Optical properties and self assembly of biotemplated gold nanoparticle chains

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Introduction

In the last decades nanotechnology has affirmed itself as one of the most promising research topic to develop advanced materials and extremely precise tools. Creating structures with precise size and shape at the nanoscale is a great challenge, together with designing materials with particular physical and chemical properties and remaining environmentally friendly and economically viable.

While enormous progress has been made in both materials science and nanofabrication in the last few years (one can think about graphene and nanowires controlled growth, for example) it is still not possible to do as good as Nature in many fields. For example, artificial molecular motors are not as nearly efficient as biological ones (like actin filaments in muscles), and peptide design can still not reach the amazing specificity of some proteins. Nature has, of course, the advantage of time. Evolutionary pressure on organisms can be seen as a hundred of millions of years long, continuous and global experiment resulting from a trial-and-error process in the creation of always more efficient, functional and complex tissues and structures. Protein folding is an amazing example of how Nature obtains high performance while minimizing the energy expense. Several biological materials have peculiar physical or chemical properties (one may think of spider's silk resistance or of the powerful neurotoxins produced by few Amazonian frogs). Moreover biological structures have a very specific functionality and show an ordered and sophisticated architecture organized from the molecular up to the macroscopic scale.

These biological structures have always been of remarkable interest, especially after the discovery of the double helix structure of DNA by Watson, Crick and Wilkins in 1953. Although chemistry and physics have provided powerful tools (as the X-ray crystallography used by Franklin to image DNA) to investigate these systems, usually they were studied exclusively per se, as part of an organism. This has changed radically within the last fifteen years, due to the realization that biological molecules, assembly and nanostructured materials operate at the same length scale, below few hundreds of nm. Since it is impossible to apply the everlasting trial-and-error approach at our level, but also on simulations (even with the most powerful calculators protein folding is still a hard code to break), it is way more convenient to mimic Nature and, when possible, directly use biological nanostructures. A better understanding of principles that regulate particular binding and specific functionalization of some macromolecules have already lead to the design and creation of materials and devices with unparalleled properties.

In particular, hybrid materials have been of great interest as ways to assemble well known materials (metals, semiconductors, some kinds of plastic) in new ways and with new methods. As an alternative to more 'traditional' top-down fabrication techniques, biomediated 'bottom-up' approaches have been proven in some cases more efficient, cheap and precise. Fabrication from nanometric 'building blocks' allows a better size and shape
Introduction

control, does not require excessive quantities of precursors and can be more easily controlled. Processes like molecular self-assembly and biomineralization have been a great starting point to mimic Nature’s work at the nanoscale. Due to the multidisciplinary nature of these studies many scientists with different formations like physicists, chemists and molecular biologists had to collaborate, but also physicians, mathematicians and engineers. Possible applications span from the medical field, where biocompatible objects can be used for transport, catalysis or sensing, to material engineering, where nanostructures with peculiar physical properties can be used to assemble nanocircuits.

It is somehow intriguing that two of the most important physicists of the 20th century, Erwin Schrödinger and Richard Feynman, got their prediction amazingly realized. The first, in his essay *What is life* in 1943 suggested how biology would have to evolve from a descriptive and phenomenological discipline to a molecular science, and the importance of entropy in biological processes. The latter, in his conference *There is plenty of room at the bottom* in 1959 theorized for the first time nanotechnology as the ability to manipulate matter at atomic level. As in 2014 science still has a long way to go before being able to realize this vision, but we can say for sure that we are on the right path.

The recent developments in plasmonic metamaterials have induced in the last few years a growing interest in controlled arrangements of nanoparticles on biological templates. Periodicity and orientation of nanoparticle assemblies, together with their shape and size, can be modified to tune the plasmonic properties of a material. Properties of these hybrid materials can be modelled and predicted, and can be used to develop devices such as biosensors or waveguides. Several self-assembly techniques and different biological templates (DNA, proteins, virus capsids) have been used as scaffolds in previous studies. Among potential biological templates, amyloid fibers are a particularly interesting class of peptide assemblies, with excellent mechanical and chemical resistance and a diameter of few nm.

In this project, lysozyme amyloid fibers have been used as templates to assemble gold nanoparticles into chains. Amyloid fibers are β-sheet rich long (up to several µm), firstly discovered and studied because their correlation with several diseases, such as Alzheimer’s and Parkinson’s diseases. This misfolded (or more precisely, alternatively folded) periodic protein arrays have recently been employed as scaffolds or molds due their unique physical and chemical properties. Gold nanoparticles have specific optical properties because their conductive nature and their size, one or two orders of magnitude smaller than visible light wavelength. Electrons in metal nanoparticles oscillate when in a variable electric field (like a lightwave), when light is in a specific wavelength range these oscillations resonate and are called surface plasmon polaritons. Because of this, gold nanoparticles extinction cross section depends on light wavelength and is maximum around 520 nm if their diameter is below 100 nm. If particles are close together, their electronic oscillations interact with each other changing the wavelength at which they resonate. Tuning this interaction allows to create structures and material with specific plasmonic properties, that can be implemented in several fields, from electronics to medicine. Achieving fabrication of these structures via self-assembly is a cheaper alternative to more classical nanolithographic techniques, even if not always as precise.

In this work the fibrillization process of Hen Egg White Lysozyme that follows its denaturation at elevated temperatures and low pH has been studied in depth, in order to find
optimal morphology in terms of the nucleation and growth. Amyloid fibers most suitable for metallization have been chosen based on parameters such as fibers length, condition and level of aggregation. Parameters such as concentration, incubation time and surface interaction have been varied in the synthesis process, along with purification techniques and seeded growth synthesis. Amyloid resilience to heat, acid environment and some chemicals has been verified. Fibers have been characterized mostly via UV-Vis spectroscopic analysis on suspensions in liquid phase and Atomic Force Microscopy after deposition on clean silicon or glass substrates. Once optimal fibers were selected they have been deposited on substrates before the metallization step. Lysozyme has a high isoelectric point and therefore its fibers carry a net positive surface charge. Gold nanoparticles capped with citrate carry a net negative surface charge on the other hand, so they attach to the fibers keeping an (average) interparticle distance between each other because of charge repulsion. Following nanoparticles grafting and characterization of the resulting hybrid structure an enhancement treatment has been finally performed to improve the metallic coating by increasing the size of the already deposited gold particles. Particles that grow closer to each other interact more strongly when under incident light, and plasmon coupling effects could be observed when enhanced samples were investigated using a spectrophotometer. AFM characterization helped to measure particles size and distance, allowing to model the optical response via simulations and better understand the plasmonic coupling along these chains.
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Chapter 1

Protein folding and amyloid fibers

Proteins are one of the keystones of biological processes: they perform a wide range of tasks, spanning from structural (like actin in muscles) to catalytic functions in metabolic reactions (enzymes). They are at the origin of cell membrane selective permeability, allow neural transmission, make DNA replication possible and allow stimuli response [1]. Because of the vast array of functions they perform, their reproducibility and their key importance for biological processes and diseases they are widely studied in both medical, biological, materials and physical sciences.

Proteins are a universal example of one of the most peculiar property of living systems: the ability to replicate with high precision and fidelity even the most complex structures. Peptide chain folding into the final three-dimensional structure is a marvel of biological self-assembly and has been one of the main topics of interest in biophysics for several decades [2].

This highly precise folding mechanism is also of particular interest also when it fails, as a result of different environmental conditions and/or mutation, leading to misfolded structures that are often the reason for several pathologies. A well known example of such is the formation of long, tubular structures called amyloids from common proteins such as insulin. Accumulation of these protein aggregates in internal organs is a hallmark of several serious pathologies like Alzheimer disease [3].

These fibers are of great interest in medical research and display interesting structural properties [4], therefore in this project amyloid structures formed from hen egg white lysozyme are used as bio-templates to create gold nanochains.

1.1 Protein structure

Proteins are biological macromolecules with an heteropolimeric nature, consisting of one (or more) chain of amino acid residues folded into a well-defined three-dimensional structure [5]. A typical protein consists of \( \approx 300 \) amino acids, and once folded is around \( \approx 5 \) nm wide.

The amino acid sequence defines the so called primary structure of the protein, the one-dimensional backbone of the molecule. Since at body temperature the chain is folded into a compact state and the relative positions of monomers are fixed the protein may be called (quoting Schrödinger) a non-periodic crystal. The shapes into which amino acid assemble (\( \alpha \)-helices or \( \beta \)-sheets/strands, turns and random coils, etc.) are called secondary
structure, while the overall disposition of these assemblies is the tertiary structure.

20 different types of amino acids occur in proteins, and they differ only for the side chain R (see Fig. 1.1). This side chain is attached to a carbon atom commonly denoted as C\(\alpha\), to which are linked a carboxyl and an amine group. Side chains are widely different, going from Glycine’s, which is just an hydrogen atom, to the cyclic structures of Proline.

![Figure 1.1: Representation of an amino acid.](image)

Amino acids can be differentiated based on the properties of the side chain, for example hydrophobic/hydrophilic. Hydrophilic ones have either charged (like Aspartic Acid) or polar (like Serine) side chains, while hydrophobic ones (like Alanine) do not. That is because hydrogen bonds occur via charge-dipole or dipole-dipole interaction of residues with polar water molecules.

The polymerization of these building units involves the creation of a peptide bond (N-C) between two amino acids (see Fig. 1.2), so the primary structure shows high stability. The peptide bond is a covalent bond, approximately 2 orders of magnitude more energetic than the hydrogen bond. The peptide chain is overall quite flexible, the average persistence length of the random coil \(l_p\) being about 2-3 nm.

![Figure 1.2: Peptide bond formation from amino acids.](image)

Since oxygen and hydrogen atoms on the peptide backbone can form hydrogen bonds, as acceptor and donor respectively, in a swollen state these bonds would be satisfied with water molecules. The most favourable way for proteins to fold is to coil into compact structures, where different residues along the chain can hydrogen bond to each other excluding water. Because of the limited directionality of the hydrogen bond only few kinds of regular structures are possible, being mostly \(\alpha\)-helices and \(\beta\)-sheets.

\(\alpha\)-helices are right handed and formed by hydrogen bonds between the N-bonded hydrogen atom of the backbone and the carbonyl oxygens of the backbone of the amino acid 4 position down in the chain. Residue chains stick outwards, conferring specific chemical properties. \(\beta\)-sheets are instead formed by parallel or antiparallel \(\beta\)-strands (extended stretches of polypeptide chain typically 3 to 10 amino acids long) forming hydrogen bonds with each other. The residue chain protrudes alternately above or below the \(\beta\)-sheet plane (see Fig. 1.3).

Elements of the secondary structure then pack together to form the tertiary three-dimensional structure. While the secondary structure is local (amino acids have contacts
only with close monomers in the chain) the tertiary is not since interaction can occur between amino acids very separated along the chain.

The main forces maintaining together protein structure are hydrophobic forces and hydrogen bonds, even if Van der Waals and electrostatic interactions contribute as well. All these forces are considerably weaker (at least one order of magnitude) than the covalent peptide bond. The only covalent bonds between non-adjacent monomers are Cys-Cys disulfide bridges: Cysteine is the only amino acid that can cross-link the polypeptide chain, forming covalent disulfide bonds between two side chains (see Fig. 1.4). The folded structure of large proteins (for example lysozyme) is typically stabilized and determined by several such bonds.

\[ -\text{CH}_2-\text{SH} + \text{HS-CH}_2 \rightarrow -\text{CH}_2-\text{S-S-CH}_2 - \]

Figure 1.4: Representation of the disulfide bridge.

### 1.2 Protein folding and misfolding

The mechanism by which a polypeptide chain folds into a specific three-dimensional protein structure is far from being fully understood, even if it is intuitive that native states of proteins almost always correspond to the structures that are the most thermodynamically stable under physiological conditions [6]. Altering those condition (like pH or, more commonly, temperature) may lead to unfolding or even misfolding of the polypeptide chain.

