Cellulose Binding Modules on Cellulosic Nanomaterials**

The author carried out the experiments, analysed the data and mostly wrote the paper. In detail sample preparation by spin coating and drop casting, AFM tip chemical functionalization. Characterization by AFM both force measurements and imaging, FT-IR experiments, WCA. Analysed and interpreted the data. Dr. H. Hähl designed the Matlab app for analyse the FD curves and helped in data analysis, interpretation of the results. B. J. M. Rooijakkers expressed and purified the fusion proteins Cel7A-CBM1 and SpyCatcher (Spy-C). Prof. M. B. Linder helped in the design of the experiments. Prof. P. Laaksonen supervised the project and the writing process.

**Publication 3: Elastic monolayer membrane via hydrogen bonding directed self-assembly of atomically precise nanoparticles**

The author carried out the experiments, analysed the data and wrote the part of the paper related to AFM indentation experiments, results and discussion. In detail, characterization by AFM both indentation measurements and imaging. Analysed and interpreted the data. Dr. H. Hähl designed the Matlab app for analyse the $F\delta$ curves and helped consistently in the interpretation of the results. Dr. A. Som prepared the samples for TEM and AFM characterization,
performed the optical and TEM characterization, wrote the paper. Prof. T. Pradeep and Prof. O. Ikkala conceived, directed the experimental research. Dr. I. Chakraborty and Dr. B. Mondal assisted in the synthesis of the silver nanoclusters in the early experiments. Prof. Päivi Laaksonen supervised the project, helped in the interpretation of the results. Dr. Nonappa supervised the project and wrote the paper. A. Griffo, Dr. A. Som and I. Chakraborty contributed equally to the manuscript.

**Publication 4: Dynamic assembly of class II hydrophobins at air-water interface**

The author performed the surface tension experiments and analysed the corresponding data. Helped in writing the manuscript. Dr. Hendrik Hähl performed the kinetics characterization, AFM imaging, analysed the data. Dr. M. Liemann expressed and purified the fusion proteins HFBI, HFBI, HFBI-dCBM, HFBI-D30N-K32Q and FpHYD5. Prof. Karin Jacobs, Dr J. Heppe, and Dr. S. Backes contributed in the design practical work of the kinetics experiments. Prof. Ludger Santen and Neda Safaridekhone performed the theoretical work. Prof. M. Linder supervised the protein design and characterization. Prof. P. Laaksonen supervised the project, planned the surface tension measurements and contributed in writing the paper.
1. Introduction

Nature is fascinating. Nature surrounds us with various functionalities and exciting materials. In natural materials, a showcase of heterogeneous components with dissimilar properties exist in close contact. The combination of those components confer to the material unique features that are very difficult to obtain for a material consisting of a single component alone, as a metal, a polymer or a protein. Taking inspiration from these materials, biomolecular components have been recently studied for building nanocomposites with low friction, high adhesiveness and mechanical performance.

In fact, biological composites show exceptional combined properties, being at the same time light-weight, stiff and tough. Among the most attractive materials in Nature showing such properties, there are nacre and bone (Figure 1). In nacre, the combination of a soft elastic matrix arranged in organic plates yields in a resilient and strong structure whereas in the bone, the structure consisting of a matrix of calcium phosphate crystals, in the form of hydroxyapatite, dispersed between structural proteins, mostly collagen, implies a greater energy-absorbing capacity. The native biomolecular building blocks are currently mimicked by novel engineered biomolecules including proteins and carbohydrates and their conjugates with synthetic molecules.

Of equal interest is another class of nanocomposites, namely self-assemblies of metal clusters consisting of inorganic cores and organic capping ligands. These assemblies are held together via supramolecular interactions and take inspiration in the supramolecular self-assembly of biological particles, such as globular proteins that can act as subunits of closed shells in the viral capsids. The development of 2D nanocluster lattices guided by supramolecular assembly is a rapidly progressing research area toward new functional materials with extraordinary mechanical properties, despite the eco-sustainable production aspect needs to be more treated and researchers are playing many effort on that. Nowadays, in fact, the request of sustainable technology is not an option anymore but mostly a necessity and is getting societal impact and political interest. In a world where plastics dominate and the ecosystem is merely compromised, a more environmentally friendly approach is requested.

In this scenario, protein engineering is a compelling tool to “create” building blocks materials starting from microbes. Microbes are extremely versatile organisms that produce proteins and, synthetic biology together with protein engineering allow their programming for creating materials according to our
needs. Already in some expositions leather-like materials made up by the mycelium of the fungus\textsuperscript{12} are accessible. For developing new materials, an examination on the potential properties of the building blocks is fundamental. Our research aims to investigate at nanoscale the properties of bio-based and biomimetic nanostructures that have the potential in novel materials. The focus in this Thesis is on the mechanical and adhesive properties as well as on the assembly mechanism of the proteinic domains proposed.

![Figure 1](image)

**Figure 1.** Sketch of the nacre architectural arrangement where inorganic platelets are separated by elastic biopolymers A) and Transmission Electron Microscopy TEM view of the microarchitecture of sheet nacre revealing the intercrystalline organic phase B). Schematics of the components of bone starting from collagen fibrils C) and light micrograph of a fractured section through a fossilized human femur D). TEM micrograph of a mineralized collagen fibril from turkey tendon E). (Adapted from Z. Tang et al. 2003, Ph. Stempflé et al. 2010, J. Sun et al. 2012, S. Weiner et al. 1998, R. Kane et al. 2013).

**Atomic Force Microscopy (AFM)** revealed, since its invention (1986) by Binnig, Quate, and Gerber\textsuperscript{13}, a fascinating tool in biophysics by which it is possible to detect for instance, adhesion and interaction forces between interfaces and the elasticity and stiffness of thin films. Here, this powerful tool is employed first to study the adhesiveness of recombinant fusion proteins (containing hydrophobin domains or cellulose binding modules) on surfaces and the conformational change of elastic coils \textit{i.e.} resilin upon pulling force. Secondly, AFM is also used to investigate the stiffness of the 2-dimensional nanoparticle lattices guided by supramolecular assembly.
In this section, the single proteins used as building blocks are first described and then their fusions are discussed for their role in bio-nanocomposites. Secondly, the bio inspired nanocluster assemblies and their function in nanomaterials science are described. Eventually, the interaction forces at surfaces and interfaces are depicted as well as the growing importance of AFM in Biophysics.

### 1.1 Genetic engineering and protein domains

Protein engineering is nowadays mentioned as a highly promising technique and its methods and applications are becoming increasingly important and widespread. With the term protein engineering is meant the design of new enzymes or proteins having new or desirable functions. It is based on the use of recombinant DNA technology to combine amino acid sequences. Recombinant DNA represents a piece of DNA that has been created by the combination of at least two strands.

The general process depicted in Figure 2 includes cloning, transformation and eventually growth, expression and purification of the proteins. Briefly, plasmids including different genes are combined with the genes of an organism such as *E. coli* or *P. pastoris*. Protein expression of the recombinant DNA will occur inside the cells, leading in the production of recombinant proteins. The final steps will include cell lysis, to extract and collect the proteins and the other components, protein purification, to separate the proteins from the other components, and gel electrophoresis, for analysing the proteins in a fluid or an extract.

Proteins that can result from the expression of recombinant DNA within living cells are termed recombinant proteins; when two or more genes that originally coded for separate proteins are joint together, recombinant fusion proteins are created. Here, through the rational design, different functional protein domains originating from different organisms can be combined.

The fusion proteins studied in this Thesis are of two types: one including adhesive (hydrophobins) and elastic coils (resilin) and the other one based on protein domains found in cellulase enzymes (cellulose binding domains).

**Figure 2.** Schematic illustration of the recombinant protein production starting from the genetic material contained inside the plasmid. The proteins are first expressed inside the organism and eventually taken out and purified. (Adapted by T. Hoffmann et al. 2012).
1.1.1 Hydrophobins

Hydrophobins (HFB) are small (≈7–10 kDa), amphiphilic proteins found in filamentous fungi, with diameter of 3 nm and remarkably high elasticity when forming a layer at air-water interface, about 0.5 Nm$^{-1}$. The primary structure is characterized by a conserved pattern of eight cysteine residues that form four intramolecular disulfide bridges. According to early hydrophobin research$^{16,17}$, hydrophobins are classified into two classes, I and II, based on differences in hydrophathy patterns and biophysical properties$^{19,20}$. Class I hydrophobin dissolves in strong acids whereas class II (Figure 3) aggregates could dissolve in aqueous dilutions of organic solvents$^{21}$. In our study, class II hydrophobins HFBI and HFBII will be investigated.

Hydrophobins can greatly adhere to surfaces and can be displayed into the formation of protective layers during fruiting body development$^{22}$, coating agents, to enhance the biocompatibility of surfaces for potential applications in biodevices i.e. promoting the serum protein adhesion$^{23,24}$.

![Figure 3. Structure of class II hydrophobin HFBI. The green part represents the hydrophobic patch and the upper part the hydrophilic side. (Adapted by G. R. Szilvay et al. 2006).](image)

Previous studies have shown how hydrophobins and its fusion proteins are suitable materials for the design of hybrid materials$^{25,26}$. For example, composite films of nanofibrillated cellulose (NFC), a hydrophobin class II fusion protein connected to two cellulose binding domains (HFBI-DCBD), and exfoliated graphene flakes were prepared and the tensile tests showed remarkably good mechanical properties$^{27}$. Functional property of hydrophobins has also been utilized for the formation of protein–nanotube composite structures, and this interaction was further used to create hybrid with gold nanoparticles (AuNPs), combining their optical and electronic functions in new ways$^{27}$.

**Self-assembly at air water interface**

Hydrophobins have appeared so far rather unique proteins for the mechanism of spontaneous 2D film formation, more properly termed as self-assembly process. The atypical assembly mechanism of class II hydrophobin HFBI has been revealed by the analysis of the lowering of surface tension by the investigation
of the shape of drops hanging from syringe needles (Figure 4 b-e). After protein assembly, the Young-Laplace equation, which is the standard method for measuring interfacial tension, was not applicable, since the drops of HFBI hydrophobin solutions had deformed profiles.

Figure 4. Schematic of the HFBI drop-surface transfer process onto a HOPG substrate A) and hanging drops of 10µg*ml⁻¹ HFBI after 60 min B) 100 µg*ml⁻¹ after 90 C) and 105 min D). Pendant drop profile shapes of HFBI overtime in water E). Trapezoid-like profile of HFBI drop on a hydrophobic solid after 30 min in ambient environment F). AFM topography images of HFBI drop-Langmuir Schaefer film G). (Adapted from G. R. Szilvay et al. 2006).

Another atypical behavior has been encountered in films drop casted on hydrophobic surfaces. In fact, the drop profile passes from a round shape to a planar one and after a while to a trapezoid-like shape (Figure 4 a, f). Those statements are clearly connected to the spontaneous formation of an elastic-like film at the interface as well as to the high coherence and elasticity of the films. It is then a relevant question to undertake the mechanism behind the self-assembly process of hydrophobins, which bring to a well-ordered hexagonal-like structure at the interfaces.