Hydrophobicity is believed to be the main force that drives the process, hydrophilic amino acids being more likely to stay outside of the folded structure while hydrophobic residues remain inside. Nevertheless a polypeptide chain has a number of possible conformations so large that searching for the most stable structure for complex peptides would require an unacceptable amount of time.

It is nowadays clear that protein folding cannot be modelled into several intermediate steps between partially folded states but it is rather a stochastic search for the most energetically convenient structure. On average native-like interactions between residues are more stable than non-native ones, so they are more persistent and the chain is able to
find its lowest-energy structure by a process of trial and error. If the energy surface (or ‘landscape’) has the correct shape only few trials are needed for the coil to find its correct shape (see Fig. 1.5). For large proteins (more than about 100 residues), one (or more) intermediate state appears to be significantly populated during the folding process and the structural properties of these intermediate states suggest that proteins generally fold into modules. Folding in other words can take place largely independently in different domains or segments of the protein. In this situation few key residues’ interactions are likely to establish a native-like fold within specific local regions or domains, and simultaneously ensuring that the folded regions then interact appropriately to form the correct overall structure [7].

Since the energy landscape is coded into the amino acid sequence it is safe to assume that natural selection favours proteins able to fold rapidly and efficiently. Protein folding is, indeed, a very rapid process: the secondary structure takes 0.1-100 µs to assemble, and the overall shape is usually reached within a second.

Figure 1.5: Schematic energy landscape for protein folding. The saddle point in the landscape corresponds to the transition state, the energy barrier that all molecules must cross if they are to fold to the native state. The yellow spheres in the representative structure are the ‘key residues’, when the native-like contacts of these residues are formed also the overall topology of the native structure is established. From [6]

In a cell, proteins are synthesized by ribosomes from the genetic information encoded in the cellular DNA. Folding can start even before the completion of the peptide chain, but often occurs in the cytoplasm. Misfolding due to inadequate interactions within and
outside the chain is not unlikely to happen, so living systems have developed a range of strategies to prevent such behaviour, such as molecular chaperones to increase the efficiency of the chain folding, but the inability of the protein to fold into a stable globular structure if key interactions do not form acts itself as a 'quality control' process by which the release of misfolded structures can generally be avoided. Failure to fold correctly or to remain correctly folded, due to mutation in the DNA code (even of a single amino acid) or modification of the surrounding environment, can lead to cell malfunctioning and hence disease. An example are amyloid deposits, intracellular protein aggregates that have a key role in many serious diseases like type II diabetes and some cancers.

1.3 Amyloid fibers

Amyloids are a wide range of protein fibrous insoluble aggregates that share specific structural traits. The biophysical definition is "an unbranched protein fiber whose repeating substructure consists of $\beta$-strands that run perpendicular to the fiber axis, forming a cross-$\beta$ sheet of indefinite length" [8]. The stacking of $\beta$-strands results in supramolecular sheets that often extend continuously over thousands of molecular units, in a structure which is unique among protein folds. In the fibers, $\beta$-sheets are densely packed, with intersheet distances around 0.1 nm (see Fig. 1.6).

These aggregates received first attention because of their association with neurodegenerative disorders such as Alzheimer’s syndrome, Parkinson’s disease and type 2 diabetes (see Fig. 1.7, 1.8), where normally soluble proteins are pathologically deposited in intracellular space in amyloid form. More than 20 proteins in the human body are able to form these fibers in vivo, and a large number have been proven to form in vitro: a surprising quantity of unrelated proteins are able to form artificial fibers under conditions where the natively folded state is thermodynamically destabilized. Moreover, functional amyloid-like materials have been discovered to serve various roles throughout nature, like in bacterial coatings, catalytic scaffolds or as adhesives. As such, amyloids can be now viewed as an alternative form of protein structure, different from the native structure but highly or-
 ganized as well and, under particular conditions, energetically favourable. Moreover, in many amyloid-related diseases the intermediate species appear to be way more toxic than the fully formed fibers. This leads to the hypothesis that amyloids could be more of an emergency solution for the organism to store toxic misfolded peptides, instead of an uncontrolled pathological behaviour of the proteins. Anyway, as said before, protein aggregation is a complex phenomenon and there will likely be multiple structural and physicochemical features of the polypeptide chain that influence the propensity for fibril formation.

Figure 1.7: APP deposit in neural tissue of a patient affected by Alzheimer’s syndrome. From WikimediaCommons.

Figure 1.8: Amylin amyloid deposit in a pancreas of a patient affected by type 2 diabetes. From abcam.

Amyloids are just one of the types of aggregates that can be formed by proteins, although their highly organized hydrogen-bonded structure gives them a unique kinetic stability. Therefore once formed such aggregates persist for long periods of time and are not easily dissolved by biological processes. It is not surprising that biological systems almost universally avoid the deliberate formation of these materials, and the exceptions are carefully regulated. Analogous $\beta$ structures are found in some spider silks, which are known for having tensile strength comparable with steel. In vitro, amyloids display heat resistance that is uncommon in biological materials, since many species resist to temperatures above 60 °C, and a great mechanical strength.

The generic amyloid structure strongly contrasts with the globular and individual native state of most proteins. Apart from their shape and size (fibers are usually 4-12 nm wide but up to several $\mu$m long) the core structure of these aggregates is mostly stabilized by interactions (mostly hydrogen bonds) between the polypeptide main chain. In some cases residues interaction can be involved in the structure, but they never play a key role like in the native folding. Since the main chain is common to all polypeptides, this might be an explanation why the amyloid structure is so common even to deeply different proteins. Anyway even if the ability to fibrillate seems generic, the circumstances under which a polypeptide chain is inclined to do so can vary depending on the protein. The aggregation rate depends on many physical and chemical factors such as charge or hydrophobicity, and since hydrophobic features and the main chain are usually inside the native structure partial (or total) unfolding (at high temperature or low pH) or fragmentation (via proteolysis) is required. Furthermore, amyloids 'building blocks’ can vary from quasi-folded native structures to (more frequently) fragments of denatured peptide chain. A common property
of many fragments is that they contain hydrophobic clusters of residues that can trigger protein aggregation.

Despite different formation conditions the kinetics of amyloid growth are generally always the same (see Fig. 1.9). Once the condition for amyloidization is achieved a period of rapid growth follows a lag phase. This behaviour is typical of nucleation-dependant processes (such as crystallization): the first visible (via AFM or SEM) structures are globular or amorphous aggregates, sometimes linked together, which later form bigger structures called ‘protofibrils’. These intermediate species have more distinctive morphologies, with short, thin, eventually curly shapes that later assemble into fibers. Once fibers are formed the spontaneous fragmentation plays a key role: because the growth occurs by addition of soluble proteins to fibril ends, the number of free ends effectively governs the overall conversion reaction. Despite the strength of amyloid structures, the possibility of spontaneous fragmentation via thermal fluctuations appears to be more likely as the length increases. The process is overall quite sensitive and even small changes in the conditions during the growth can lead to differences in growth, and heterogeneous populations can exist even within the same species.

![Aggregation scheme of amyloids](image)

Figure 1.9: Aggregation scheme of amyloids. The transformation of monomeric amyloid peptide goes through a variety of toxic oligomeric stages before creating a mature fibril. Adapted from [9]

As mentioned before, amyloid hierarchical structure and highly organized \( \beta \)-sheet composition are the reason of their peculiar mechanical properties. AFM nanoindentation probe measurements and bending experiments show that the Young’s modulus of a single fiber is between 1-20 GPa [10] (higher than human bones) and can vary up to an order of magnitude if measured radially or axially.

The tendency of amyloids to readily self-assemble without external energy input (such as ATP), together with their robust properties make them interesting for the synthesis of artificial biomaterials as well as biotemplates for hybrid organic-inorganic nanostructures.
1.4 Lysozyme

Lysozyme (also known as muramidase or glycosidase) is an enzyme of biological significance common in nature and found in abundance in several internal organs as spleen and liver, and in tears, saliva, mucus, milk and eggs. Although it is responsible for the start of digestion in the mouth it is mostly used as a non-specific defense against gram-positive bacteria and some fungi, being for this reason a major component of the innate immune system of many species. More specifically, this enzyme binds itself to the polysaccharides chains on bacteria or fungus cell membrane, catalysing the hydrolysis of the glycosidic bond inducing stress on the molecule. The target cell membrane is therefore lysed, leading to cell death. Lysozyme is a small hence highly stable protein, containing only 129 amino acid residues and weighting $\sim 14.7$ kDa. Because of its essential role in the immune system and of its availability, it has been subject of extensive research regarding its structure and function.

Figure 1.10: 3D model of hen egg white lysozyme. Carbon atoms are outlined in gray, oxygen atoms in red, nitrogen atoms in blue, sulfur atoms in yellow.

Figure 1.11: 3D model of hen egg white lysozyme secondary structure. In green are $\alpha$-helices and in red $\beta$-sheets. Disulfide bridges are in yellow.

Lysozyme was first characterized and named in 1922 by Alexander Fleming, who accidentally discovered it by sneezing into a Petri dish containing a bacterial culture [11]. In 1965 David C. Phillips determined the protein structure via X-ray crystallography, making lysozyme the first enzyme ever investigated this way. The protein structure contains five $\alpha$-helix regions and five antiparallel $\beta$-sheet regions, as shown in Fig. 1.11. Linking these secondary structures, a number of beta turns and a large number of random coils make up the remainder of the polypeptide backbone. The protein is kept in shape, apart from hydrophobic interaction and hydrogen bonds (see Fig. 1.11), by four disulfide bonds involving eight Cysteine residues (Cys6-Cys127, Cys30-Cys115, Cys64-Cys80 and Cys76-Cys94).

This enzyme is largely used for biochemical and pharmaceutical applications owing to its antibacterial and antifungal properties, as well as a food preservative. The most
used (and cheaper) type is Hen White Egg Lysozyme (HEWL), extracted from chicken egg white, since 3% of its protein content is made up of this enzyme. Although only 40% identical in sequence, HEWL structure is highly analogous to human lysozyme and shares similar properties.

1.4.1 Lysozyme amyloidization

Lysozyme is an interesting amyloid forming protein, since in its native state it has a high isoelectric point (pI=11.35) that confers it a high solubility in neutral and acidic aqueous solutions [12]. It is a very stable protein too, circular dichroism measurements indicate that its secondary and tertiary structures do not change significantly in acidic solution down to pH 0.64 [13].

Human lysozyme has been shown to form amyloid brils in vivo in individuals suffering from nonneuropathic systemic amyloidosis, all of whom have point mutations in their lysozyme LYS gene. Amyloid deposits form in different tissues containing the variant but not wild-type lysozyme. Formation of such deposits is usually slow, but is generally fatal by the fifth decade of life, and the only effective treatment at present involves transplantation of damaged organs.

In vitro both wild-type human and HEWL can be induced to aggregate into amyloid fibrils when incubated at pH 2.0 and at high temperature (57°-65°) [14] or even at neutral pH if moderate concentrations of protein denaturants are present in solution. A lag time of ∼ 48 h is expected before any aggregates become visible. It has anyway been shown that reduction of the four disulfide bonds in HEWL causes the protein to form amyloid fibrils more readily than the fully native protein, supporting the principle that the ready conversion of a protein into the amyloid structure requires at least partial unfolding prior to aggregation into fibrils.

Despite the high stability of the protein, harsh incubation conditions including acidic pH and elevated temperature break the peptide chain in fragments deriving from the partial acid hydrolysis of the protein at Asp-X (and X-Asp) peptide bonds. The composition of
the fibrillar fraction appears to be essentially complementary to that of the soluble fraction of the same samples, suggesting a differential partitioning of protein fragments between the fibrillar and soluble fractions. Therefore some of these fragments assemble into the well-defined fibrillar structures during the incubation period, while others remain in solution. Most of the α-domain remains soluble, while the majority of fragments detected into de fibrils encompass the region 49-101 of the HEWL peptide chain [15]. This region of the chain is crosslinked by two disulfide bridges (Cys64-Cys80 and Cys76-Cys94) and contains one out of five α-helices of the native HEWL secondary structure and 2/3 of its β-strands. Predictions confirm that fragment 50-101 (or 50-87/88-101) has a significantly higher aggregation propensity than that of any of the other fragments produced by the acid hydrolysis.

At pH 2.0, fibers form also at lower temperatures (T>37°C), and fibrils containing full-length protein are then also formed. At higher temperatures (>80°C), spherical aggregates start to form instead of fibers, probably because that under such conditions the protein is already almost completely unfolded. At higher pH, fibril formation slows down considerably, and does not occur at all above pH 4.0. Concentration appears to be another important parameter (below 2.5 % by weight fibrilization does not occur at all), but the lag time of about 48h appears to be constant and parameter-independent. This means that the governing process for the fibril formation is the structural change of single protein molecules caused by a prolonged exposure to a temperature close to the midpoint of the unfolding transition.

The high percentage of amine (-NH$_2$) and carboxylic acid (-COOH) moieties provides a high potential for functionalization and covalent grafting to substrates or particles. The amine provides also a good affinity for oxidized silicon, making lysozyme fibers very suitable for technological applications and AFM measurement.
Chapter 2

Biotemplated metallization

Since the invention of the Scanning Tunnelling Microscope in 1981 the research effort in nanoscience has kept increasing. Feynman’s predictions in 1959 proved to be amazingly true, and nowadays nanotechnology is one of the most important topics both for basic and applied research. Nanotechnology has been the key to construction of smaller and smaller microchips, and opened new paths in material science. Nanodevices are used also in medicine, passively as sensors and actively to deliver specific drugs targeting.

Reaching the physical limits of the traditional ‘top-down’ approach to nanofabrication, the ‘bottom-up’ one has become of remarkable importance, conjugating both physical and chemical methods. This approach allows to focus on quality over quantity, and often to synthesize structures otherwise impossible to obtain. Systems that are able to build themselves through self-assembly are of particular interest, and wonderful examples can be found in Nature.

Several organism are able to assemble nanometric structures through biomineralization, showing great flexibility but also high specificity. For this reason bioinspired and bioinorganic materials can be a new approach to nanofabrication techniques.

Specifically, the growing interest in nanoplasmonics has started the interest in controlling the deposition of metal nanoparticles on biological materials, using them to tune the properties of the assembly. The resulting hybrid materials could be optimized to be used in biosensors or as waveguides.

Due to their high resistance to heat and mechanical stress, amyloid fibers are interesting biotemplates, especially for metallization. Furthermore lysozyme’s high isoelectric point also allows to use electrostatic interaction for self-assembly along these fibers.

2.1 Bottom up approaches

The most common top-down approach to nanofabrication involves lithographic patterning techniques using short-wavelength optical sources. An important advantage of this approach is that the parts are both patterned and built in place, so that no assembly step is needed. Optical lithography is nowadays widely used in industrial processes (first of all the fabrications of microchips), reaching dimensions below 100 nanometres. Reaching smaller resolution is difficult because of the physical limits given from the wavelength and the numerical aperture of lenses. Several alternatives are possible, for example involving extreme ultraviolet and X-ray sources or advanced photomasks, at the cost of increasing consider-
ably the processing cost. In general, top-down techniques are cheap, efficient and relatively easy to use, but while they work well at the microscale it becomes increasingly difficult to apply them at lower scales. Moreover, excluding rough techniques as milling, deposition and etching are planar, making difficult to construct three-dimensional objects [16].

For these reasons the self assembly of nanometric structures is an important complement to most of the top-down techniques. The basic idea is to use physical or chemical interactions at the nanoscale in order to assemble complex structures starting from basic, simple units, sometimes as small as molecules. Since practically all the structures in living beings are created in such fashion it is not surprising that a lot of these approaches mimic biological processes.

Figure 2.1: Conventional engineering techniques hardly allow the production of structures smaller than 20 nm. The limits of regular bottom-up approaches are in the range of 2-5 nm. The gap is fulfilled by molecular components and colloidal metal nanoparticles or semiconductor materials.

A large number of processes have been developed to create nanoparticles, from condensation of atomic vapours on surfaces to coalescence of atoms in liquids (as with Turkевич method). Oriented crystal growth enables the construction of nanowires directly on surfaces (see Fig. 2.2). More advanced processes mimic the nutrient transportations between cells using inverse micelles as templates. Lipid molecules in a nonaqueous solution have their polar (hydrophilic) ends pointing inward to form a hollow core, which size can be tuned and can be used to produce size-selected nanoparticles of semiconductor, magnetic, and other materials [17]. An example of self-assembly that achieves (partially) control over formation other than size is the growth of quantum dots. Indium gallium arsenide (InGaAs) dots can be formed by growing thin layers of InGaAs on GaAs in a way that the compressive strain in the InGaAs layer results in repulsive forces that trigger in the formation of isolated quantum dots. After the growth of multiple layer pairs, a fairly uniform spacing of the dots can be subsequently achieved [18]. Nanoparticles shape can be manipulated as well, regulating nucleation and more importantly crystallographic growth direction via the use of surfactant or other chemical compounds able to bind to crystals only in specific crystal directions.

Ordered systems can be created via the bottom-up approach as well. Techniques as the Langmuir–Blodgett compression allows to mechanically control single monolayers of fluid or colloid, thus controlling with good precision the particle density on a surface.
2.2 Biomineralization and bioinorganic materials

Local forces, as the electrostatic interaction, can be used as well to bind a type of 'building block' to another, or to arrange them periodically into a structure (even in three dimensions). Also simpler methods, such as layer by layer deposition, can be implemented up to a surprisingly high degree of precision, for example creating ordered patterns of perpendicularly-crossing nanowires [20].

As stated before, biological systems often show a refinement hard to achieve technologically. Most of the molecules that regulate our body processes foil themselves due to internal interactions (as shown in Chap. 1), and display a wide range of architectures in order to cover a huge number of well-defined functions. Some of these act as building blocks to create structures with remarkable mechanical properties, such as actin fibers in muscles, or really precise databases such as DNA. These structures can also be used as scaffold for more complex nanostructures. The specific functional groups that are present on biomolecules can then be used to trigger specific cohesion between structures, even inorganic ones if correctly functionalized [21]. Processes as metallization or electrodeposition can then be applied to create conductive objects. Several biological structures have been used for this purpose, and recently peptides and DNA sequences have been engineered to fit precise needs in nanofabrication.

Figure 2.2: SEM picture of Au-catalysed (In,Ga)As-(Ga,Mn)As core-shell nanowires grown on GaAs(111)B substrate. From [22].

Figure 2.3: 3D photonic crystal: self assembly of SiO$_2$ nanoparticles. From [23].

2.2 Biomineralization and bioinorganic materials

Several organisms are able to produce inorganic or bioinorganic structures. This process is called biomineralization and it is a widespread phenomenon, since it is encountered in all six taxonomic kingdoms. Organisms and minerals can vary sensibly, going from calcium phosphate and carbonate in the skeleton of vertebrates to silicates in more primitive lifeforms such as algae and diatoms. Mineralized tissues are usually used for structural support or defence, but there are some surprising exceptions, as Fe$_3$O$_4$ used as magnetic sensor in magnetotactic bacteria.

In nanoscience the structures of these materials are fascinating because their growth is highly controlled from the nanometer up to the macroscopic level, resulting in complex architectures that provide multifunctional properties. Most industrial nanofabrication pro-
cesses need stringent conditions (like high pressure, temperature or pH) and have an overall low efficiency. Reproducibility is often an issue as well, since fine control of agglomeration is all but easy. On the other hand several organisms are able to assemble analogous structures under mild conditions and with incomparable precision and efficiency. In these biological processes the mineral grows on a biological template that directs the crystal nucleation, controls growth and morphology. This template is usually proteic and leaves a low residue in the final product: by weight, molluses shells are 99-96% calcium carbonate, and only about 1% protein [24].

![Diatom frustule](image1.png)

**Figure 2.4:** Salt water centric diatom frustule. Diatoms are microscopic unicellular algae that produce intricate silica cell walls. From Dennis Kunkel Microscopy

![TEM image of magnetotactic bacterium](image2.png)

**Figure 2.5:** TEM image of a magnetotactic bacterium. The chain of magnetite (Fe3O4) nanoparticles is along the long axis of the cell. From [25].

Since the molecular mechanisms behind these processes are not yet fully understood, attempts at protein-mediated materials biotemplating have involved the empirical use of alternative biological templates, rather than exactly copying well-characterized natural routes. Because of their conductive, plasmonic and in some cases magnetic properties, metals are interesting candidates for deposition on biological scaffolds.

One of the first attempts use crystalline bacterial cell-surface layers (S-layers) as substrate. S-layer is a glycoproteic envelope present on most bacteria, and can be easily isolated and reconstructed in vitro to generate two-dimensional monomolecular protein arrays with a pore size in the range 2–6 nm. This substrate was used both for in situ nucleation of gold nanoparticles [26] and patterning of nanoparticles by chemically linking the particles to repetitive protein-binding sites in the layer [27].

More generally, electroless plating techniques from the microelectronics industry have been proved a good starting point for the construction of hybrid structures. Electroless plating uses autocatalytic chemical reactions to deposit metallic layers or particles without the use of external electric fields. These techniques were originally developed to enhance or alter specific material properties such as hardness or conductivity, and treatments that are compatible with biological matter (room temperature, circumneutral pH, ..) have been successfully implemented also on organic scaffolds. Not only proteins have been used: lipids, virus structures and DNA molecules have been investigated as well [28]. For example, DNA is a versatile candidate, because of its ability to attach to specific binding sites (depending on the bases sequence), its negative surface charge and the easiness of its reproduction. Nanowires created growing gold on DNA show linear ohmic behaviour, and
2.3 Amyloid fibers as biotemplates

One of the limits of biotemplated fabrication is fragility of the biological materials under conditions incompatible with life. Biological structures that do not suffer under thermal stress and at a high or low pH, like amyloid fibers, are therefore of remarkable interest. The huge number of proteins that have been proven able to form amyloid fibers represents an advantage as well, offering a lot of options when looking for the best template.

Several studies have used amyloids as biotemplates for metallization. For example amyloids formed from the N-terminal and middle region of Sup35p, a prion determinant from *Saccharomyces Cerevisiae*, have been used to create gold and silver nanowires [31]. Small (<2 nm) gold nanoparticles have been covalently cross-linked to the fibers and then coated with silver. From 10 nm wide fibers, 60 nm wide nanowires are obtained, which display low resistance and ohmic behaviour. A similar procedure had been used in the past on DNA as well, but it is unstable under the conditions (10 pH, > 60°) necessary for industrial metallization.

Amyloids have also been used as a mold, since their cylindrical structure often presents a hollow core. Silver ions can be reduced using 2,2,2-trifluoroethanol (TFE) inside the core of fibers, forming a metal wire. The fiber can later be removed using a protease enzyme (See Fig. 2.6). Silver nanowires obtained this way are very thin (1-2 nm) and up to 2 µm long [32].

![Figure 2.6](image-url) Scheme of casting silver nanowires within amyloid scaffolds. From [32].

2.4 Electrostatically driven HEWL amyloids gold metallization

In this project 15 nm diameter gold nanoparticles were bound to HEWL amyloid fibers via an electrostatically-driven self assembly mechanism. This process differs considerably from the chemically induced grafting techniques, since electrostatic interactions need a shorter time to develop than chemical bonds and can also reach higher energy. Lacking

silver nanowires can be assembled in similar fashion [29]. Binding to specific sites on DNA can be used for creating ordered arrays of nanoparticles. 2D grids made of DNA strings can be deposited with gold nanoparticles, that arrange in a periodic square lattice. The interparticle spacing can be tuned by acting on the DNA template [30].