Surface modification with hydrophobins

Due to their amphiphilic nature, hydrophobins can adhere to both hydrophilic and hydrophobic surfaces (Figure 5). Of great interest is the adhesion of HFBI and HFBII to hydrophobic surfaces by their hydrophobic patch. For example hydrophobin HFBII conjugated with a fluorine-containing compound was used to fluorinate the surfaces of poorly reactive hydrophobic polymers in a more environmentally friendly manner. In fact HFBII, via electrostatic interactions, enables the otherwise inefficient binding of a phosphate-terminated perfluoropolyether onto polystyrene, polypropylene, and low-density polyethylene surfaces. Of similar interest was the investigation of the change in the wettability properties of hydrophobic surfaces as teflon, improving in this way the cell adhesion and the biocompatibility. Finally, hydrophobin coated surfaces can enhance the lubrication in biological systems as a result of a combination of
tenacious attachment of the water molecules to the charges on such molecules. For such all applications, knowing the adhesion force of a single molecule on hydrophobic or hydrophilic surfaces is a fundamental question to answer; for instance, it can help for the choice of the surface type on which adhere the hydrophobins.

1.1.2 Resilin-like proteins

Resilin is an elastomeric protein found in arthropods and especially in insects (Figure 6 a-c). It is identified as a product of the *Drosophila melanogaster* gene that is composed of 4 exons that encode 4 functional segments and plays a crucial role for the jumping mechanism and the flight of bugs. By secondary structure analysis, resilin appears as an intrinsically unstructured or disordered protein (Figure 6d). The conformational disorder and flexibility derive from the high content of glycine and proline residues, and contribute to its mechanical features. The characterizing feature of resilin is its capacity to be deformed reversibly without loss of energy. It can reach long extension under little force applied and, if completely hydrated, shows a perfect rubber-like behavior, with a rather low stiffness, an extensional length up to three times and a compression of one-third of its original length. Earlier studies have reported a Young’s modulus value of 0.6–0.7 and 0.9 MPa for resilins respectively in the elastic tendons of dragonflies and locust ligaments.
Resilin-like polypeptide (RLP) has been generated alone or as a fusion protein with elastin and collagen like molecules (REC), by genetic engineering and cloning techniques with controlled amino acids sequences. Because of its properties, RLP has been applied as a building block in composites or in material science in general\textsuperscript{43}. It also finds potential uses in tissue engineering and in the medical field\textsuperscript{44–46}. In fact, recent studies reported that it is multi-functional and multi-stimuli responsive; including temperature, pH, moisture, ion and photo-responsive with tuneable photo-physical properties\textsuperscript{48}. Furthermore, recombinant resilin was found to retain properties in equilibrium between a structured globular protein and a denatured protein\textsuperscript{47}. When engineered with cellulose binding modules, RLP can potentially be used for cellulose crosslinking. Recent studies\textsuperscript{48} show that the employment of the modified resilin in cellulose hydrogel and nanopaper increase their modulus of stiffness through a cross-linking effect.

**PH responsiveness of resilin-like proteins**

The pH responsiveness of resilin make it an attractive material for sensor applications. Earlier work has shown a relation between conformational changes and pH (Figure 7). It has been reported that by tuning pH from 5 to 12 the protein conformation tunes from globular to extended coil where stronger protein–water interaction is observed\textsuperscript{49}. Moreover, at elevated pH, the unordered secondary structure of resilin goes through a change towards a more ordered β turn structure\textsuperscript{50}. The main causes of the extended “brush like” resilin conformation,
at pH higher than the isoelectric point (IP), reside into the hydration and the electrostatic repulsion of the charged groups.

![Figure 7](image)

**Figure 7.** Sketch of the conformational changes of resilin associated to pH tuning. (Adapted from M. Y. Truong *et al.* 2010).

### 1.1.3 Cellulose binding modules

Cellulose binding modules (CBMs) are protein domains contained in cellulase enzymes which are able to degrade cellulose into sugars. Cellulases are secreted by certain organisms such as fungi. CBM protein module can anchor the actual enzyme to cellulose selectively.

Cellulose binding modules are classified into different families according to the amino acid sequence similarity. Family I CBMs are small (typically 36 amino acids), protein modules with two highly conserved disulfide bridges and asymmetric shape with one side serving as the binding surface. The three aromatic residues of Tyrosine (Tyr) of the down face of CBM1 family I, represent the driving interaction for binding to cellulose (**Figure 8**). In addition to the stacking of aromatic residues via π-electron interactions, hydrogen bonding is involved in forming affinity and specificity between protein and cellulose.

![Figure 8](image)

**Figure 8.** Side view of cellulose binding module interacting with a cellulosic surface. The green patch of the CBM represents the three aromatic residues of Tyr interacting with the cellulose chains.
Structural studies showed that the CBM binding mainly occurs between the aromatic rings and the pyranose rings exposed on the cellulose crystalline face\textsuperscript{54,55}, because of the coincidence of the spacing of the three aromatic residues with the spacing of every second glucose ring on a cellulose chain\textsuperscript{56,57}.

Binding protein modules much alike CBMs or Chitin Binding Modules (ChBMs), can also exist at the interfaces of biological composite structures such as nacre\textsuperscript{58} and squid beak\textsuperscript{59}. These can play a very important role in adsorbing impacts through the breakage of their intramolecular bonds, or in promoting adhesion at the interface of these hybrid materials (see Figure 1). Therefore, they can be particularly interesting for the design of composites of high mechanical resilience and as a tool in bioengineering\textsuperscript{60}. Previous studies showed how coupling engineered proteins containing CBMs as interlinking architectures with stiffer materials can tune the mechanical properties of the designed architecture\textsuperscript{7}.

### 1.1.4 Fusion proteins role in nanocomposites

By coupling of protein modules and their interlinking architectures, nature inspired composites having better mechanical properties than the single components, can be created\textsuperscript{7}. A common example includes collagen, that can act as soft energy-dissipative domain between hard reinforcing domains, binding them together in a perfect synergy and involving hidden lengths and sacrificial bonds\textsuperscript{61–63}. At the same way, the modular fusions will increase the strength and the toughness through stress distribution (Schematics of Figure 9).

![Figure 9](image.png)

**Figure 9.** Schematic illustration of calcium mediated sacrificial bonds of collagen filaments in the bone that might involve multiple weak bonds in parallel A). Sketch representing CBMs connected by short linker bound to CNF fiber and stress-strain graph showing how increasing the amount of fusion proteins bring an higher elastic module B). Sketch of engineered fusion proteins which the terminus can bind on NFC and HOPG surfaces C). Force distance curve showing how sacrificial molecules increase the stiffness and the toughness of a material D). (Adapted from P. Laaksonen et al. 2011, G. E. Fantner et al. 2005, G. E. Fantner et al. 2006, S. Varjonen et al. 2011).
Fusion proteins involving CBMs and hydrophobins at the two termini separated by different linkers have been studied so far for crosslinking cellulose nanofibers (CNF) or to inter-linkage sandwich like systems done by graphene and CNF. The choice of the mentioned domains and surfaces is due to the high binding affinity of hydrophobins and CBMs respectively for hydrophobic surfaces and cellulose.

1.2 Supramolecular assembly of nanoparticles in 2D nanocomposites

Biomimetic materials include not only the protein based structures but also different types of Nature inspired arrangements i.e. organic-inorganic ones. The latest are inspired by the exquisite self-assembly of proteins into multi-level architectures, such as 1D microtubules, 2D bacterial surface layers (S-layers), and 3D virus capsids. Supramolecular chemistry has appeared a valid approach for building hierarchical self-assembled structures based on building blocks, in which the physical forces involved can be mainly hydrogen bonding, ionic interactions, metal coordination and π-stacking.

In colloidal self-assembly, which is the spontaneous aggregation of ordered array of colloid particles, stability is controlled by a delicate balance between the attractive and repulsive interaction forces between the particles. In fact, the spontaneous packing to 2D colloidal crystals occurs if the interactive sites of the constitutive units imply a suitable broken symmetry, indeed, if the units show no directionality but isotropic interactions, 3D structure instead than 2D are observed.

More than for colloids, the assembly of noble metal nanoclusters in 2D or 3D materials represents a more emerging field because those nanoparticles, especially in the size range <10 nm, have unique optical, magnetic, catalytic, as well as potential biological applications. However, for their inert but instable nature, those structures need to be stabilized against aggregation by protecting ligands. The first pioneering approach was done in 1981 on monodisperse gold 55 (Au55) cluster having triphenyl phosphine (PPh3) ligands (Au55(PPh3)12Cl6) but PPh3 protected ligand revealed an instable nature. After that, the synthesis and application of more stable ligand protected noble metal nanoclusters has been widely explored. Furthermore, the embedding matrix of organic molecules on the nanoparticles attains remarkable mechanical properties. In the current study, 2D ligand protected silver nanoclusters (AgNCs), are designed.

1.2.1 Hydrogen bond-directed assembly

Hydrogen bond is a partially electrostatic bond that occurs between two electronegative atoms as N, O or fluorine bridged by a hydrogen. It has a certain relevance in Nature as for example in the DNA base pairs where the two
strands of a DNA helix are kept together thanks to the H-bonds occurring between complementary nucleotides. Furthermore, each base can also form H-bonds with the water of the surrounding environment. Metal nanoparticles can be capped with ligand of self-assembled monolayers (SAM) having carboxylic termini that can easily form H-bonds. This permits to build an architecture in which the driving force keeping together the two dimensional silver nanoclusters (AgNCs) is the H-bond, that is the strongest interaction between the not covalent ones.

Hydrogen bond-driven architectures have revealed unique properties in terms of dynamics and hierarchical assembly\textsuperscript{74–76}. Furthermore, compared to systems in which the building blocks are kept together by vdW interactions, hydrogen bonds driven architectures rise superior mechanical features as better strength and elasticity.

![Figure 10](image)

**Figure 10.** Crystal structure of Ag\textsubscript{44}-pMBA\textsubscript{30} A) and Packing of the clusters in same layer in the crystal, showing the possibility of a 2D assembly B) Views of the p-MBA ligand-ligand binding between two neighboring silver nanoparticles (L2-bundles lateral assembly), both located in the same layer of the super lattice and views of the p-MBA ligand-ligand binding between two neighboring silver nanoparticles, located in neighboring layers of the super lattice (L3-bundles interlayer H-bond C).