In the end the great interest in the production of conductive nanostructures for application in electronic has been the best motivation to research new biotemplated materials so far, and amyloid fibers are emerging as good candidates to fill this role.
the presence of specific functional groups on the protein fiber and the metal nanoparticle to attach the latter on specific sites the procedure may be not as accurate as others, but allows anyway the assembly of a well-ordered periodic structure. The high isoelectric point of lysozyme (11.35), caused by the large abundance of basic amino acid residues, make the fibers optimal for electrostatic functionalization. Moreover, because of the periodic structure of amyloid fibers, these positively charged residues, such as lysine (Lys) and arginine (Arg), are periodically disposed as well, granting an homogeneous distribution of charge on the fiber’s length.

Gold nanoparticles are capped with citrate, dissolving trisodium citrate ($Na_3C_6H_5O_7$), which plays also the role of reducing agent during synthesis. Citrate capping gives gold nanoparticles negative surface charge, making them suitable for grafting on the positive amyloid fibers, and ensuring the colloidal stability of the gold suspension thanks to charge repulsion.

Subsequent deposition of fibers and nanoparticles is done on borosilicate glass or Si/SiO$_2$ silicon oxide substrates. These surfaces are cleaned with piranha solution, which also provides activation. Activated silicon based surfaces have a (macroscopically) uniform distribution of hydroxyls group, that are negatively charged [33]. This surface charge is responsible for the adhesion of the fibers on the surface (specific fibers position are given from the local charge distribution) and prevents citrate capped gold nanoparticles to deposit on the surface itself.

As shown in [34] particle disposition on fibers is mostly driven by interparticle repulsion than by particle-fiber interaction. Particle spacing is not function of the particles diamenter only, it is also dependant on the pH and the salt and citrate concentration in the solution used for deposition.
Chapter 3

Gold Nanoparticles 1-dimensional arrays

Metal nanoparticles (NPs) have been used for hundreds of years to stain glass, but the first scientific investigations started only in the 19th century. Since then they have been extensively studied, and eventually became a keystone of modern nanosciences.

These particles, with diameters ranging roughly between 1 and 100 nanometers, are at the boundary between molecules and extended solids. They are complex many-electron systems, where reduced sizes and quantum confinement of electrons and phonons give birth to fascinating new effects, potentially tunable with particle size and shape [35].

In metals, conduction band electrons can couple with an oscillating electric field, and the curved surface of sub-wavelength nanostructures exerts an effective restoring force on the driven electrons, allowing a resonance leading to field amplification both inside and in the near-field zone outside of the particle. This resonance is called localized surface plasmon (LSPR) and can be excited by direct light illumination, in contrast with volume and surface polaritons. Because of this nanoparticles have highly wavelength-dependent optical properties, that still makes them a great subject of research for their potential technological application. They are used from electronics to catalysis, and find applications also in biosciences and medicine.

Since LSPRs in metal nanoparticles interact with each others if they are close enough, organized arrays of these structures have been an interesting topic of research. These arrays (usually 1- or 2-dimensional) can display features largely different form the parts they are composed of, and, especially if obtained by self-assembly, might find applications in many fields, including plasmon waveguides or SERS spectroscopy [36].

3.1 Gold nanoparticles

Gold is a metal widely used to create nanoparticles, and probably the first ever to be used so. Synthesis of colloidal gold (a suspension of gold nanoparticles in a fluid, usually water) is known, or at least used without much understanding of the process, since ancient times, usually to stain glass with an intense ruby red colour. An example is the Lycurgus cup, a 4th century glass cage cup made of dichroic glass: the cup looks red if illuminated from the back (absorbance) but green if illuminated from the front (reflectance) (see Fig. 3.1). This is because of the gold and silver nanoparticles in the glass which have the plasmon
absorption peak in the visible green range, around 520 nm. Also silver has been used for similar purposes, having also a plasmon absorption peak in the visible range, giving the glass a bright yellow color. Despite the synthesis technique being refined during the 17th century and a photographic process that used colloidal gold being invented in 1842, the first scientific work, by Michael Faraday, dates from 1857. In 1908 Gustav Mie’s solution to Maxwell’s equations for the extinction spectra of spherical particles of arbitrary size finally explained the peculiar optical properties of gold nanoparticles.

Figure 3.1: Two pictures of the Lycurgus cup, illuminated from the front and from the back.

In the last two decades, gold nanoparticles proved to be still a great subject of research for their potential technological applications in a rapidly growing range of disciplines. In electronics, with the miniaturization of the components, they can be used to connect elements in an electronic chip, but NPs can also be designed for use as conductors from printable inks to electronic chips [37]. Due to their refractive index dependent optical properties they are used in a variety of sensors, for the detection of pollutants or biological molecules via surface-enhanced Raman spectroscopy. Because of their scattering properties they are used as probes in biological imaging as well [38]. In chemistry they are used as catalysts in a number of chemical reactions [39], and for the selective oxidation on the nanoparticle surface. They are also convenient components for sub-wavelength optical devices [40] and for nonlinear optics [41].

More recently interaction of metal nanoparticles with biological matter started to be investigated, and nowadays many biomedical applications exist. Gold nanoparticles have been used in diagnostics to detect biomarkers in a variety of diseases [42] and in lateral flow immunoassays (for example in home pregnancy tests), but also for therapeutic agent delivery, since the large surface area-to-volume ratio of nanoparticles makes them well suited for coating with hundreds of molecules [43]. Near-IR absorbing particles, that produce heat when excited at wavelengths in the 700-800 nm range, have been used to kill cancer cells by rapid heating [44].

3.2 Optical properties

Interaction of light with small objects strongly depends on their size and shape, as well as their composition and the composition of the medium in which they are embedded.
When light illuminates a metal nanoparticle, two different processes occur: scattering and absorption. Scattering occurs because in a metal electrons are free to move, so they are set in an oscillatory motion from the incident light wave, generating radiation. If the particle transfers the energy of the exciting light into another energy source (usually heat) the light is said to be absorbed. The total extinction is the sum of the contributions of both scattering and absorption.

Solving the problem of scattering and absorption of light by a small particle for its cross section implies solving Maxwell’s equations with the correct boundary conditions. An exact solution exists for spherical and cylindrical objects, as shown in 1908 by Gustav Mie [45], while the complete derivation of Mie theory is given by Bohren and Huffman. The approach of the Mie theory is to expand the internal and scattered fields into a set of normal modes described by vector harmonics. The solution is derived imposing continuity of the tangential electric and magnetic field at the particle’s surface, where there is a discontinuity in the dielectric function $\varepsilon$ and the magnetic permeability $\mu$. In most cases, the relative magnetic permeability of the materials is close enough to 1 to be neglected.

![Figure 3.2: Schematic representation of the problem. A particle with dielectric function $\varepsilon(\omega)$ is embedded in a medium with dielectric constant $\varepsilon_m$, and illuminated by a plane wave $\vec{E}_0$, which generates an electric field and magnetic field inside the particle. The particle radiates a scattered field in all directions, that varies with the incident wavelength.](image)

For a spherical, homogeneous and isotropic particle in a homogeneous medium under an applied electromagnetic plane wave the exact solutions for scattering, extinction and scattering cross sections are:

$$\sigma_{sca} = \frac{2\pi}{k^2} \sum_{n=1}^{\infty} (2n+1)(|a_n|^2 + |b_n|^2)$$  \hspace{1cm} (3.1)

$$\sigma_{ext} = \frac{2\pi}{k^2} \text{Re}(a_n + b_n)$$  \hspace{1cm} (3.2)

$$\sigma_{abs} = \sigma_{ext} - \sigma_{sca}$$  \hspace{1cm} (3.3)

since extinction power is the sum of the scattered and absorbed power. The coefficients $a_n$ and $b_n$ are given by

$$a_n = \frac{m\psi_n(mx)\psi_n'(x) - \psi_n(x)\psi_n'(mx)}{m\psi_n(mx)\xi_n'(x) - \xi_n(x)\psi_n'(mx)}$$  \hspace{1cm} (3.4)
Chapter 3: Gold Nanoparticles 1-dimensional arrays

\[ b_n = \frac{\psi_n(mx)\psi'_n(x) - m\psi_n(x)\psi'_n(mx)}{\psi_n(mx)\xi'_n(x) - m\xi_n(x)\psi'_n(mx)} \]  

(3.5)

in which \( x = kR \) (\( R \) is the radius of the particle) is the size parameter, \( m = \sqrt{\frac{\varepsilon}{\varepsilon_m}} \) and \( \psi \) and \( \xi \) are the Ricatti-Bessel functions of order \( n \). The prime indicates derivative to the parameter in parenthesis.

The complex dielectric function \( \varepsilon = \varepsilon_1 + i\varepsilon_2 \) is related to the particle’s complex refractive index \( \tilde{n} = n + ik \), whose real and imaginary parts describe the phase and amplitude of an electromagnetic wave in matter, i.e. the phase velocity in the material and amount of absorption loss in the propagation. Under assumption of non-magnetic material (\( \mu \approx 1 \)), \( \varepsilon = \tilde{n}^2 \). The dielectric functions of most metals were measured in the 1960s and 70s [46], the measurements for gold by Johnson and Christy in 1972 are considered the most reliable, and used in this thesis (see Fig. 3.3).

![Figure 3.3: Real and imaginary parts of the bulk dielectric function \( \varepsilon(\omega) \) of gold. From [46].](image)

3.2.1 Quasi-static approximation

A much easier approach can be used if one considers small particles (\( R < 100 \text{ nm} \)), if \( R \ll \lambda \) (the particle is much smaller than the wavelength of light in the surrounding medium). In this approximation, called the Rayleigh limit, the phase of the harmonically oscillating electromagnetic field is practically constant over the particle volume, so the spatial field distribution can be calculated assuming the particle in an (instantaneously) electrostatic field. This quasi-static approximation valid for sub-wavelength spheres is equivalent to retaining only the first term of the Mie’s power series expansion.

A homogeneous, isotropic sphere of radius \( R \) and (complex) dielectric constant \( \varepsilon(\omega) \) (assumed as a constant number, i.e. at given wavelength in the calculations) is surrounded by a non-absorbing isotropic medium with (real) dielectric constant \( \varepsilon_m \). The sphere is immersed in a static electric field \( \vec{E} = E_0\hat{z} \). The electric field \( \vec{E} = -\nabla \Phi \) can be calculated via the potential \( \Phi \), that satisfies the Laplace equation \( \nabla^2 \Phi = 0 \).