A conceptually simple two dimensional noble metal nanocluster (NCs) system can be created using H-bonds as driving forces for assembly. The nanoclusters are capped with para-mercaptobenzoic acid (pMBA) and para-aminobenzoic acid (pABA) as ligands and can form H-bond directed self-assemblies of atomically precise nanoclusters.\textsuperscript{77} The NCs system that was studied in this work by AFM indentation (publication 3) consists of nanoparticles contains 44 silver
atoms (Ag44) and 30 4-mercaptopbenzoic acids (pMBA). Each pMBA can interact with the other ligands by 2 H-bonds, forming 60 bonds in total. The hydrogen bonding occurs in bundles of ligands, which are known as L2 and L3 (see Figure 10). L2 represents bundles of two H bonds on the 2D membrane and L3 means bundles of 3 H bonds between the different sheets (the 2D membranes in the z-direction). There are 12 L2 (24 pMBA) bundles and 12 L3 bundles (36 pMBA). Therefore, each nanoparticle in the 2D membrane contributes to 24 H-bonding

1.2.1 Stiffness and elasticity of suspended two dimensional materials

Two dimensional sheets of nanoparticle clusters are seen as materials of great interests, because of the unique properties that 2D materials possess compared to 3D or bulk materials. Due to their thickness and flexibility, they usually have high Young’s modulus and fracture behavior that can find applicability for example in foldable electronics. Resistance to mechanical failure is another potential feature vital in lubricant industries.

![Figure 11](image)

**Figure 11.** AFM indentation on MXene (atomically thick materials consisting of transition metal carbides, nitrides, or carbonitrides known for their good conductivity) flakes suspended on a supportive patterned grid A) and related Force-indentation curves B,C). The inset of Figure B represents the broken sheet upon cycles of indentation. From A. Lipatov et al. 2018. Reprinted with permission from AAAS.

Furthermore, many NCs sheets reported high affinity and adhesion to a wide range of substrates making them good candidates also in microelectronics. Similar 2D inorganic materials revealed also easier in tuning the electronic, magnetic and optoelectronic properties by strain engineering. For all these reasons the study and the investigation of new 2D materials and their properties is important. However, for their atomic thickness and planar nature, the study of the mechanical properties of those two dimensional systems, including both nanoclusters or metallic films i.e. molybdenum disulfide (MoS2) or graphene, is quite demanding. One of the best techniques that involves high precision at nanoscale is the AFM indentation (Figure 11).
1.3 Interaction forces

Interaction forces determine the behaviour and describe the affinity between similar or dissimilar systems (i.e. protein/surfaces, particle/surface, surface/surface) each other in contact. They can be of different nature: intermolecular, covalent or ionic. The main interaction forces involved in the studied systems are discussed here. The surface properties and geometry of the interacting systems as well as the nature of the medium and the distance between the interacting partners is determining for define the interaction forces involved between two systems.

1.3.1 Intermolecular forces of the macromolecules studied

Intermolecular forces (IMF) are the forces which mediate interaction between molecules, including forces of attraction or repulsion which act between molecules and other types of neighbouring particles, i.e. atoms or ions. Attractive intermolecular forces are here displayed since they play a key role in adhesion. Interactions between biopolymers, like between any macromolecules or colloidal particles, are a combination of different types of forces, where one interaction may dominate over another at certain distances.

1.3.2 Adhesion forces on proteins-surfaces

Adhesion is the tendency of dissimilar particles or surfaces to cling to one another. The intermolecular forces responsible for such phenomenon can be of different nature and are distinguished as covalent, electrostatic, H-bonds, dispersive. Chemical adhesion refers to the covalent or ionic bonds that the atoms at the interfaces or two separate surfaces can form. They are responsible of chemisorption on surfaces. In covalent forces, the bonds are characterized by the sharing of the electrons between the two or more atoms so that the discrete nature of the atoms is lost. They operate over very short distances of the order of interatomic separations (0.1–0.2 nm). The Coulomb interaction indeed is the force acting between two electric charges. It is very strong, the force needed to break the ionic bond is ~3 nN that is similar to the force needed to break a covalent one. Coulomb interactions are long range forces. The so named dispersive forces belong to the van der Waals forces and are responsible of physisorption mechanisms. Dispersion forces are attractive forces resulting from instantaneous or induced dipoles in atoms or molecules due to the electrons motion around the nucleus. Hydrogen bond happens when a hydrogen atom in one molecule is attracted to an atom of nitrogen, oxygen, or fluorine in another molecule. H-bonds are specific, short-range, and directional non bonded interactions. Distance of 2–4 Å between hydrogen-bond donor and acceptor were found. Eventually, ad-
hesion can also be defined as a geometry controlled process, especially for biological attachment systems, due to the “contact splitting” principle according to which, keeping the same contact area, multiple contact points show higher adhesion than a single contact point.

### 1.3.3 Interaction at the surfaces

**Hydrophobic interaction**
Hydrophobic interaction describes the unusually strong attraction between hydrophobic molecules and surfaces in water, often stronger than their attraction in free space. There is no bond associated to hydrophobic interaction, rather it is an entropic phenomenon, which arises primarily from the rearrangement of H-bond configurations in the overlapping solvation zones as two hydrophobic species come together. Therefore it is of much longer range than any typical bond. It is stronger than the van der Waals attractions.

**Hydration forces**
The hydration forces are short-ranged forces (1-3 nm) that arise when water molecules bind to the hydrophilic surfaces by charge-dipole and dipole-dipole (hydrogen bonding) interactions. The strength of repulsion depends on the energy required to remove the water of hydration, or the surface-adsorbed species containing water molecules, from the surfaces when they approach each other.

**Specific interactions**
Specific interactions can lead to specifically oriented associations of like molecules or to preferential association of unlike molecules. Such effects can be due to molecular geometry (molecular shape or “topology”) and/or physico-chemical effects (specific bonds) that cooperate, i.e. hydrogen bonding, van der Waals, electrostatic and hydrophobic interactions (Figure 12). They are particularly common in the interactions between biological molecules.
1.4 Atomic force microscopy in biophysics

Atomic Force Microscope (AFM) has appeared in the last decade one of the most accurate tools for detecting physics interactions at a single molecular/atomic level. In detail, Force Spectroscopy (FS), consisting of a technique that studies the interactions between individual molecules, is the major technique applied in the current work. With the same technique, according to the system studied and by an opportune choice of the tip functionalization, it is possible to get a wide spectrum of information as the ones listed below. Many researchers belonging to the biological field chose FS to study for example the specific ligand-receptor binding or the understanding of cell surface biology and cellular recognition phenomena\textsuperscript{87–89}. The technique in the specific case is often renamed Single Cell Force Spectroscopy. Larger entities, such as cells, are also investigated for their stiffness by the analogous technique\textsuperscript{90,91} or alternatively bacterial ad-
hesion is investigated according to the surface roughness \(^{92,93}\). Still inside the biological field, the membrane structure can be studied \(^{94,95}\) by AFM as well as the conformation and the unfolding phenomena in human body proteins \(^{96}\) and engineered proteins. \(^{97}\) Last advances included also works on structure and mechanics of viral capsids \(^{98}\). In polymer science indeed, polymer chains desorption is investigated on different surfaces \(^{99,100}\) and in biomimetic, biopolymers are further studied \(^{101}\). The forces ranges mostly from pN - nN for specific binding protein-protein, protein-surfaces, cell-surface etc. Table 1 contains the mentioned research lines in FS science, some of the main research groups and their key publications associated.

Table 1. Research interests and related research groups in FS

<table>
<thead>
<tr>
<th>Area of interest</th>
<th>Some of the main research groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand-receptor Binding Cell-surface biology</td>
<td>D. Müller(^{102}), D. Alsteens(^{103}), Y. Dufrêne(^{104})</td>
</tr>
<tr>
<td>Cell stiffness, cell adhesion, lignin, bacterial adhesion, bioadhesion</td>
<td>I. Sokolov(^{105}), Alvaro San Paulo(^{91}), M. Österberg(^{106}), K. Jacobs(^{107})</td>
</tr>
<tr>
<td>Membrane structure, lipids, lipidic membrane, DNA, RNA</td>
<td>D. Müller(^{108}), S. Scheuring(^{109}), R. Berger(^{110}), L. R. Morata(^{111})</td>
</tr>
<tr>
<td>Single molecules and protein unfolding and conformations</td>
<td>H. Li(^{112}), F. Rico(^{96}), M. Nash(^{97}), M. Rief(^{113}), F. Oesterhelt(^{114}), H. Gaub(^{115}), Lorna Dougan(^{107}),</td>
</tr>
<tr>
<td>Mechanics of viral capsides</td>
<td>W. H. Roos(^{116})</td>
</tr>
<tr>
<td>Polymer desorption, biomimetics</td>
<td>H. Hansma(^{101}), K.G. Blank(^{117}),</td>
</tr>
<tr>
<td>Friction</td>
<td>M. Ruths(^{118}), R. Bennewitz(^{119})</td>
</tr>
<tr>
<td>Polymer desorption</td>
<td>J. Vancso(^{99}), T. Hugel(^{120})</td>
</tr>
</tbody>
</table>

The introductive section sheds light on the features and the properties of those that will be the systems studied in the current work. The description starts with the main protein domains examined (hydrophobin, resilin, and cellulose binding modules), and describe their role in nanocomposites due to the enhanced affinity toward specific surfaces such as cellulose or hydrophobic ones. Hence, the discussion turns to silver nanoclusters capped by ligands OH terminated, that can assembly with H bonds through supramolecular bioinspired
interactions. Two different biomimetic approaches are here employed to produce synthetic protein-based materials and nano-hybrid systems. For those materials, the mechanical features and the interaction forces of main interest are deeply studied with AFM.
2. Aim of the study

The focus of the Thesis is to yield a deep understanding at single molecular level of the forces involved at the surfaces and/or interfaces of the chosen hybrid materials to propose new assembly strategies of biopolymers or hybrid nanoclusters toward fabrication of high-performance functional composites.

In the first part of the Thesis, the surface forces and adhesive features of engineered fusion proteins having hydrophobins (HFB) or cellulose binding modules (CBMs), with the attention also on the conformational structures of elastic coils like domains of resilin, are investigated at molecular level. The aim was to study new candidates for the design of biomimetic composites. The HFB assembly and strong binding of HFB on hydrophobic surfaces as well as CBM binding to different cellulosic substrates were shown and quantified. Resilin was chosen and studied for its elastic properties associated to conformational changes in hydrated state.

In the second part instead, the attention is turned on high performance nanomaterials consisting of a 2D silver nanoclusters array where the elasticity was monitored in order to investigate new enhanced assembled nanostructures. It was demonstrated that ligand capped silver nanoclusters self-assembled by H-bonds, show high elastic modulus.

The driving tool employed in the current study was Atomic Force Microscope (AFM) that was employed both for detecting morphological features of the materials and for detecting the interactive forces and the elastic responses of hybrid structures by the analysis of the so named Force Distance Curves.
3. Materials

In this chapter, the materials used in the current study are displayed. The main materials include fusion proteins containing hydrophobins, resilins, cellulose binding modules and SpyCatcher domains. The expression of the mentioned fusion proteins is described. Hence, the synthesis of the silver nanocluster hybrids is described. More information are accessible in the attached Publications 1-4.

3.1 Engineered Fusion proteins

3.1.1 Production, Expression, and Purification of the dCBM−RLP−HFBI Fusion Protein

Synthetic genes encoding Drosophila melanogaster Rec1-resilin (RLP) and the T. reesei HFBI and CBMs were codon-optimized for T. reesei and synthesized. The T. reesei CBHII (cellulbiohydrolase II; Cel6A) and CBHI (cellulbiohydrolase I; Cel7a) CBMs were separated by a linker. A C-terminal Strep-tag (WSHPQFEK) was added after the HFBI to the protein to enable detection of the protein and affinity purification. The synthetic gene fragments were cloned into an expression plasmid containing cbh1 promoter, secretion carrier and terminator, hygromycin selection marker, and targeting sequence for the cbh1 locus. dCBM− RLP−HFBI was expressed as a CBHI carrier protein fusion with a KEX2 protease cleavage site, NVISKR, between the carrier and the dCBM−RLP−HFBI protein. The ligation mixtures were transformed into Escherichia coli by electroporation, and colonies were selected on the kanamycin agar plates. The final expression construct pMIs was digested with KpnI-XhoI, and the 10 kb integration fragment was isolated and transformed into protoplasts of T. reesei strain M658 carrying deletions for extracellular proteases. Transformed colonies were selected based on the hygromycin resistance (125 μg*mL⁻¹) and carried out. The culture was started by inoculating 1 × 10⁷ spores into a 50 mL growth medium. The T. reesei fungus secreted the
The fusion protein dCBM-RLP-HFBI was purified by aqueous two-phase separation (ATPS). The samples from ATPS purification were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Gel Code Blue Stain, Pierce) and after blotting to nitrocellulose filters by western blotting. After separating the proteins in the SDS-PAGE gel, they were transferred to a nitrocellulose membrane using a turbo blotter (Bio-Rad). The dCBM-RLP-HFBI expression level in the culture supernatant was determined to be 260 mg/L on day 5 of the culture. The culture supernatant containing the fusion protein was processed and purified via ATPS and reversed-phase chromatography and was around >97% pure. From 1 L of culture supernatant, 60 mg of dCBM-RLP-HFBI was recovered after freeze-drying.

### Expression and Purification of SpyCatcher-Cel7a

The Smt3-SpyCatcher-SAligner-Cel7A-His6 plasmid was cotransformed into chemically competent BL21(DE3) E. coli cells with CyDisCo plasmid pMJS20523 (which was employed to ensure the formation of disulfide bridges in the CBM1 domain). One colony was picked and used to grow a preculture in LB medium supplemented with kanamycin (50 mg L\(^{-1}\)) and chloramphenicol (35 mg L\(^{-1}\)), overnight at 37 °C with shaking. The cells were then harvested by centrifugation (24 471 g (rotor radius 15.2 cm), 4 °C, 10 min), removal of the media and resuspended in lysis buffer (20 mM NaH\(_2\)PO\(_4\), 20 mM imidazole, 500 mM NaCl, 100 μg mL\(^{-1}\) fresh lysozyme, 20 μg mL\(^{-1}\) DNase I, 20 μg mL\(^{-1}\) MgCl\(_2\), protease inhibitor cocktail (Sigma-Aldrich) at 4 °C. After 30 min shaking at 4 °C, the cell suspension was further lysed by running it 2 times through an EmulsiFlex-C3 homogenizer (Avestin, Inc.), after which the cell debris was removed by centrifugation (24 471 g, rotor radius 15.2 cm, 4 °C, 25 min). The resulting lysate was incubated with the protease Ulp1 for 1 h at room temperature to cleave off the Smt3 domain. The proteins were subsequently purified using a GE healthcare ÄKTA Pure LC system with HisTrap IMAC columns. SpyCatcher-His6 ("plain" SpyCatcher protein) was produced using the same method, only omitting the CyDisCo plasmid and the cleaving with Ulp1 (no Smt3). The pure proteins were verified by SDS-PAGE analysis with Coomassie Blue staining and MALDI-TOF mass spectrometry (Bruker). Protein concentrations were determined by measuring the absorption at 280 nm using a Varian Cary 50 U-vis spectrophotometer, corrected for background and calculated using the extinction coefficient, predicted based on a protein sequence by ProtParam.

### Expression and purification of HFBI, HFBII, HFBI-dCBM, HFBI-D30N-K32Q and FpHYD5

HFBI, HFBII, HFBI-dCBM, HFBI-D30N-K32Q and FpHYD5 were produced using recombinant strains of _T. reesei_ and purified by a two-phase extraction and RP-HPLC. All the information are displayed in references\(^\text{121-123}\).

HFBI and HFBII (HFBI: 7.5 kDa, HFBII 7.2 kDa) naturally occur in _T. reesei_, FpHYD5, that is a glycosylated hydrophobin, indeed stems from _Fusarium_.


graminearum. From its total molecular weight of 9.2 kDa, the polypeptide consists of 7.5 kDa, indeed the glycan structures attached to the protein weights about 1.7 kDa. HFBI-dCBM (18.5 kDa) is a fusion protein in which HFBI is interconnected with two CBMs by a short linker. HFBI-D30N-K32Q is indeed a HFBI mutant having two neutral residues near the hydrophobic patch instead the two charged present in the wild type.

3.2 Preparation of ligand capped two dimensional silver nanoclusters (AgNCs)

Ag44 cluster was synthesized starting from a solution of AgNO3 in DMSO and water (4:7 volume ratios) under constant stirring. MBA was then added to the mixture and the solution was kept under stirring and 50% cesium hydroxide CsOH was further added dropwise till the cloudy nature of thiolates became clear and a greenish yellow color appeared. Then NaBH4 in water was added dropwise. Slowly the color changed to deep brown and after 1 h it became deep red which confirmed cluster formation. The crude clusters were purified using DMF which precipitated the clusters and the mixture was centrifuged. After removing the centrifugate, the clusters were extracted in citric acid containing DMF solution. This is to acidify all the protons of the carboxylic acid which made the cluster soluble in DMF. Extracted cluster was again precipitated using toluene and centrifuged. Similarly, the acidification step was repeated one more time. After this, the precipitate was re-dissolved in DMF to get the purified cluster solvent drying process at air-water interface was used to create the cluster assembly.
4. Methods

In this section the methods used during our study are described. The main focus is directed toward Atomic Force Microscope and the related techniques applicable with this tool. The other main supportive techniques used include, Quartz Crystal Microbalance with Dissipation Monitoring, Surface Tension measurements, Infrared Spectroscopy and Water Contact Angle.

4.1 Atomic Force Microscope

Atomic Force microscopy (AFM) has been used so far to study the morphological features and physical interactions of a wide range of surfaces and materials, from biomolecules to alloys. The working principle is based on the interaction of a cantilever having a tip mounted and a surface of interest (Figure 13). In detail, the forces acting between the tip and the surface cause the cantilever deflection that is monitored from the position of a reflected laser beam on a photodiode. A detector will receive this signal transforming it in an electrical response, which is analysed in terms of a force distance curve or morphological image. Multimode 8 and FastScanBio AFM have been used in the current work.

![Figure 13. Schematic illustration of the components of AFM and the working principle while carrying imaging and FS experiment. The insets of the schematic show a morphological image of cellulose nanofibers deposited on mica and a typical force distance graph with](image-url)
the related approaching and retraction cycles. (Adapted from M.L. Hughes et al. 2016 and by Messersmith et al.).

### 4.1.1 Force distance curves

The entire force measurement typically exhibits two curves that are usually identified as approach and retraction curves and typically takes a couple of seconds on a rate from hundreds of nm·s⁻¹ to tens μm·s⁻¹. The retraction curve is recorded from the moment in which the cantilever starts to retract from the sample until the final detachment (inset of Figure 13).

The trigger force $F$ can be determined from the deflection of the cantilever $z_l$ and the spring constant $k$ by the Hookes law:

$$F = kz_l$$

The AFM tip, that is usually functionalized, can be used in order to “fish” single molecules and to measure parameters as the stretching (if the molecule of interest is coiled), the length of the molecules at its maxima extension and its conformations. Furthermore, the contour length $L$ and persistence length $P$ can be easily accessed by fitting the force-distance (FD) curves.

![Figure 14](image.png)

**Figure 14.** Schematic illustration of a force distance curve and sketch of the approaching and retraction cycles. By fitting the slope of the approaching curve stiffness and elastic modulus can be detected. The height and the area of the peak in the retraction curve refer respectively to the rupture/detachment force and work of adhesion A). Schematic of the indentation on a surface with an AFM tip and the related curves obtained which the slope can change according to the stiffness of the material indented B,C). Example of a structure suspended on a holey support, in order to minimize the effect of the subsurface phenomena D). (Adapted from C. Roduit et al. 2009).
The approach curve (Figure 14 a) instead is recorded while the cantilever approaches the sample (contact point) and deflects according to the set trigger force. Typically, it can show the nature of the interaction, i.e. whether repulsion or attraction is involved.

The approach curve reveals also information that are connected to the stiffness or softness of the material. After the contact point, the cantilever indents the surface of interest and the deflection slope depends on the hardness of the layer. Usually less steep is the slope, the softer is the layer\textsuperscript{124,125}. For a stiff sample, the cantilever deflection equals the piezo movement in z direction. Instead, for a soft sample, the tip indentation will lead to a smaller deflection and result then in a flatter force curve having a smaller slope (Figure 14 c). To calculate the indentation, the measured deflection of the force curve has to be subtracted from a force curve on a stiff sample, where there is no (or at least a negligible) indentation.

Usually, a cantilever is used to apply a small force at the centre of a nanostructure suspended over a tiny groove\textsuperscript{126,127}. The usage of holey substrates in which the material of interest is suspended can avoid a misanalysis of the mechanical properties due to the effect of subsurface (Figure 14 d).

**Single Molecule Force Spectroscopy (SMFS) experiments**

*Force distance measurements.* The first system studied includes hydrophobin and resilin fusion proteins, whose adhesive features and conformational changes were studied by Force Spectroscopy (Publication 1). The experiments were conducted in two ways: (1) between the silanized AFM tip and the protein-functionalized cellulose surface, and (2) silanized surface and proteins on the cellulose-functionalized AFM tip (Figure 15 a and b).

![Figure 15](image.png)

*Figure 15.* Schematic of two different experimental set up. In A) the molecules of interest are bound to the tip whereas in B) the molecules are bound to the surface. (Adapted from A. Griffo et al. 2017).

The experiments were carried out in a buffer solution at room temperature with a Bruker Dimension FastScan Bio instrument. Each experiment consisted of at least 500–600 single force/distance curves. The force measurements were carried out using a z-range of 500 nm, a scan rate of 1 Hz, a MLCT-D cantilever with a spring constant of 0.03 N m\(^{-1}\), and a relative force trigger of 0.1 V.

In our second system studied, where the interaction forces of cellulose binding modules were investigated (Publication 2) the experiments were carried out
with a Bruker Multimode 8 instrument, sample immersed in 10 mM PBS buffer, using MLCT-D cantilevers (0.03 N m\(^{-1}\) spring constant). The force measurements were carried out using a ramp size of 500 nm, a scan rate of 0.5 Hz, a forward and reverse velocity of 500 nm s\(^{-1}\), and a relative force trigger of 0.1 V.