Due to the symmetry of the problem, the solution is of the form

\[ \Phi(r, \theta) = \sum_{l=0}^{\infty} \left[ A_l r^l + B_l r^{-(l+1)} \right] P_l(\cos \theta) \]  

(3.6)
3.2 Optical properties

Figure 3.4: Schematic representation for the quasi-static approximation. The electric field $\vec{E}$ in $P$ can be evaluated as a function of $r$ and $\theta$

where $P_l(\cos \theta)$ are the Legendre Polynomials of order $l$, and $\theta$ the angle between the position vector $\vec{r}$ at point P and the z-axis (see Fig. 3.4). Under the requirement that potentials remain finite at the origin, the solution inside and outside the sphere can be written

$$
\Phi_{\text{in}}(r, \theta) = \sum_{l=0}^{\infty} A_l r^l P_l(\cos \theta)
$$

(3.7)

$$
\Phi_{\text{out}}(r, \theta) = \sum_{l=0}^{\infty} \left[B_l r^l + C_l r^{-(l+1)}\right] P_l(\cos \theta)
$$

(3.8)

The coefficients $A_l$, $B_l$ and $C_l$ can be determined via the three boundary conditions, one at $r \to \infty$ and two at the sphere surface $r = R$. For $r \to \infty$ it is required that

$$
\Phi_{\text{out}} \to -E_0z = -E_0 r \cos \theta
$$

(3.9)

i.e. at infinity the field is the same as the one of the incident wave. So $B_1 = E_0$ and $B_l = 0$ for $l \neq 1$. The equality of the tangential component of the electric field at the sphere surface demands that

$$
-\frac{1}{R} \frac{\partial \Phi_{\text{in}}}{\partial \theta} \bigg|_{r=R} = -\frac{1}{R} \frac{\partial \Phi_{\text{out}}}{\partial \theta} \bigg|_{r=R}
$$

(3.10)

Likewise, the equality of the normal component of the displacement field implies

$$
-\varepsilon_0 \varepsilon(\omega) \frac{1}{R} \frac{\partial \Phi_{\text{in}}}{\partial r} \bigg|_{r=R} = -\varepsilon_0 \varepsilon_m \frac{1}{R} \frac{\partial \Phi_{\text{out}}}{\partial r} \bigg|_{r=R}
$$

(3.11)

These two boundary conditions lead to $A_l = C_l = 0$ for $l \neq 1$, and the potentials are evaluated via calculations of the remaining coefficients $A_1$ and $C_1$

$$
\Phi_{\text{in}} = -\frac{3 \varepsilon_m}{\varepsilon + 2 \varepsilon_m} E_0 r \cos \theta
$$

(3.12)

$$
\Phi_{\text{out}} = -E_0 r \cos \theta + \frac{\varepsilon - \varepsilon_m}{\varepsilon + 2 \varepsilon_m} \frac{E_0 R^3 \cos \theta}{r^2}
$$

(3.13)
A physical interpretation can be given to Eq. 3.13: $\Phi_{\text{out}}$ is given by the superposition of the applied field and that of a dipole located at the particle center. Therefore, the equation can be rewritten as

$$\Phi_{\text{out}} = -E_0 r \cos \theta + \frac{\vec{p} \cdot \vec{r}}{4\pi \varepsilon_0 \varepsilon_m r^3}$$

(3.14)

introducing $\vec{p}$ the virtual dipole moment of magnitude proportional to $E_0$ given by

$$\vec{p} = 4\pi \varepsilon_0 \varepsilon_m R^3 \frac{\varepsilon - \varepsilon_m}{\varepsilon + 2\varepsilon_m} E_0$$

(3.15)

Finally, the polarizability $\alpha$ of a small sphere of sub-wavelength diameter in the electrostatic approximation, defined by $\vec{p} = \varepsilon_0 \varepsilon_m \alpha \vec{E}_0$, is:

$$\alpha = 4\pi R^3 \frac{\varepsilon - \varepsilon_m}{\varepsilon + 2\varepsilon_m}$$

(3.16)

It is evident that polarizability had a resonance enhancement when $|\varepsilon + 2\varepsilon_m|$ is at minimum, which can be approximated to

$$\text{Re}[\varepsilon(\omega)] = -2\varepsilon_m$$

(3.17)

if $\text{Im}[\varepsilon(\omega)]$ is small or slowly varying around the resonance frequency. This expression is called the Fröhlich condition, and the associated mode in an oscillating field is the dipole surface plasmon. In vacuum (or air), for a Drude metal (free electron gas), the condition is satisfied at $\omega_0 = \frac{\omega_p}{\sqrt{3}}$, with $\omega_p$ the plasma frequency of the metal. The resonance redshifts as the refractive index $n$ of the medium increases, since $\varepsilon_m = n^2$.

Introducing oscillation in the electrostatic field is equivalent to switching to an incident plane wave $\vec{E}(r, t) = \vec{E}_0 e^{-i\omega t}$. For the condition $R \ll \lambda$ the particle can be represented as a dipole, allowing time-varying fields but neglecting spatial retardation effects over the particle volume. The oscillating field induces an oscillating dipole moment $\vec{p}(t) = \varepsilon_0 \varepsilon_m \alpha \vec{E}_0 e^{-i\omega t}$, with $\alpha$ from the quasi static approximation. The scattering of the sphere can be therefore as radiation from a point dipole. The total electric and magnetic fields in the radiation zone of a dipole can be written as

$$\vec{E}(t) = \frac{1}{4\pi \varepsilon_0 \varepsilon_m} \left\{ \frac{k^2}{r} (\hat{n} \times \vec{p}) \times \hat{n} + \frac{1}{r^3} \left[ 3\hat{n} (\hat{n} \cdot \vec{p}) - \vec{p} \right] \right\} e^{ikr-i\omega t}$$

(3.18)

$$\vec{H}(t) = \frac{ck^2}{4\pi} (\hat{n} \times \vec{p}) e^{ikr} \left( 1 - \frac{1}{ikr} \right) e^{-i\omega t}$$

(3.19)

with $\hat{n}$ the unit vector in the chosen direction, and $k = \frac{2\pi}{\lambda}$ the wave number.

The scattering and absorption cross sections can be calculated via the Poynting-vector determined with Eq. 3.18 and 3.19

$$\sigma_{\text{abs}} = k \text{Im}[\alpha] = 4\pi k R^3 \text{Im} \left[ \frac{\varepsilon - \varepsilon_m}{\varepsilon + 2\varepsilon_m} \right]$$

(3.20)

$$\sigma_{\text{sca}} = \frac{k^4}{6\pi} |\alpha|^2 = \frac{8\pi k^4 R^6}{3} \left| \frac{\varepsilon - \varepsilon_m}{\varepsilon + 2\varepsilon_m} \right|^2$$

(3.21)

In the small particle approximation, the scattering efficiency, scaling with $R^6$ is dominated by the absorption efficiency, that scales with $R^3$. For very small particles, scattering is
then almost negligible as compared to absorption. Scattering and absorption are both resonantly enhanced when the Fröhlich condition is met, at the dipole particle plasmon resonance. That goes for extinction as well, which explicit expression for a sphere of dielectric constant \( \varepsilon = \varepsilon_1 + i\varepsilon_2 \) is:

\[
\sigma_{\text{ext}} = \sigma_{\text{abs}} + \sigma_{\text{sca}} = 12\pi k \varepsilon_m \frac{3}{2} R^3 \frac{\varepsilon_2}{(\varepsilon_1 + 2\varepsilon_m)^2 + \varepsilon_2^2}
\]

(3.22)

Figure 3.5: Gold nanoparticle extinction cross section (evaluated with 3.22) of radius \( R = 15 \) nm embedded in media of different refractive index \( n \), and of different radii \( R \) embedded in a medium of refractive index \( n = 1.265 \).

Up to this point, no explicit assumptions regarding the material from which the nanoparticle have been made. The formulas are indeed both valid for dielectric and metallic particles, although the results in the two cases are hugely different because of the big difference in the dielectric function \( \varepsilon(\omega) \) between metals and dielectrics. The quasi static approximation does not take retardation into account, so the resonance is independent of particle size. Actually the resonance is redshifted for larger particles, and the scattering peak generally lies further to the red than the absorption peak. The absorption spectrum displays a plateau for wavelengths lower than the resonance, which is caused by the absorption of the bounded d-band electrons.

When the size of the particle becomes comparable to the wavelength of the light the phase of the electric field cannot be considered constant in the particle, inducing a retardation effect. The retardation of the propagation of the electric field in the metal implies a further redshift of the surface plasmon resonance.

### 3.3 Plasmon coupling in 1-dimensional nanoparticle arrays

For single spherical metallic nanoparticles the localized plasmon resonance can be red-shifted by increasing the particle size. In particle ensembles a further shift is expected due to interactions between local modes. These interactions mostly are of dipolar nature, so in a first approximation a group of particles can be treated as an ensemble of interacting dipoles.

In disordered ensembles, like concentrated nanoparticles suspended in a fluid, effective medium approximation are usually well suited to describe the optical response of the
ensemble. The most common is the Maxwell-Garnett approximation [47], that calculates an effective dielectric constant $\varepsilon_{\text{eff}}$ considering exact dipole-dipole interaction. For the Maxwell-Garnett equation:

$$\frac{\varepsilon_{\text{eff}} - \varepsilon_m}{\varepsilon_{\text{eff}} + 2\varepsilon_m} = f \frac{\varepsilon - \varepsilon_m}{\varepsilon + 2\varepsilon_m}$$

(3.23)

with $f$ the filling factor, i.e. the volume fraction of the nanoparticles. For high values of $f$ further corrections are required, such as in Bruggeman Theory [48].

$$f \frac{\varepsilon - \varepsilon_{\text{eff}}}{\varepsilon + 2\varepsilon_{\text{eff}}} + (1 - f) \frac{\varepsilon_{\text{eff}} - \varepsilon_m}{\varepsilon_{\text{eff}} + 2\varepsilon_m} = 0$$

(3.24)

In ordered metal nanoparticle arrays a different approach can be used. The ideal situation is a 1-dimensional array of spherical nanoparticles of radius $R$ with interparticle distance $d$. To have the dipolar approximation to be fully justified the further assumption $R \ll d$ will be assumed, so the particles can be treated as point dipoles. In this approximation two regimes can be distinguished depending on the order of magnitude of the interparticle distance $d$.

For large separations ($d \sim \lambda$) far-field dipolar coupling dominates, whose distance dependence is $d^{-1}$. In this case narrow peaks (widths less than 1 nm) appear when the incident wavelength is close in value to the interparticle distance [49]. These peaks are caused by the long range dipolar far-field interactions resulting from a rapid variation in the dipole sum as the wavelength is varied close to the interparticle separation.

At smaller separations (assuming $d \ll \lambda$) near-field interactions with a distance dependence of $d^{-3}$ dominate, and the chain of nanoparticles can be treated as an array of point

![Figure 3.6: Schematic representation of the transverse and longitudinal polarization modes.](image1)

![Figure 3.7: Numerical simulation of the local electric field for a chain of 9 gold nanoparticles ($R = 10$ nm) with incident electric field normal and parallel to the chain.](image2)
dipoles interacting via their near-field. The response of the chain is largely dependant on the angle and the polarization of the incoming light, since induced dipoles are parallel to the oscillating electric field. Transverse modes are induced by light perpendicularly incident on the chain (since \( \vec{E} \perp \vec{k} \)), with polarization parallel to the chain itself, while longitudinal modes are induced by light with polarization perpendicular to the chain (see Fig. 3.6). Intuitively one can see interparticle dipole-dipole coupling will lead to shifts of the spectral position as compared to the one obtained for an isolated particle. Also, strong field localization in the gaps between particles occur [50]. This field localization is due to a suppression of scattering into the far-field via excitation of plasmon modes in particles along the chain axis, mediated by near-field coupling.

This near-field coupling in linear nanoparticle chains is of great interest, mainly because the transverse and longitudinal modes supported by the chains are no longer localized on the individual nanoparticles but can propagate along the chain axis, allowing electromagnetic energy transport. For this reason nanoparticle chains could be used as waveguide structures smaller than the diffraction limit of the excitation light. Coupling of light to propagating surface plasmons then makes it possible to direct energy along a chain, maybe allowing also to bend light around corners or split it into two or more branches using particular geometries, such as T-shaped structures.