**Mathematical model.** The model chosen to fit the recombinant proteins studied for both the systems described in the previous section (Publications 1 and 2) was the Worm-like chain model (WLC). The model is applicable for semi-flexible polymers. It incorporates the entropic elasticity of a molecule and envisions a continuously flexible isotropic rod\(^{128}\). It is adopted well in chains having units (segments/monomers/amino acids) with persistence length within a few orders of magnitude of the polymer length. The formula used is:

\[
F(z) = \frac{k_BT}{l_P} \left( \frac{z}{l_c} + \frac{1}{4(1-z/l_c)^2} - \frac{1}{4} \right)
\]

where \(l_P\) is the persistence length, \(l_c\) is the contour length, \(z\) the extension length, \(k_B\) the Boltzmann constant and \(T\) is the temperature. This model is suitable for single and double-stranded DNA\(^{129,130}\) and unstructured proteins\(^{131}\).

**Tip functionalization.** For the hydrophobin and resilin fusion protein study (Publication 1), MLCT-D cantilevers (Spring constant 0.03, resonance Frequency 15 kHz) were silanized with octadecyltrimethoxysilane (OTS) to make them hydrophobic. In detail, the AFM probes were first plasma-cleaned for 3 min and then left immersed for 15 min in a solution of OTS added to a bicyclohexyl and CCl\(_4\) in a molar ratio of 479:1. Finally, the probes were rinsed with chloroform and dried in air. Functionalized tips were used within a week from the preparation.

For the study on cellulose binding modules interactions forces (Publication 2), tip functionalization of MLCT-D cantilevers previously plasma treated for 3 minutes consisted of three steps (Figure 16). First the silicon nitride tip was silanized (overnight in toluene in presence of TEA as catalyst) with an alkyne terminate silane (3.4 kDa), which then reacted with the azide group of a SpyTag (ST) terminated peptide via click chemistry reaction forming a triazole (overnight in water, in presence of sodium ascorbate and copper sulfate pentahydrate). Hence, the ST reacted with the SpyCatcher (Spy-C) terminus of the Cel7A fusion protein 1\(\mu\)M, that is casted on the ST coated tip for 15 min and rinsed with phosphate buffer saline 10 mM (PBS). PBS has been described as one of the best buffers to quickly activate the SpyTag-SpyCatcher reaction. The protein casting was performed 30 min before each AFM experiment, whereas the silanized and SpyTag-functionalized tips were prepared within a week before the experiment.
**Sample preparation.** For the hydrophobin and resilin fusion protein study (Publication 1) the samples have been prepared by drop casting of protein solution on a previously spin coated cellulose sample and rinsing with buffer after 15 minutes, or alternatively the protein was drop casted on a previously cellulose dip coated probe. In detail, the cellulose surfaces were prepared on the silicon oxide surface by converting trimethylsilyl cellulose (TMSC) into cellulose\textsuperscript{132,133}. 50 μL of 1 mg mL\textsuperscript{-1} TMSC\textsuperscript{134} dissolved in hexane were spin coated at a spinning speed of 5000 rpm for 60 s on silicon oxide 0.5 × 0.5 cm\textsuperscript{2}. Hence, TMSC-coated surface was exposed to 12 M hydrochloric acid for 2 min. The silanized samples were prepared as described for the tip functionalization.

For the study on cellulose binding modules interactions forces (Publication 2) all the cellulosic surfaces studied (cellulose from TMSC, cellulose nanocrystals by sulphuric and hydrochloric acid hydrolysis, chitin nanocrystals) were prepared by spin coating from suspensions containing the molecules or nanocrystals. Spin coating is a procedure that uses centrifugal force to mechanically adsorb a thin film on the top of a flat surface. The thickness of the film can be controlled by the rotational speed as well as by the concentration, viscosity of the solution and solvent.
AFM indentation experiments

AFM indentation measurements. The AFM indentation experiments were performed with a Bruker Multimode 8.0 Instrument, in air at room temperature using Scansyst cantilevers (spring constant 0.4 N m⁻¹, curvature radius 2 nm). For each cantilever, the spring constant was calibrated by thermal methods. In the force curve measurements, the approaching and retracting velocity was kept at 500 nm s⁻¹, scan rate at 0.5 Hz and scan size at 250-500 nm.

The experiments were carried out each time on AgNCs thin film formed on a QUANTIFOIL TEM holey grid that consisted of a gold grid on which a thin carbon layer presenting a holey pattern was deposited (Figure 17). All the samples were previously checked by TEM analysis and the measurements were performed with increasing indentation force until the rupture of the suspended film (10-20 nN).

![Figure 17. Schematic, AFM image and optical microscope image of AgNCs freestanding film deposited on top of the holey carbon QUANTIFOIL grid A). AFM images before B) and after C) many indentation cycles.](image)

Mathematical model. For the study of the elastic modulus on atomically precise silver nanoclusters AgNCs (Publication 3) the mathematical model used, was within the framework of elasticity theory for homogeneous membranes:

\[ F = k\delta + \frac{\pi Eh}{3R^2} \delta^3 \] (3)

where \( h \) is the film thickness, \( R \) the hole radius, \( \delta \) the indentation depth and \( k \) the spring constant that depends on the pre-strain of the film and is used as fitting parameter. The model is applicable when \( \delta/h \gg 1 \) and the tip diameter is small compared to the film radius.

Sample preparation. The purified clusters described in section 3.2 were first precipitated using toluene and then dissolved in pentanol. This solution was spread over the surface of milli-Q water taken in a beaker. The thin film
formed at the water-air interface was transferred to a mesh grid with holey carbon support film (CF-Quantifoil) for characterization.

Morphological analysis
For the hydrophobin and resilin fusion proteins study (Publication 1) topographical images were recorded by AFM in the tapping mode using Bruker FastScanBio. For measurements in air, the images were scanned using silicon cantilevers (Olympus) with a resonance frequency of 300 kHz and a force constant of 26 N m$^{-1}$. For the measurements in liquid environment, FastScan-D and SNL cantilevers having resonance frequencies of 110 and 65 kHz and force constants of 0.25 and 0.35 N m$^{-1}$, respectively, were used.

For the study on cellulose binding modules interactions forces (Publication 2) topographical images were recorded by AFM in the tapping mode in air using Multimode 8. The images were scanned using silicon cantilevers (HQ:NSC, tip radius 8 nm, MikroMasch) with a resonance frequency of 325 kHz and a force constant of 40 N m$^{-1}$.

For atomically precise Ag nanoclusters (Publication 3), AFM images were recorded using the Quantitative Nanomechanical Mapping (QNM) Mode. The technique is based on oscillating the cantilever as in the simple tapping mode, but differently from it, operates in non-resonant mode and the oscillation is at frequencies well below the cantilever resonance. Scansyst cantilevers having resonance frequency of 70 kHz and a force constant of 0.4 N m$^{-1}$ were used for the imaging.

For the study on hydrophobin self-assembly mechanism at air-water interface (Publication 4) AFM images were recorded either in Tapping mode and in QNM-mode using a Bruker FastScanBio with Nanoscope V controller. SNL-B probes having nominal spring constant of 0.12 N m$^{-1}$ were used for the imaging.

4.2 Quartz crystal microbalance with dissipation monitoring

QCM-D was used for the hydrophobin and resilin fusion proteins study (Publication 1), in order to investigate the consistence and the organization of the proteins and the cellulose on the top of the silicon surface. The working principle consists of using acoustic waves generated by oscillating a piezoelectric, single crystal quartz plate for measuring mass$^{36}$. The Q-sensor consists of a piezoelectric crystal sandwiched by two gold electrodes; when an alternating electric field is generated the crystal starts to oscillate at its resonance frequency. The frequency of the oscillation changes as the mass on the sensor changes, therefore the technique is highly sensitive to the mass of deposited layers as well as of the wetting liquids at their surface. Cutting of the AC current will generate a decay of the crystal oscillation which the rate is proportional to the energy dissipation of the oscillator (Figure 18 a-c). The decay rate or Dissipation factor is related to the elasticity and viscosity of the molecular layer on the sensor. From the decay curve, the resonance frequency $f$ and the energy dissipation $D$ are extracted$^{37}$. 
For the viscoelastic nature of the protein film of hydrophobin and resilin fusion proteins studied (ΔD >0) the Kelvin Voigt model was applied. It can be described as

$$G = G' + iG'' = \mu + i2\pi f \eta 1$$

where $\mu$ is the shear elasticity, $\eta$ the viscosity, $G^*$ is complex shear modulus, $G'$ is the storage modulus, and $G''$ is the loss modulus.

The samples for QCM-D analysis were prepared by spin coating of the sensor as described for the SiO$_2$ samples used in AFM analysis.

The QCM-D measurements were performed in flow mode using a Q-Sense E4 instrument (Q-Sense, Sweden). AT-cut quartz crystals, with a fundamental frequency of 5 MHz, were purchased from Q-Sense. The measurements were carried out at 22 °C using a flow rate of 100 μL min$^{-1}$. 0.1 mg*ml$^{-1}$ of protein solution was injected and the buffer was exchanged from 5 (sodium acetate buffer) to 11 (phosphate buffer).

### 4.3 Surface Tension measurements

*Surface tension measurements* have been performed for the study on hydrophobin self-assembly mechanism at air/water interface (*Publication 4*), in order to understand deeply the self-assembly process of hydrophobins. Surface tension represents the work necessary to increase the surface area of a liquid due to the cohesive forces between the molecules. Water has a higher surface...
tension value compared to many others liquids since the cohesive forces between the molecules are dominated by hydrogen bonds. Surface tension values are in fact depending on the intermolecular forces between liquids, therefore they vary according the nature of the liquid or the presence of solutes as surfactants. Physically it is reported as $\gamma$ and it is measured in force per unit length: $\gamma = J^* m^{-2}$.

The surface tension values were detected with a tensiometer (Sigma, Attention) using the Wilhelmy plate. The technique is based on the geometry of fully wetted plate in the liquid phase. The complete wetting is ensured by the choice of the material (usually iridium-platinum). The Wilhelmy plate is first dipped into the liquid and then pulled back to the ‘zero depth of immersion’, that represents the position recorded at the first contact \textbf{(Figure 19)}. When the plate returns to this position, the force it registers is used for the surface tension calculation according to the equation:

$$\gamma = \frac{F_G}{L \cos \theta}$$

where $L$ is the length of the plate, $\theta$ is the contact angle (that for the completed wetted plate is 0) and $F_G$ is the gravitational force recorded.

\subsection*{4.4 Fourier Transform Infrared Spectroscopy}

\textit{Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR)} has been used for the study on cellulose binding modules interactions forces \textbf{(Publication 2)} in order to prove the chemical functionalization of SiO$_2$ surfaces (with the reaction steps that were then applied to functionalize the AFM probes) as well as to estimate the crystallinity of the cellulosic surfaces studied.

The \textit{ATR sampling technique} has been invented in 1960 and in conjunction with FT-IR permits to examinee samples in liquid or solid state without need of further preparation. The working principle of ATR consists of a beam of
infrared light that goes through the crystal and reflects the internal surface of the crystal forming an evanescent wave that propagates into the sample (Figure 20). The number of reflections depends on the angle of incidence. The beam is then collected by a detector. A fundamental working condition of the technique is that the crystal has a higher refractive index compared to the sample studied, otherwise the light is lost to the sample. In the current study ATR-FTIR with germanium crystal was used (Perkin Elmer instrument).

![Figure 20. Total internal reflection at the interface of ATR crystal. (Adapted from A. Voilley et al. 2011)](image)

The basic principle of Infrared Spectroscopy involves the interactions between infrared radiation and the molecules studied that adsorb frequencies according to their structure. When the molecule adsorbs a vibration between 10.000 and 100 cm\(^{-1}\), the energy absorbed by the molecule is converted in vibrational energy causing two type of vibrations: stretching or bending. When those vibrations cause a variation of dipole moment, the IR vibration happens. In order for a vibrational mode in a sample to be "IR active" in fact, it must be associated with changes in the dipole moment (Figure 21).

![Figure 21. The oscillation of a molecule, even if it is nonpolar, may result in an oscillating dipole that can interact with the electromagnetic field. Here we see a representation of a bending mode of CO\(_2\). (Adapted from P. Atkins, J. d. Paula Fifth Edition 2009.)](image)

4.5 Water Contact Angle

*Water contact angle WCA technique* has been used both in the study on hydrophobin and resilin fusion proteins than for the study on cellulose binding
modules interactions forces (Publications 1 and 2) to characterize the surfaces before and after the functionalization steps and to verify the desired films/molecular attachment.

With the term contact angle is conventionally meant the angle where a liquid-vapour interface meets the solid. Water contact angle can quantify the wettability and the hydrophobicity/hydrophilicity of a surface and is one of the most used techniques in surface science for its simplicity and reliability. By definition, a surface is hydrophobic when θ > 90° and hydrophilic when θ < 90°. Since each coating has a different nature, the technique can be used to see if the surface treatment is successful or not.

The model describing the WCA theory is the Young equation that relates the surface tension between three phases: solid liquid and gas. The WCA of a liquid on a solid is then predicted by the three surface energies involved according to the following equation:

\[ \gamma_{SG} = \gamma_{SL} + \gamma_{LG} \cos(\theta) \]  

where \( \gamma_{SG}, \gamma_{SL}, \) and \( \gamma_{LG} \) are the surface tension respectively at the solid vapour, solid liquid and liquid vapour interface (Figure 22).

![Figure 22. WCA recorded on a cellulose surface and schematics of the surface tensions involved in the calculation.](image)

Interestingly, the contact angle can also be related to the work of adhesion \( W_A \) through the Young–Dupré equation:

\[ W_A = \gamma_L \left( 1 + \cos(\theta) \right) \]

where \( \gamma_L \) is the surface tension of the liquid.
5. Results and Discussion

The following section highlights and summarizes the most important findings of the Thesis. The main target of the research was to detect the mechanical properties i.e. adhesion, binding affinity and elastic modulus of engineered fusion proteins and silver nanocluster composites.

5.1 Bioadhesion of engineered protein domains

Nature has provided many protein-based adhesives having waterproof excellent performance starting for example from mussel proteins to the adhesives secretes by sandcastle worms\textsuperscript{1,143,144}. Hydrophobins secreted by the fungi\textsuperscript{145}, cellulose binding modules found in carbohydrate active enzymes\textsuperscript{60} squid beak proteins from chitin\textsuperscript{146}, collagen in the human body and elastin-like polymers in nacre, are many other examples of existing functional proteins. The deep knowledge of the adhesive mechanism, the interaction forces and the affinity of those natural proteins for specific materials, represents a key into the design of new high performance protein-based composites. A beautiful example of protein based composite materials is given by the work carried by the artist Maurizio Montalti, that managed to create a range of objects (i.e. chairs and shoes in Figure 23) with some very unique characteristics, with the development of a controlled method to grow fungi on all kinds of materials, in collaboration with a team led by Professor of Microbiology Han Wösten, in Utrecht University. From a materials science point of view, the development of synthetic engineered proteins inspired by the existing ones, is a focal point to build new bio-based materials at larger scale.

Figure 23. Chair, shoes and other small objects consisting of fungus that adheres on different kind of materials. Picture taken at Micropia museum in Amsterdam.
5.1.1 Hydrophobin fusion proteins as elastic and adhesive termini

Adhesion force
Hydrophobin is the first protein domain studied in Publication 1. In detail, the adhesive forces associated to the HFBI hydrophobic patch were here quantified on a model hydrophobic surface of octadecyltrichlorosilane (OTS). The hydrophobic patch present on one face of the hydrophobin (Figure 3) is well known for its strong affinity toward hydrophobic surfaces. This is the reason why OTS has been chosen as model surface. The fusion protein could potentially be employed as interlinking connector between inorganic/cellulosic platelet to build a composite mimicking the pattern of nacre (Figure 1). The adhesion force of HFBI on OTS represent the fundamental and basic step before to move on the design of bioinspired composites. In detail, HFBI domain was here connected with a short linker (PGASTSTGMGPGG) to two cellulose binding modules (CBMs) that cooperatively physisorbed on a cellulose coated surface (Figure 24 a and inset c).

The adhesion force between HFBI functionalized sample and OTS coated tip, was measured by Force Spectroscopy and reached values between 50-200 pN per molecule with a maximum at 86 pN. These values are within the range polymer/protein-surface interaction detected for single molecules\textsuperscript{47–150}, but slightly higher compared to adhesion of proteins \textit{i.e} ferritin, immunoglobulin G, nebulin protein fragment, myosin and silk on hydrophobic surfaces\textsuperscript{151,152}, strengthening the concept of high hydrophobin adhesion toward hydrophobic surfaces and making it a candidate for composites having hydrophobic building blocks.

![Figure 24](image-url)  
*Figure 24.* Cartoon of HFBI-dCBM fusion protein A) and histograms representing contour length B) and adhesion force between hydrophobin and octadecyltrichlorosilane coated tip C).

The adsorption mechanism of HFBI on CH$_3$-terminated surfaces was also investigated by previous computational studies stating that the HFBI adsorbs preferentially with its hydrophobic patch on CH$_3$-SAM\textsuperscript{153}, Li \textit{et al.}\textsuperscript{112} indeed, quantified the hydrophobin class II HFBI binding on hydrophilic surfaces to be lower (48-50 pN) than onto hydrophobic surfaces (120-140 pN); value that
closely matches with the one reported in our current study. Another interesting observed phenomenon was that, the cohesive forces and self-assembly between hydrophobins molecules, enhanced the binding force^{112}.

With the linker used (PGASTSTGMGPGG) to connect the HFBI domain to the CBMs termini (Figure 24 a), in the fusion protein studied, there is not much work of rupture associated to the process (85 E/KT). Furthermore, at the pulling force used, HFBI results to be a stable domain (no protein unfolding has been observed) and the total length of the HFBI-dCBM matches with the theoretical estimated values. By fitting the peak attributed to the HFBI-OTS interaction and applying the worm-like chain model, the contour length distribution falls at 12 nm, which results to be equal to the HFBI-linker-CBMI-linker-CBMII theoretical length.

*The role of cohesive and electrostatic forces in self-assembly*

The described adhesive features of HFBI and the high coherence and elasticity of the films, are related to the self-assembly mechanism of those unique class of proteins. For the study on hydrophobin self-assembly mechanism at air-water interface (Publication 4), the assembly at air/water interfaces has been investigated via ellipsometry and surface tension measurements, and aspects such as steric hindrance and electrostatic charges of different type of hydrophobins have been addressed.

First, in order to study the role of the strong lateral interactions between the proteins, both the wild-type HFBI and HFBII and the fusions having bulky side chains and having glycosylated portions to the proteins (respectively HFBI-dCBM and FpHYD5) were compared. The side group of HFBI-dCBM and the glycosylation of the FpHYD5 were hypothesized to enlarge the size of the proteins limiting the close contact between hydrophobin domains and believed to inhibit the crystal formation at the air/water interface. In fact, while the adsorption kinetics for wild-type HFBI and HFBII did not follow the classical Langmuir-type trend, in HFBI-dCBM and FpHYD5 the kinetics became more Langmuir-type^{154}. The addition of bulky attachments to the protein body altered the adsorption kinetics strongly; both the saturation value of the adsorbed amount and the area per protein and the time evolution of the adsorption rate changed. The main reason is that the steric hindrance restricts the strong cohesive interconnections in the monolayer (Figure 25 a and c).

![Figure 25. Kinetics adsorption A) and surface pressure B) of HFBI (cyano), HFBII (red), HFBI-dCBM (pink) and FpHYD5 (blue) at 0.5 µM. Sketch of assembly of hydrophobin with the two CBMs as side groups. The side groups interfere on the assembly process.](image-url)
Also the surface pressure measurements revealed significant differences (Figure 25b). HFBI-dCBM, showed not only the lowest molecular density measured by ellipsometry but also lower surface pressure than the wild type HFBI and HFBII. FpHYD5 indeed, despite presented molecular density similar to the WT, revealed a much lower saturation surface pressure. This is because of the different chemical composition associated to the presence of glycan groups in the hydrophilic part of the protein that may increase bound water molecules in the inter-phase layer and the solubility.

Secondly, also lateral Coulombic interactions played a role in the self-assembly process. Two type of experiments were carried out with the wild types HFBI and HFBII and a mutant (named HFBI-neutral) where two charged amino acid on the hydrophobic patch have been replaced with two neutral ones[55]. In one experiment the kinetics of the HFBI, HFBII and HFBI-neutral were compared. In another one, the ionic strength of the buffer solution was varied while keeping the protein structure constant. In all the experiments an enhanced adsorption rate due to reduced electrostatic interactions – either by screening or by exchanging charged side-groups was observed leading to the notion that the electrostatic interactions between the proteins, that are repulsive, decelerates the adsorption kinetics (Figure 26a and b).

A closer look to the assembly process of hydrophobins proteins is presented by an AFM study. By the images is clear that the mechanism of assembly provides
the formation of clusters that grew over time and which the initial density matches with the final one. When the charges were screened, at higher ionic strength, an increase of the protein cluster velocity was observed. Furthermore at higher ionic strength also the number of clusters was lower than the one observed when there was no charge screening (Figure 26c). This involves a higher nucleation barrier in presence of screening. The presence of supramolecular assemblies in presence of charges has been already observed for a HFBI variant in presence of NaCl\textsuperscript{156}, so this process is also attributed to the solution behavior of hydrophobins. This AFM study confirmed that the repulsive interactions have a decelerating effect on the assembly mechanism of the studied hydrophobins.

5.1.2 Resilin as energy dissipating interlinkage connector

A similar fusion protein to the one described in section 5.1.1 was expressed, but the linker was replaced by a resilin like polypeptide (RLP) (Figure 28b). Here, a deep analysis of the resilin behavior under tensile strength and at two different pH is discussed. Resilin, for its elastomeric properties and its ability to dissipate energy, is one of the most promising natural linker for bio-composites.