### 3.3.1 Coupled Dipole Model

A first model to explain the behaviour of an ordered, 1-dimensional collection of spheres under incident light can be derived starting again from the dipole approximation. The general framework for modelling the optical response of a collection of spheres involves self-consistent solution of the response of each particle to the incident field and the scattered fields of the other particles. The assumption that the particles respond primarily to the electric dipole component of the local field is justified as long as \( R \ll \lambda \) and \( \mu \approx 1 \) (non-magnetic material). This approximation assumes also coherent excitation of all the spheres. Therefore, as long as the component nanospheres are not spaced too closely, the response of an aggregate to electromagnetic radiation can be determined by self-consistent solution of the electric dipole polarizations, \( \vec{P}_m \), of each sphere in the field of the incident light and the sum of the dipole fields of the other particles, as shown in [51]. Assuming \( N \) particles, and the \( m \)-th one has polarizability \( \alpha_m \) and center in \( \vec{r}_m \)

\[
\vec{P}_m = \alpha_m \cdot \vec{E}_{loc}^m
\]

with \( \vec{E}_{loc}^m = \vec{E}_{loc}(\vec{r}_m) \) the local electric field, sum of the incident field \( \vec{E}_{inc}^m = \vec{E}_{inc}(\vec{r}_m) = \vec{E}_0 e^{i(\vec{k} \cdot \vec{r}_m - \omega t)} \) and the retarded fields of all the other \( N - 1 \) dipoles, shown in 3.18. From now on the time dependence will be omitted in the calculations. \( \alpha_m \) is the polarizability of the single sphere calculated in quasi-static approximation, as shown in Eq. 3.16

\[
\vec{E}_m = \vec{E}_0 e^{i\vec{k} \cdot \vec{r}_m} - \sum_{n \neq m} \frac{e^{ikr_{mn}}}{r_{mn}} \cdot \left\{ k^2 \hat{r}_{mn} \times (\hat{r}_{mn} \times \vec{P}_n) + \frac{ikr_{mn} - 1}{r_{mn}^2} \cdot [3\hat{r}_{mn} \cdot \vec{P}_n \cdot \vec{P}_n] \right\}
\]
where \( \vec{r}_{mn} = \vec{r}_m - \vec{r}_n \) is the vector between the \( m \)-th and the \( n \)-th particles, \( r_{mn} = |\vec{r}_{mn}| \) and \( \hat{r}_{mn} = \frac{\vec{r}_{mn}}{r_{mn}} \). Substituting Eq. 3.26 into 3.25

\[
\vec{E}^{\text{inc}}_m = \alpha^{-1} \vec{P}_m + \sum_{n \neq m} A_{mn} \cdot \vec{P}_n
\] (3.27)

with \( A_{mn} \) the matrix of the coefficients from Eq. 3.26. The explicit expression of \( A_{mn} \), which is useful for computational purposes, is, from [52],

\[
A_{mn} = \frac{e^{ikr_{mn}}}{r_{mn}} \left[ k^2 (\hat{r}_{mn} \hat{r}_{mn} - \mathbb{1}_3) + \frac{ikr_{mn}}{r_{mn}^3} (3\hat{r}_{mn} \hat{r}_{mn} - \mathbb{1}_3) \right]
\] (3.28)

where \( \mathbb{1}_3 \) is the identity \( 3 \times 3 \) matrix and \( \hat{r}_{mn} \hat{r}_{mn} \) is a dyadic tensor defined as \( \hat{r}_{\mu \nu} = \hat{r}_\mu \hat{r}_\nu \) (\( \mu \) and \( \nu \) are Cartesian components).

In matricial form, Eq. 3.27 becomes

\[
\vec{E}^{\text{inc}} = A' \vec{P}
\] (3.29)

where \( \vec{E}^{\text{inc}} \) and \( \vec{P} \) are \( 3N \) vectors and \( A' \) a \( 3N \times 3N \) symmetric matrix, constructed from the \( 3 \times 3 \) interparticle interaction matrices \( A_{mn} \) with additional terms \( \alpha^{-1} \) along the diagonal.

Solving this set of \( 3N \) complex linear equations allows the polarization vector \( \vec{P} \) to be obtained and, consequently, the optical properties, like the extinction cross section, to be calculated.

\[
\sigma_{\text{ext}} = \frac{4\pi k}{|\vec{E}_0|^2} \sum_{n=1}^{N} \text{Im} \left\{ (\vec{E}^{\text{inc}}_n)^* \cdot \vec{P}_n \right\}
\] (3.30)

It is remarkable that the dipole field calculated in Eq. 3.26 consists of two parts, the first of which varies as \( 1/r \) and the second of as \( 1/r^3 \) for small \( r \). The second part is from electrostatic contributions, and effectively depends on \( \frac{3 \cos^2 \theta - 1}{r^3} \), where \( \theta \) is the angle between the incident field \( \vec{E}^{\text{inc}} \) and the interparticle vector \( \vec{r}_{mn} \). On the other hand the first term represent the radiative dipole field that varies as \( \frac{\sin^2 \theta}{r^3} \). Both terms are modulated by the retardation factor \( e^{ikr} \), which implies that when \( r \) is comparable to or larger than \( \lambda/(2\pi) \) (about \( > 100 \text{ nm} \) for plasmon resonances in the visible), the field is strongly modulated and can even change sign.

### 3.3.2 1-dimensional arrays

Further simplifications can be introduced in the model in order to explain the optical response of a chain of metal nanospheres. All the particles in the system can be considered identical (i.e. \( \alpha_m = \alpha \ \forall \ m \)), and the 1-dimensional array can be considered a perfectly straight chain with regular spacing between dipoles. The interparticle distance vector then becomes

\[
\vec{r}_{mn} = (m-n)d \cdot \hat{l}
\] (3.31)

where \( \hat{l} \) is the unit vector in the direction of the chain. Incident light is considered normal to the chain \( (\vec{k} \perp \hat{l}) \), so the phase of the incident field can be assumed constant along the chain. In other words, this means that

\[
\vec{k} \cdot \vec{r}_m = 0 \quad \Rightarrow \quad \vec{E}^{\text{inc}}_m = \vec{E}_0 \ \forall m
\] (3.32)
To understand this result, the problem can be restricted to the two limit cases, i.e. incident light with polarization parallel or perpendicular to the chain. In the first case (longitudinal mode) the surface plasmon resonance redshifts, while in the second (transverse mode) it blueshifts.

**Longitudinal mode**

In the first case the incident field $\vec{E}^{inc}$ is parallel to the unit vector of the chain $\hat{l}$. Therefore the polarization vector $\vec{P}_n$ is parallel to $\vec{r}_{mn}$. This implies that

$$\hat{r}_{mn} \times \vec{P}_n = 0$$ (3.33)

$$\hat{r}_{mn} \cdot \vec{P}_n = P_n$$ (3.34)

For the straight chain of dipoles the $1/r$ dependant dipole radiation contribution becomes zero, and dipoles interactions are only electrostatic:

$$A_{mn}\vec{P}_n = \frac{e^{ikr_{mn}}}{r_{mn}^3}(ikr_{mn} - 1)(3\vec{P}_n - \vec{P}_n)$$ (3.35)

Eq. 3.27 then becomes a one dimensional equation, since only the vectors’ component (the one along the chain direction) is not null:

$$E^{inc}_m = E_0 = \alpha^{-1} P_m + \sum_{n \neq m} 2\frac{e^{ikr_{mn}}}{r_{mn}^3} (ikr_{mn} - 1) P_n$$ (3.36)

and the problem is reduced to a set of $N$ equations.

Under the assumption of identical particles and regular spacing stated above

$$E_0 = E^{loc}_{m} + \frac{2\alpha}{d^3} \sum_{n \neq m} e^{ikd|m-n|} |m-n|^3 (ikd|m-n| - 1)E^{loc}_{n}$$ (3.37)

Since the dipole interaction is short-ranged (in this case it depends only on $1/r^3$), only the neighbour particles up to a certain order will effectively contribute to the electric field in $\vec{r}_m$. If the chain is long enough most of the particles will behave as if the chain was infinitely long. The limit $N \to \infty$ can then be used to model long 1-D arrays, neglecting the extremities. In this approximation every particle ‘sees’ infinite particles ahead and behind itself, so the local field $E^{loc}_{m} = E^{loc} \forall m$. So, for normal incident light

$$E_0 = E^{loc} \cdot \left[ 1 + \frac{4\alpha}{d^3} \sum_{n=1}^{+\infty} \frac{e^{ikdn}}{n^3} (ikdn - 1) \right]$$ (3.38)

$$= E^{loc} \cdot \left[ 1 + \frac{4\alpha}{d^3} (ikd\text{Li}_2(e^{ikd}) - \text{Li}_3(e^{ikd})) \right]$$ (3.39)

$$= E^{loc} \cdot \left[ 1 + \frac{4\alpha}{d^3} L_n(kd) \right]$$ (3.40)

where $\text{Li}_n(t) = \sum_{n=1}^{+\infty} \frac{t^n}{n^2}$ is the polylogarithm function of order $n$. The series always converges since $kd$ is a positive real number. It is now possible to calculate the extinction.
cross section of a nanoparticle in the infinite chain (it is not possible for the whole chain since it would be, obviously, infinite). For Eq. 3.30

\[
\sigma_{ext} = \frac{4\pi k}{|E_0|^2} \text{Im} \left[ E_0 \cdot \alpha E^{loc} \right] \tag{3.41}
\]

\[
= \frac{4\pi k}{|E_0|^2} \text{Im} \left[ E_0 \cdot \alpha E_0 \frac{1}{1 + \frac{4\pi^2}{d^2} L_a(kd)} \right] \tag{3.42}
\]

\[
= 4\pi k \cdot \text{Im} \left[ \frac{\alpha}{1 + \frac{4\pi^2}{d^2} L_a(kd)} \right] \tag{3.43}
\]

**Transverse mode**

In the second case the polarization vector \( \vec{P}_m \) is normal to \( \vec{r}_{mn} \), so

\[
\hat{r}_{mn} \cdot \vec{P}_n = 0 \tag{3.44}
\]

Given the identity \( \vec{a} \times (\vec{b} \times \vec{c}) = \vec{b}(\vec{a} \cdot \vec{c}) - \vec{c}(\vec{a} \cdot \vec{b}) \)

\[
A_{mn}\vec{P}_n = \frac{e^{ikr_{mn}}}{r_{mn}} \left\{ k^2(\vec{r}_{mn} \cdot \vec{P}_n) - \vec{P}_n(r_{mn} \cdot \hat{r}_{mn}) + \frac{ikr_{mn} - 1}{r_{mn}^2}(-\vec{P}_n) \right\} \tag{3.45}
\]

\[
= -\frac{e^{ikr_{mn}}}{r_{mn}} \left( k^2 + \frac{ikr_{mn} - 1}{r_{mn}^2} \right) \vec{P}_n \tag{3.46}
\]

Again, Eq. 3.27 then becomes a one dimensional equation, and following the same procedure used above

\[
E_0 = E^{loc} \cdot \left[ 1 - \frac{2\alpha}{d} \sum_{n=1}^{+\infty} e^{ikn} (kd)^2 \frac{1}{n^2} - \frac{2\alpha}{d^3} \sum_{n=1}^{+\infty} e^{ikn} \frac{ikdn}{n^3} - 1 \right] \tag{3.47}
\]

\[
= E^{loc} \cdot \left[ 1 - \frac{2\alpha}{d} (kd)^2 L_1(e^{ikd}) - \frac{2\alpha}{d^3} \left( idkL_2(e^{ikd}) - L_3(e^{ikd}) \right) \right] \tag{3.48}
\]

\[
= E^{loc} \cdot \left[ 1 - 2\alpha \left( \frac{L_0(kd)}{d} + \frac{L_2(kd)}{d^3} \right) \right] \tag{3.49}
\]

The first series always converges since \( kd \) is a positive real number \( \neq 0 \) (\( d > 2R \), otherwise there would be contact between spheres). Again, the extinction cross section of a single particle is:

\[
\sigma_{ext} = 4\pi k \cdot \text{Im} \left[ \frac{\alpha}{1 - 2\alpha \left( \frac{L_0(kd)}{d} + \frac{L_2(kd)}{d^3} \right)} \right] \tag{3.50}
\]

In both cases the polarizability \( \alpha \) is 'corrected' into an effective polarizability, \( \alpha_{eff} \), that depends also from interparticle spacing and not only the radius of the particle.