Adhesion force and entanglement
The adhesion force on OTS silane self-assembled monolayer was studied by recording hundreds of force distance curves. The experimental procedure was analogous to the one described in the subsection Single Molecule Force Spectroscopy (SMFS) experiments (page 24). Briefly, the RLP fusion protein (dCBM-RLP-HFBI) having on one terminus HFBI and on the other one two CBMs connected by a short linker was bound to a cellulose coated surface by physisorption of CBMs on cellulose. The experiments were performed at two different pH: 5 and 11. While at pH 5 the force distribution was close to the one observed for HFBI-dCBM (Figure 24c), at pH 11 the values reached forces of 600 pN, meaning that the resilin coil interfered on the adhesion mechanism.

At pH above the isoelectric point (IP) of resilin 9.6, the conformation of the resilin module is affected. Due to the negative charges on the resilin backbone, the amount of bound water is higher, causing a transition from the globular structure to an extended coiled one. This causes also a softer and swollen layer of the extended coil or brush-like conformations. Previous QCM-D studies on a RLP protein bound on gold showed the analogous conformational changes\textsuperscript{49}.

The higher rupture forces observed in our experiments are associated mainly to the resilin entanglement leading to simultaneous attachment of several hydrophobins on the hydrophobic OTS. In fact, the simultaneous attachment of several hydrophobins on the tip/surface is not surprising due to the rather long and flexible RLP linkers (compared to the short one described at page 24), which can easily entangle. In earlier work, the unordered secondary structure of the resilin was proved to turn toward a more ordered β turn structure\textsuperscript{50}.
at higher pH. This further explains the strong bundling that could be a reason for the simultaneous attachment of several hydrophobins/higher binding force.

Often in *Single Molecule Force Spectroscopy (SMFS)*, higher forces are associated to the simultaneous detachment of two or more molecules\(^\text{157}\). Higher forces associated to more molecules are also recognizable when the corresponding persistence length (P) is lower than the usual value of 0.36-0.38 nm associated to a single amino acid. P values around 0.005 are in fact attributed to the stretching of more filaments\(^\text{158}\).

A closer look to the plots Adhesion Force vs Persistence Length (\(F_A/P\)) and Adhesion Force vs Contour Length (\(F_A/L_c\)) permitted to individuate three populations of curves when the dCBM-RLP-HFBI binding was studied at pH 11 (*Figure 27*). For the \(F_A/L_c\) scattered data, in population 1, \(F_A\) was nearly constant for different \(L_c\) with very low scattering (blue circles), and in populations 2 and 3, \(F_A\) depends nearly linearly on \(L_c\) (green and purple triangles).

For the \(F_A/P\) scattered data indeed it was obvious that especially population 3, for its low P values, could not be associated for individual polypeptide chains; rather it was interpreted to result from entangled protein chains, which the adhesion force will depend on the possible attachment points, that are likely not only the hydrophobin end-domains. Population 1 was clearly attributed to the stretching of a single chain. Population 2 indeed showed shifted higher contour length values of population 3 and P values between the two other populations. Such behavior may be suitable to a situation in which proteins were attached to the tip and not to the surface but instead entangled with other surface attached proteins. The less numerous number of curves showing such behavior, support this assumption.

---

**Figure 27.** Plots of adhesion force vs contour length A) and adhesion force vs persistence length B) describing the different behavior of RLP. Schematic of the 4 different situations when the silanised tip fishes the RLP during a *Force Spectroscopy* experiment.
**Stretching of the resilin-like-protein**

A further study on the structural and conformational changes of the resilin-like-protein was carried out by the analysis of the contour length observed for the FD curves recorded at the different pHs. Contour length is described as the length of a polymer chain at its maximum physically possible extension. Therefore, by fitting the peaks recorded, the length associated upon stretching at a certain pH attempts the pH related conformational changes. At pH 5 two mode of detachment were observed: at c.a. 35 and 90 nm. If we compare those number to the estimated ones of 32 (folded) and 136 (extended), it is clear that the first distribution matches with the coiled structure, indeed the second one hint to a partial stretching of the chain. At the pulled force used in fact, the intramolecular interactions are too strong to permit the stretching of the whole chain.

At pH 11 the situation was more complex; four different contour length distributions were observed: 64, 133, 149, and 243 nm (Figure 28). As already mentioned, above the IP, the RLP is supposed to swell and to assume brush-like conformation. The 64 nm is the swollen coil, indeed the mid values 133 and 149 nm, that represent the majority of the measurements, are close to the fully extended state (136 nm). The distribution at around 243 nm is most likely due to entanglement (discussed in the previous section) of the RLP, or stretching of cellulose chains. At pH 11, cellulose swells notably, which was observed in the topography measured by AFM and the QCM-D dissipation data (Figure 29).

![Figure 28](image)

**Figure 28.** Histogram representing the different distributions of contour length for the RLP at pH 5 (blue) and 11 (red) and FD curves for resilin at pH 5 (last on the bottom) and 11 (the others) A). Sketch of the resilin coil at pH 5 and 11 B).

**Dissipated energy**

The FD curve areas were integrated in order to quantify the work of rupture associated to the unbinding phenomena. The values are reported in Table 2 and present a significantly higher rupture work at pH 11. Despite, as discussed,
not all the values correspond to the stretching of a single molecule, it is clear that the work required to stretch the resilin was relevant. Values were in fact much consistent than the ones observed for HFBI-dCBM, where the protein domains were connected by the short linker. Resilin is well known to its dissipative ability and high resilience\textsuperscript{38}. It adsorbs energy when deformed elastically and release it upon unloading. In its hydrate state a resilience value of 92\% was recorded\textsuperscript{34}.

### Table 2. Overview of the AF, CL and P recorded for HFBI-dCBM and dCBM-RLP-HFBI

<table>
<thead>
<tr>
<th>Surface</th>
<th>Rupture force ± standard error (pN)</th>
<th>Contour length ± standard error (nm)</th>
<th>Persistence length ± standard error (nm)</th>
<th>Work of rupture ± standard error (E/kT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCBM-RLP-HFBI on Cellulose at pH 5</td>
<td>162 ± 4</td>
<td>29 ± 3</td>
<td>0.04 ± 0.001</td>
<td>485.3 ± 9</td>
</tr>
<tr>
<td>HFBI-dCBM on cellulose at pH 5</td>
<td>86 ± 1</td>
<td>12 ± 1</td>
<td>0.2 ± 0.01</td>
<td>84.7 ± 5</td>
</tr>
<tr>
<td>dCBM-RLP-HFBI on Cellulose at pH 11</td>
<td>438 ± 36</td>
<td>64 ± 5</td>
<td>0.04 ± 2</td>
<td>6725 ± 29</td>
</tr>
<tr>
<td></td>
<td>133 ± 2</td>
<td>149 ± 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>243 ± 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose at pH 5</td>
<td>483 ± 3</td>
<td></td>
<td></td>
<td>1004 ± 6</td>
</tr>
<tr>
<td>Cellulose at pH 11</td>
<td>159.95 ± 5</td>
<td>18±1</td>
<td>0.12±0.1</td>
<td>270 ± 48</td>
</tr>
</tbody>
</table>

The last observation in our resilin system concerns the swelling and softness features, derived by bound water molecules when RLP is in its hydrate state. This was already partially discussed and is here further confirmed by the observation of the dissipation values, thickness and absorbed mass by QCM-D analysis. Dissipation was associated with a viscoelastic response while the increase of thickness was because the swollen state of resilin. The frequency change overtime was also observed and revealed an increased adsorbed mass when the pH was tuned at 11, associated to the higher bound water in these conditions (Figure 29).

As control, the same experiments (AFM and QCM-D) were also repeated simply with spin-coated cellulose and with spin-coated cellulose and the hydrophobin fusion protein HFBI-dCBM absorbed on it. As expected HFBI was not affected by changes in pH. In fact, the section analysis at two different pH did not show consistent differences. Similarly, the dissipation observed by tuning the pH has the rather modest value of 2*10\textsuperscript{-6} ΔD mostly associated to the cellulose swelling. In fact, the same value of 2*10\textsuperscript{-6} ΔD is observed by tuning the pH for the cellulose layer. The analysis of the thickness eventually, reported a value of 1.5-2.0 nm that is associated to a monolayer formation\textsuperscript{49}. 
A qualitative observation of the approaching curve of the RLP system showed at pH 11, a less steep slope compared to experiments performed at pH 5 (Figure 30), suggesting that the layer indented was softer and extended to longer distance at pH 11. AFM images at pH 11 present higher roughness as well as higher section profiles, which supports the Force Spectroscopy observations.

Figure 29. AFM height images of cellulose, hydrophobin and resilin like polypeptide RLP fusion protein respectively at pH 5 A, C, E) and 11 B, D, F). QCM-D frequency G), dissipation H) and thickness I) data showing respectively the adsorbed mass, the swelling and the dissipation of the cellulose (red), HFBI (yellow) and RLP (black) fusions.

Figure 30. Approaching AFM indentation curves for RLP at pH 5 (blue) and 11 (red) D). Steeper is the slope stiffer is the layer. The red curve highlight the softness of resilin at pH 11.
5.1.3 Cellulose binding module adhesives for cellulosic nanomaterials

Cellulosic materials revealed very attractive due to their properties. Cellulose is the most abundant material in Nature as well as biodegradable and biocompatible\textsuperscript{160}. Researchers are moving in the direction of cellulose applicability in \textit{i.e.} medical stitches\textsuperscript{161} and breast prosthesis\textsuperscript{162}. A great interest is growing even between material scientists since cellulosic materials showed good mechanical properties such as strength and stiffness\textsuperscript{163}. These properties can be enhanced with the use of protein based materials such as resilin\textsuperscript{48,164} and first of all cellulose binding modules,\textsuperscript{165}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{cellulose_binding_modules.png}
\caption{AFM height pictures of cellulose from trimethylsilyl cellulose (TMSC) A), chitin nanocrystals (ChNCs) B), cellulose nanocrystals obtained by hydrochloric acid hydrolysis (CNC\textsubscript{HCl}) C) and cellulose nanocrystals obtained by sulfuric acid hydrolysis (CNC\textsubscript{H2SO4}) D).}
\end{figure}

For the study related to cellulose binding modules (\textbf{Publication 2}), the interaction forces of fusion proteins with cellulose binding modules (CBMs) were quantified on cellulose surfaces differing for crystallinity and chemical composition. In detail, four model surfaces were employed (\textbf{Figure 31}): amorphous cellulose (converted by HCl hydrolysis from TMSC), cellulose nanocrystals from HCl hydrolysis (CNC\textsubscript{HCl}), cellulose nanocrystals from H\textsubscript{2}SO\textsubscript{4} hydrolysis (CNC\textsubscript{H2SO4}) and chitin nanocrystals (ChNCs).

\textit{The cellulosic surfaces and their affinity toward CBMs}

The binding mechanism of CBMs on cellulose involves the three $\pi$-electron rich tyrosine groups that are exposed to the binding face of the protein (\textbf{Figure 8}), therefore the main interactive forces involved are $\pi$ stacking, together with hydrogen bonds. The interaction force recorded by \textit{Force Spectroscopy} on the different cellulosic surfaces reached a value around 40–50 pN but, some
differences are revealed between the surfaces. The binding force recorded on amorphous cellulose was 44 pN and the one recorded on CNC$_{\text{HCl}}$ 51 pN (Figure 32).

From the first comparison of the different surfaces, it is intuitive to think that the morphology did not play a crucial role. Despite cellulose from TMSC is supposed to be highly amorphous, it contains short crystalline regions that may contribute to the binding of the CBM domain.

An estimation of the crystallinity of the cellulosic surfaces involved, was carried by applying the theory of Nelson O’Connor according to, by the ratio of the intensity of the peaks recorded at 1372 and 2900 cm$^{-1}$ (1375/2900 cm$^{-1}$), the crystallinity index (TCI) is detected. The wavenumber range considered was between 1200-1400, because it contained several bands affected by the amorphous content of the sample rather than by the lattice type. The results are reported in Table 3.

**Table 3.** Estimated Values of TCI for the Surfaces Studied

<table>
<thead>
<tr>
<th>Surface</th>
<th>TCI (1375 cm$^{-1}$/2900 cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>0.1</td>
</tr>
<tr>
<td>CNC$_{\text{HCl}}$</td>
<td>0.5</td>
</tr>
<tr>
<td>CNC$_{\text{H$_2$SO$_4$}}$</td>
<td>0.7</td>
</tr>
<tr>
<td>ChNCs</td>
<td>-</td>
</tr>
</tbody>
</table>

The modified and unmodified cellulose nanocrystals, exhibited interaction forces with the CBM of respectively 39 and 51 pN (Figure 32). The lower force recorded for the modified CNCs find its explanation in the limited binding ability in presence of substitution. In fact, earlier work indicated that the spacing of the three aromatic residues coincides with the spacing of every second glucose ring on a cellulose chain. Therefore, the binding occurs between the aromatic rings and the pyranose rings exposed on the (110) crystalline face of cellulose. The CNC$_{\text{H$_2$SO$_4$}}$ crystals used report a sulfur content of 0.33% S, hence 6–7% of the hydroxyls groups of the cellulose surface are substituted by sulfate groups and this set a limitation into the binding.

Secondly, also electrostatic repulsion may be associated to the presence of sulfate groups. Approaching FD curves have been analyzed for both the nanocrystals, and, when chemically substituted, the repulsion extended to 30 nm, supporting our statement (Figure 33).
Figure 32. Comparison of the Gaussian distributions for the interaction forces recorded between cellulose binding modules and the following cellulosic surfaces: cellulose from TMSC (red), CNC\textsubscript{HCl} (violet), CNC\textsubscript{H2SO4} (green), ChNCs (yellow) and the corresponding FD curves B). Values are normalized.

Eventually, also short range forces, as the hydration, may weaken the described interaction. There is more bound water to the negative SO\textsubscript{3} groups of CNC\textsubscript{H2SO4} than the neutral polysaccharide CNC\textsubscript{HCl} and hence a higher hydration repulsion effect. However, due to the very short distance action range of the force and the overlap of all the repulsion interactions in the force distance curves, no assumptions can support this statement.

Figure 33. Overlap of 120 FD approaching curves representing the interaction between the SpyCatcher-Cel7A-CBM1-CNC\textsubscript{HCl} (black dots) and SpyCatcher-Cel7A-CBM1 -CNC\textsubscript{H2SO4} (red dots). The larger separation range for the interaction with sulfate-derivatized CNCs is explained by a higher repulsive effect due to the negative charges on the nanocrystals. (Adapted from Griffo et al. 2019).

Chitin nanocrystals were the last surface examined. The distribution showed the lowest interaction force (30 pN) revealed in our study, corroborating the assumption that the binding strength follows the substrate specificity, since cellulose is the primary substrate. Chitin surfaces in fact, revealed quite strong
interaction forces when the domain is the chitin binding module as previously reported\textsuperscript{172}.

*The role of SpyCatcher in molecular binding*

The SpyCatcher (Spy-C) domain was used in the study on cellulose binding modules interactions forces (Publication 2) to bind our molecule of interest to a polymer coated tip, then as step of the functionalization of the AFM probe. SpyCatcher is a protein fragment derived by the splitting of immunoglobulin like collagen molecule in two fragments, the SpyTag and the SpyCatcher. Those domains can bind forming a strong covalent isopeptide bond between Lys and Asp\textsuperscript{173}. A value of 2 nN has measured by FS to break the covalent bond. SpyCatcher domain can therefore be used together with its complementary fragment as tag to bind different protein blocks and forming more complex systems.

SpyCatcher has been also found to bind to graphene surfaces as reported in Figure 34 (data not published). This is very interesting since introduce the concept of immobilization and selectivity to the desired analyte molecules\textsuperscript{174}.

![Figure 34. Sketch A) and QCM-D Δf and ΔD adsorption graphs for SpyCatcher (Spy-C) binding on a graphene coated silicon sample and after SpyTag green fluorescent protein injection B). AFM height pictures of graphene coated SiO$_2$ C, left) and St-GFP-ST – Spy-C – graphene coated SiO$_2$ C, right).](image)

In fact, SpyCatcher adsorbed to a surface can potentially bind selectively an analyte of interest, if this is engineered to contain the complementary SpyTag peptide. SpyTag-SpyCatcher system has been already studied in order to grow a layer-by-layer protein based nanofilm to make complex architectures that could potentially be used for therapeutic protein delivery\textsuperscript{175}.

However, it is not yet clear if the SpyCatcher once bound on a surface loses its activity. Figure 34 showed that once that Spy-C was bound to graphene, it has a weak adsorption for SpyTag-Green Fluorescent Protein-SpyTag (ST-GFP-ST) fusion protein. The reason could be related to steric hindrance of the
recombinant protein used having two SpyTags on the termini, that could induce the protein to assume a lying position. Alternatively, SpyCatcher activity could be affected once the protein was bound on surface.

5.2 Silver Nanoclusters arrays

In the last part of the Thesis the interest is directed from the design of bio-based nanocomposites to smart organic-inorganic nanostructures with a bio-inspired approach. A new array of OH-terminated ligands capped silver nanoclusters was designed and the elasticity of a nanocluster monolayer upon stretching was measured.

5.2.1 High performance elastic 2D-materials

For the study of the elastic modulus on atomically precise silver nanoclusters AgNCs (Publication 3) an AFM tip mounted on a cantilever has been used to indent a 2D array of AgNCs, and by the fitting of the approaching/loading/indentation curves the elastic modulus was extracted. The novelty of the proposed system is that, contrarily to most of the systems where the inter-ligand interactions are of van der Waals type, here the driving force that guided the assembly is the hydrogen bond. Furthermore, since, as previously described, 24 H-bonds are involved on the xy plane, the system is supposed to be highly stable and strong.

The elastic membrane of silver nanoclusters and 4-mercaptobenzoic acids (pMBA) ligands was suspended on a patterned holey carbon QUANTIFOIL grid with holes of 1.2 μm of diameter. Since the rim of the hole was 20 nm high the film was literally suspended of 20 nm up to the carbon support. The indentation was performed on the middle of the membrane and the response was evaluated by the observation and analysis of the Fδ curves.

Figure 35. AFM height pictures of suspended two dimensional silver nanoclusters (AgNCs) membrane before A) and after B) breakage upon many indentation cycles.
The equation chosen, described in the experimental section (page 28), does not include the tip radius, rather it is assumed to be a point-like force. Therefore, the choice of a sharp tip having 2 nm of nominal radius points to a more accurate estimation of the Young Modulus $E$.

All obtained indentation curves were linear in force, $F$, for small indentations, $\delta$, but turned nonlinear/cubic as $\delta$ significantly exceeded the membrane thickness, $h$ (Figure 36 a and b). Since for the large indentation displacement reached, the cubic term dominated, the pre stretch, which for small $\delta$ depend on $k$ and cannot be determined a priori, can be neglected and $E$ was then calculated\textsuperscript{126,178}.

On the basis of those considerations, the $E$ values determined for tens of Ag-membranes, fell in a range between 10-30 GPa with the highest probability at 15 GPa (Figure 37). The AFM tip was pushed against the membrane with gentle cycles of indentation of 10 nN and the load was progressively increased until the rupture of the layer (Figure 35). The absence of hysteresis between approaching and retraction cycles indicated an elastic reversible response of the film without energy dissipated. Furthermore, any significant difference in the slopes between the indent and retract slopes was not observed, supporting the absence of a plastic behaviour\textsuperscript{126}. Hysteresis was only observed for $\delta<0$ because of the adhesion tip-surface.

![Figure 36](Image)

**Figure 36.** Overlap of $F\delta$ curves recorded on the same spot of a hole applying increasing force A) and on three different covered holes B).

If we consider that for each nanoparticle 12 H bonds were involved in the binding, the obtained $E$ modulus appears reasonable and not extremely high. Wang et al.\textsuperscript{179} reported for gold nanoparticles array in which the driving assembly was guided by vdW forces, a value of 19 GPa. This suggest that possibly not always all the H-bonds are formed between the particles. Moreover, another contribution in the force may be given by the core-core vdW interactions, especially since the ligands are short in length. The benzene electron rich system of the thiophenol indeed may interact by $\pi$-stacking with the carbon support limiting any friction phenomena.
These set of experiment confirmed so far that a 2D material in which two dimensional silver nanoclusters (AgNCs) were bound together by H-bonds rose unique elasticity and stiffness values. The system is a potential candidate for the development of new materials but, more properties need to be studied.
6. Conclusion and future perspectives

Bioinspired materials have been so far a branch of interest for many scientists. The comprehension of the arrangement of the complex architectures existing in Nature is not only fascinating but allows to aim at the design of artificial natural materials or more precisely bio-based structures.

The current study focussed on molecular interactions whose understanding is essential for being able to create new hybrid materials to replace those that are not based on sustainable raw materials. With the help of the powerful tool of genetic engineering, new synthetic fusion proteins with selected features were designed. Here the study was fundamental and limited to deeply understand the nanoscale interactions at single molecular level or on atomically thick composites films. Once identified the better candidates for the new biobased materials, the production can be scaled up and the study could be moved at meso and macro scale.

In Publication 1, we identified dCBM-RLP-HFBI as a promising candidate for making energy dissipative composites, by the calculation of the adhesion force of the HFBI fusion protein and by a deep focus on the resilin features and properties. The response on the molecular level to the change in pH was striking.

In Publication 2, the attention was instead moved on cellulose and its interactions with proteins. Cellulose is a versatile material for many applications and it is very abundant in Nature. The quantification of the affinity force of a protein domain secreted by cellulase enzymes revealed fruitful in terms of thinking to new cellulosic composites. The focus will be, in the near future, to use linkers that could behave as sacrificial bonds.

In Publication 3 the attention was moved to nanoparticle systems, whose hierarchical structure was inspired by Nature. Supramolecular assembly was the driving force that kept together highly elastic ligand capped nanoparticles.

Eventually with publication 4 we went back to the fundamental question of protein assembly and shed light on the unique features that hydrophobins present. The study of hydrophobin assembly revealed how proteins structure, chemistry and size can affect the monolayer formation.

As final remark the wish is to incentive and inspire research on the direction of the “thinking Bio” for a more sustainable society.
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