The most general solution, developed in [53] and reported in [51], is

\[
\alpha_{eff} = \frac{\alpha}{1 - S\alpha} \tag{3.51}
\]
3.3 Plasmon coupling in 1-dimensional nanoparticle arrays

where \( S \) is the retarded dipole sum

\[
S = \sum_{n \neq m} \left[ \frac{(1 - ikr_{mn})(3\cos^2 \theta - 1)e^{ikr_{mn}}}{r_{mn}^2} + \frac{k^2 \sin^2 \theta e^{ikr_{mn}}}{r_{mn}^3} \right]
\] (3.52)

This model predicts a significant redshift of the surface plasmon resonance for longitudinal modes (parallel polarization), while a less intense blueshift is expected for transverse modes (perpendicular polarization). Experiments using far-field extinction spectroscopy have proven this prediction true [54]. Anyhow, due to the strong scaling of the interaction strength with \( d^{-3} \), particle separations in excess of \( \sim 6R \) are sufficient to recover the behaviour of essentially isolated particles.

At smaller distances, dipole-dipole interactions are not anymore the only interactions to consider, and higher modes (usually quadrupole and octupole) arise.

3.3.3 Finite chains

So far nanoparticle chains have been assumed infinite. For a small number of particles the optical response is greatly different, tending asymptotically to the infinite chain behaviour as the number increases. Starting from a dimer (a couple of identical particles at a fixed distance), and adding particles keeping the interparticle distance constant to form a linear chain results in an increasingly red-shifted (longitudinal) plasmon resonance of the chain. However, both experiments and calculations have demonstrated that the plasmon shift saturates. The infinite chain limit is typically reached for chains consisting of about 10 nanoparticles, since near-field interactions scale as \( d^3 \) and become negligible for larger separations [55].

Figure 3.8: SEM images of chains composed of one, two, three, and four gold nanoparticles, and scattering spectra of chains that contain an increasing number of nanoparticles. Particles have axes of 102 nm and 74 nm and chains were produced via electron-beam lithography. From [56]

The surface plasmon polariton propagates in the chain with finite group velocity, so retardation effects may occur in finite chains. This is not an issue when considering infinite chains, since every particle ‘sees’ the same number of neighbours ahead and behind itself. That is of course not true in a finite chain. It has been shown that phase retardation effects are indeed important if the nanoparticle size exceeds 70-100 nm: the plasmon
resonance peak wavelengths are always red-shifted, but do not follow a monotonic trend versus the particle number (see Fig. 3.8) [56]. In order to explain certain effect the quasi-static model must be extended including the retardation effects, for example retardation is responsible for the splitting of the dispersion relation for transverse modes into two anticrossing branches [57].

When interparticle distance is small enough to make the multipolar plasmon modes increasingly important, other effects can occur in finite chains. Far-field radiative coupling has to be considered, and nondegenerate coupled plasmon modes can become subradiant [58]. Subradiant modes suffer smaller radiative losses compared with the super-radiant dipole mode, therefore excitation at energies corresponding to these modes leads to longer propagation distances. Although several theoretical studies have investigated plasmon propagation in nanoparticle chain waveguides and propagation lengths of several µm have been calculated [59], [60], the effective construction is still problematic and only a few experimental realizations have been reported so far [61].
Chapter 4

Materials and methods

4.1 HEWL fibers preparation

Hen Egg White Lysozyme fibers have been prepared starting from the native protein. The procedure is well known in literature [14], but has been investigated considerably in this project in order to obtain fibers optimal for metallization.

4.1.1 Starting solution

The starting solution was prepared by dissolving lyophilized Sigma Aldrich Hen Egg White Lysozyme (HEWL)® (L6876) in DI water, at a concentration of 25 mg/mL. Despite the good purity of the product (99.1%) purification is recommended to remove small protein monomers/oligomers from the solution, to prevent any interference with the process’ kinetics.

The HEWL solution was therefore transferred in Spectra/Por 50 kDa Dialysis Membrane® [62]. A pH 4, 200 mg/L Sodium Azide (NaN₃) buffer solution was used as dialysate, in an approximate 100:1 buffer to sample volume ratio. To achieve the necessary purity the buffer is changed three times at two hours intervals, and then left overnight. After dialysis the HEWL solution was acidified down to pH 2 adding a small volume of 1M HCl.

The HEWL solution gains volume during dialysis due to osmotic pressure on the membrane. Light absorbance of a peptide is mostly caused by his aromatic amino acid content, which is defined and fixed for every protein. The native HEWL extinction coefficient at 281.5 nm is $\epsilon = 2.65 \, \text{L} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ ( [63]). The new concentration was then evaluated using a spectrophotometer, measuring the absorbance of the solution at 281.5 nm and using Lambert-Beer law:

$$A = l \cdot \epsilon \cdot C \quad \Rightarrow \quad C = \frac{A}{\epsilon \cdot l} \quad (4.1)$$

with $A$ the absorbance, $l$ the path length (the cuvette size) and $C$ the HEWL concentration. Measuring the absorbance of the solution at different dilution and running linear regression on the data provides a good estimation ($< 1 \text{mg/mL}$ uncertainty) of the concentration of the purified HEWL solution.
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4.1.2 Incubation and storage

Once purified HEWL solution was diluted down to the desired concentration (usually 5, 10 or 15 mg/mL) with pH 2 sodium Azide buffer and transferred to eppendorf tubes for incubation. Two types of containers have been used in this project: low-adhesion plastic tubes of 1.5 mL volume, and glass tubes of ~3 mL.

Tubes were then sealed and immersed in a water bath set at 60°C, for several days. The growth was monitored, and could be stopped letting the sample cool down.

Once fibers were fully grown the suspension was purified via dialysis in 50 kDa bags in order to remove any small aggregate left in the sample. The suspension was then stored in the fridge at 2°C after been diluted 4 times with DI water in order to reach optimal coating densities.

Amyloid fibers are remarkably stable, and can be preserved in this fashion for several months and possibly more than a year.

4.2 Gold nanoparticles synthesis

Citrate-capped gold nanoparticles with mean diameters of 15 nm were synthesized using variants of the Turkевич method. The precursor solution was prepared by diluting chloroauric acid (HauCl₄) at 0.01%. 100 ml of solution was heated to boiling point, then 5 ml of 1% sodium citrate (Na₃C₆H₅O₇) solution was quickly added. The solution changed colour from pale yellow to intense red within 5 minutes. The solution was kept boiling and vigorous stirring for 30 minutes before cooling down gradually to room temperature, in order to reduce all the gold left in solution.

Figure 4.1: Extinction spectra of 15 nm gold nanoparticles suspension.

The absorbance spectra of the particle suspension was then acquired, and fitted with Eq. 3.22 to verify that the particle size was as expected. Concentration can be calculated on the basis of size (since the amount of gold in the starting solution is known), assuming all the gold has been reduced. Refractive index can be roughly estimated but was used also as a parameter. The fitting approach is not completely reliable but is a good first control.
of the synthesis’ result. AFM characterization is a good complement to check particle sizes. Both optical and microscopic investigation confirmed that the synthesis worked as expected, producing nanoparticles with diameters slightly lower than 15 nm.

4.3 Fibers deposition and metallization

In order to study amyloid fibers with AFM, the structures have to be immobilized on a solid and flat substrate. Two kinds of substrates were used: silicon oxide and borosilicate glass. The latter also allowed UV/Visible spectroscopy investigation, which is essential to determine the optical properties of the gold nanoparticle arrays. Metallization was then achieved by attaching gold nanoparticles on the deposited fibers. Gold nanoparticles’ size could finally be augmented using a gold enhancement protocol.

4.3.1 Substrate activation

The 1 cm² silicon (100 crystal orientation with ∼ 10 Å native oxide layer) and 2.5 cm diameter, 0.13 mm thick borosilicate glass substrates used in this project were extensively cleaned in order to avoid any possible contamination. Silicon substrates were cleaned via piranha solution, consisting in sulfuric acid (H₂SO₄, 98%) and hydrogen peroxide (H₂O₂, 30%) at a 3:1 ratio. The reaction is a sulfuric-acid boosted conversion of hydrogen peroxide (a relatively mild oxidizing agent) into one sufficiently aggressive species to dissolve elemental carbon. This transformation can be viewed as the energetically favourable dehydration of hydrogen peroxide to form hydronium ions, bisulfate ions, and, transiently, atomic oxygen:

\[
\text{H}_2\text{SO}_4 + \text{H}_2\text{O}_2 \rightarrow \text{H}_3\text{O}^{+} + \text{H}_2\text{SO}_4^- + \text{O}
\]  

Piranha solution is a strong oxidizing agent that reacts quickly with organic matter, leaving the substrate clean. It also hydroxylates the substrates, making them hydrophilic and more importantly charging them negatively, adding hydroxyl (-OH) groups on the surface. Substrates were submerged in piranha solution for 20 minutes, before being extensively rinsed with DI water and blown-dried in a nitrogen flow.

Because of its reactivity and since its preparation is an exothermic reaction (the solution start to boil a few seconds after the reagents have been mixed), piranha solution is extremely dangerous. It has to be handled with proper protections and never close to organic reagents. Its disposal must be done properly, allowing the solution to cool down and degrade prior to storing it as hazardous waste.

Glass surfaces were cleaned with aqua regia, in order to remove metallic surface contaminants. Aqua regia was prepared mixing three parts of hydrochloric acid (HCl, 37%) and one part of nitric acid (HNO₃, 65%); aqua regia cleans the glassware leaving no chemical traces that could spoil surface modification. Aqua regia quickly loses its dissolving power but however remains a strong acid, and must be also used and disposed with care. The solution was prepared right before use to increase its effectiveness, and glass substrates were submerged for 20 minutes. After DI water rinsing the same piranha activation protocol used for silicon substrates was applied.
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4.3.2 Fibers deposition

Fibers are bound strongly to the substrate by electrostatic interactions (fibers are positively charged while the substrate is negatively charged), which prevents any displacement caused by interaction with the AFM tip. Binding can be influenced by parameters such as pH, temperature, and humidity, but it has proven strong enough in the majority of times.

5 µL of fiber solution was placed on silicon substrates (10 µL on glass), and incubated for 30’. In order to prevent drying during the incubation, the samples were placed in a Petri dish with a small amount of DI water. The samples were then blown from a side with nitrogen, rinsed with DI water, and then dried in a nitrogen flow, so as to avoid AFM tip contamination with loose protein monomers.

Substrates may be heated after deposition in order to improve the fibers attachment, slightly denaturing the protein.

4.3.3 Fibers metallization

Citrate capping provides gold nanoparticles with a negative surface charge. Parameters such as pH, ionic strength and citrate concentration have been proven to impact on the deposition of the particles on fibers. The protocol used in this project was optimized in previous studies [34].

The gold nanoparticle suspension was diluted with 1 mM trisodium citrate buffer with pH 5.0, in a 1:1 ratio. 5 µL (or 10 µL for glass surfaces) of the resulting nanoparticle suspension was deposited on lysozyme fiber-modified surfaces and incubated for 1 minute. The sample was then dried with DI water or citrate buffer and then rinsed with a low-pressure nitrogen flow.

Several nanoparticle batches were tested, both lab-made and commercial. In some cases purification was needed in order to achieve an acceptable deposition on fibers.

4.3.4 Gold enhancement

In order to increase the size of particles deposited on fibers a gold enhancement protocol was used on several samples [64]. This protocol allows for the reduction of gold dissolved in a solution only at the surface of existing gold seeds, allowing nanoparticles growth but avoiding the metallization of the bare substrate and fibers. This was achieved using a surfactant (CTAB) as capping agent. Cetyltrimethylammonium bromide (CTAB) interacts with the gold surfaces allowing the reducing agent (ascorbic acid) to precipitate gold on the particles.

1.6 mL CTAB 100 mM was added into 8 mL of DI water, followed by 48 µL chloroauric acid (HAuCl₄). The solution was stirred gently until its colour shifted (CTAB complex). 0.95 mL ascorbic acid 400 mM was then added. 200 µL of solution was dripped over the metallized fibers samples, and incubated for a variable time (5-120 minutes). Samples were finally rinsed with DI water and dried in a nitrogen flow.

4.4 Purification techniques

Dialysis and centrifugation techniques have been important in this project, both for fiber synthesis and metallization: lysozyme was purified before incubation, and later fibers had
4.4 Purification techniques

Commercial batches of gold nanoparticles needed purification as well, in order to eliminate big agglomerates.

4.4.1 Centrifugation

Centrifugation was both used for removing short fibers and monomers from fibers batches and to remove agglomerates from commercial gold nanoparticles samples. Fibers were centrifuged for 4 minutes at 10000 rpm, then the supernatant was removed and acidified (pH 2) DI water was added. Using a pipette the fiber deposit was dispersed back into water, and the procedure was repeated 3 times. This process was proved useful to improve the quality of the samples for metallization, leaving almost only fibers longer than 100 nm.

Centrifugation was also useful to purify commercial gold nanoparticles batches. These batches contain surfactants in order to reduce agglomeration. Centrifuging the suspension for 2 minutes at 2000 rpm was sufficient to remove the bigger aggregates and homogenize the suspension.

4.4.2 Dialysis

Dialysis devices from Spectrum Laboratories ® were used to remove small protein monomers /oligomers, and to purify gold nanoparticle batches. Cellulose ester 16 mm wide tubes with a molecular weight cut off of 50 kDa were used, and sealed with dialysis clips.

Dialysis purification uses the movement of molecules by selective and free diffusion through a porous, semi-permeable membrane against a concentration gradient. Particles diffuse from a high concentration volume to a lower one until equilibrium is reached. The membrane pores set a size limit on which particles can diffuse, allowing separation by weight. Several parameters affect (and therefore may be used for tuning) the dialysis rate, such as pH, temperature, viscosity, membrane surface area and thickness, electric charge, etc.

Diffusion is described by Fick’s first law:

\[ \vec{j} = -D \cdot \nabla c \]  

where \( \vec{j} \) is the particle flux, \( D \) its diffusion coefficient and \( c \) its concentration. If more than one specie is present in solution, the net flux will be the sum of all the species’ fluxes.

The solution than needs to be purified (known as the dialysate) and a buffer solution are divided by a porous membrane. Molecules that are small enough are drawn to the other side because of the concentration gap while big molecules are kept in the dialysate by the membrane. To facilitate (and quicken) the diffusion process it is better to use a large buffer/dialysate volume ratio (around 100:1 for the dialysis used in this project), and to replace the buffer solution several times.

Dialysis was optimal to purify lysozyme before incubation, but proved not completely effective in separating small fragments from long fibers. In that case centrifugation was much more effective and less time consuming. Centrifugation was also proved more effective to purify nanoparticles suspensions. However dialysis is an important tool to regulate ionic strength when needed.
4.5 UV/Visible Spectroscopy

UV/Vis absorbance spectroscopy is an optical spectroscopy technique that, as the name suggests, operates in the near ultraviolet-visible spectral region. Many molecules absorb light when the energy of the photon matches the energy difference between two electronic states, therefore showing a particular spectrum since only photons at particular wavelengths are absorbed. Absorbance spectroscopy is also a useful tool when investigating metallic nanostructures. Because of the interaction of the conduction band electrons with the electric field of the incident light, as shown in Cap. 3, metallic nanoparticles have particular extinction cross-sections that depends on their material, shape and size.

![Absorption Spectroscopy Schematic](image)

**Figure 4.2:** Absorption spectroscopy schematic. Light with different wavelengths is focused on a sample, upon striking photons that match the energy gap of the molecules present are absorbed. Other photons transmit unaffected and, if the radiation is in the visible region (400-700 nm), the sample color is the complementary color of the absorbed light.

In this project an Ultrospec 2100 spectrometer was used, to determine the different concentrations of the purified protein solutions, to verify the outcome of the Turkевич synthesis, and finally to study the optical properties of fibers in solution and metallized fibers on glass substrates. Samples were placed between a light source and a detector, the apparatus recording the degree of absorption of each sample as the ratio between the emitted and the detected light, as a function of wavelength.

1 mL quartz cuvettes were used as container for liquid samples in the spectrophotometer, due to the low absorbance of the material in the spectral range used. Special glass cuvettes, with hermetic caps, were used both to incubate lysozyme and to record its spectra at different incubation times. This allowed easier measurements and prevented perturbation of the sample due in and out pipetting.

An *ad hoc* support was designed for glass substrates, and a holed screen was positioned in front of the sample. The area covered by metallized fibers can vary from sample to sample, so limiting the light flow to a small, selected area is a good way to obtain a quantitative comparison. To obtain more statistic ans significant information, several spectra were recorded, varying the position of the screen, and then averaged.
4.6 Atomic Force Microscopy

Atomic Force Microscopy is a high-resolution type of scanning probe microscopy, with height resolution down to fractions of nanometers. It is the successor of the Scanning Tunnelling Microscope (STM), invented in the 1981 by Binning and Rohrer. STM measures the tunnelling current between a sharp tip and the sample, therefore restricting its usage only to conductive samples. AFM instead measures the forces developing between a tip and a sample, allowing scanning of every kind of solid material. Binning developed the idea, and the first implementation was competed in 1986 in collaboration with Quate and Gerber. Only three years later the first commercially available model was released.

AFM is used in almost every facility that research in the nanofield, because of its versatility and relatively low cost. Almost every sample which roughness does not exceed few µm can be characterized, no matter its hardness, stiffness or conductivity. While other scanning probe techniques such as STM needs high vacuum to work, AFM is able to work also in air and, in some cases, in liquid environments with good precision. Several parametrizations and different operating modes (contact, non-contact, tapping) can be applied to better suit the sample’s nature and the information needed. Variations can be used to also map the electric potential or the conductivity. While developed and used mostly for imaging, AFM can also be used to probe the mechanical properties of the sample or to cut, bend or detach soft materials. While created as a characterization tool mainly in the material science area, AFM has proven useful also in the biophysical and biomolecular research. DNA strands and several proteins have been imaged, and the technique has proven very useful also for studying hybrid bioinorganic materials.

4.6.1 Working principle

Atomic Force Microscopes use cantilevers with sharp tips at their ends that is used to scan the surface of samples. When the tip is close enough with the surface, interaction forces between the two lead to a deflection of the cantilever according to Hooke’s law. Therefore the distance between the tip and the sample can be quantified from the force exerted on the cantilever

\[ F(\Delta z) = -k \cdot \Delta z \]  

(4.4)

where \( \Delta z \) is the displacement in the vertical direction and \( k \) the Young modulus of the cantilever.

Forces that cause the deflection are usually Van der Waals forces, or, depending on the apparatus and the sample, the interaction can be caused by mechanical contact, capillarity, chemical bonding, electrostatic interactions, or, in specific setups, magnetic or Casimir forces. Dominant interatomic interactions when measuring in vacuum or air have a Lennard-Jones shaped potential:

\[ V_{LJ}(z) = 4\epsilon \left[ \left( \frac{\sigma}{z} \right)^{12} - \left( \frac{\sigma}{z} \right)^{6} \right] \]  

(4.5)

where \( \sigma \) is the finite distance at which the inter-particle potential is zero and \( \epsilon \) the depth of the potential well. At large distances the potential (and therefore the interaction force) is of course null. At distances in the scale of few Å the electronic orbitals of atoms on the tip and on the substrate would overlap, but that is impossible due to the Pauli Exclusion
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Principle and results into a strong repulsive force. At distances of a few nm, Van der Waals interactions dominate and the total force is attractive.

![Schematic representation of an Atomic Force Microscope.](image)

The silicon or silicon nitride (Si$_3$N$_4$) cantilevers are a few µm long and the sharp tip that protrudes at their ends have a radius of curvature of few nm. These probes are manufactured with high-precision microelectromechanical systems. Cantilevers are usually assembled into a holding plate of a few mm$^2$, allowing to handle them with tweezers and perform a rapid substitution of the probe in the (not infrequent) case the tip scratches on the surface and breaks. Cantilever deflection is measured with a laser beam, focused on the back of the cantilever and collected from a position-sensitive detector (PSD) consisting of two closely spaced photodiodes. Angular displacement of the cantilever results in one photodiode collecting more photons than the other, producing an output signal which is proportional to the deflection. Beam path is maximized in order to amplify the angle variations, thus increasing resolution.

The scanning process is performed by a ceramic piezoelectric scanner, which expands and contracts proportionally to an applied voltage. The scanner is actually composed of three independent piezo electrodes, in order to manipulate the position of the sample (or the probe) with high precision in all three spatial directions. A setpoint voltage value is selected at the start of a measurement accordingly to the sample’s nature. Scanning then is performed line by line, taking a certain number of steps in the $x$ direction before taking one in the $y$ direction. A feedback circuit keeps the tip-surface force (and therefore distance) constant, thus obtaining the topography of the sample by measuring the voltage needed by the $z$ piezo to keep the cantilever in place. Voltage is converted into height using a calibration done on a known surface, that has to be repeated from time to time since piezos lose sensitivity over time.
4.6.2 Imaging modes

Atomic force microscopes are often used in three different modes, according to the nature of the tip motion. In contact mode the tip, once contact with the sample is achieved, is dragged across the surface. Topography is obtained using the feedback signal required to keep the cantilever at a constant deflection. The $z$ piezo moves the cantilever up and down following the sample’s features. In order to minimize noise and drift (this technique measures a static signal), low stiffness cantilevers are used to boost the deflection. Contact mode is always used with the tip at a close distance from the surface, having an overall repulsive force in order to avoid the tip to spring harshly towards the sample in case of a sharp valley. Contact mode is the most suitable to characterize sharp surfaces with large and sudden changes in the vertical direction. This mode can be optimized to achieve atomic resolution, and allows high scan speed. The drawback is that in this mode the tip is most prone to get damaged, and images suffer from a poor lateral resolution due the existence of of lateral forces between tip and sample. Soft samples may also get damaged by the force exerted by the tip. A variation of this mode, called Force Modulation Microscopy, allows to measure the hardness of the surface and characterize it mechanically. This is achieved by rapidly moving the tip up and down while pressing it into the sample.

Under ambient conditions most surfaces are covered with a thin water layer, and this meniscus can cause the tip to stick to the surface. Dynamic contact (or tapping) mode allows to avoid this problem, driving the cantilever to oscillate near its resonance frequency, with an amplitude $> 100$ nm. When the tip comes close to the surface, interaction forces perturb the oscillation and decrease the amplitude. A feedback circuit adjusts the height to maintain the amplitude set as the cantilever is scanned over the sample. Imaging is done by mapping the voltage used to drive the vertical movements of the piezoelectric scanner. This mode is considerably slower than contact mode but offers higher lateral resolution and minimizes tip scratching risk (and therefore damaging of both probe and sample). This mode is gentle enough to map fragile biological structures (like lipid bilayers) and to leave unchanged the conformation of single molecules.

![Image](image.png)

Figure 4.4: Plot of tip-sample interaction against distance and measurement modes.

Non-contact mode imposes no contact at all between the tip and the surface. The cantilever oscillates at its resonance frequency as in the tapping mode, but with an amplitude $< 10$ nm. Van der Waals forces, (which are strongest from 1 nm to 10 nm above the surface) or any other long-range forces reduce the resonance frequency of the cantilever. A constant oscillation amplitude is maintained by a loop system, and the resonance frequency
decrease is measured to map the surface. This mode is optimal for scanning soft matter due the very low interaction between tip and sample.

4.6.3 Experimental setup

Two microscopes have been employed to scan the samples in this project, a JPK\textsuperscript{®} NanoWizard\textsuperscript{®} 3 Bioscience AFM and an Agilent Technologies\textsuperscript{©} 5500 AFM/SPM microscope, exposed to ambient air. Bruker’s Sharp Microlever, MSNL-F model triangular silicon nitride tips were used for characterization. Tips have an average nominal radius of 2 nm, with resonance frequency in the range of 110-120 kHz and spring constant of 0.6 N/m for characterization in tapping mode. Samples were electrically grounded before characterization in order to avoid any electrostatic charge stacking that could bias the measurement. No accurate temperature control was needed because the samples have proven quite stable and no significant drifting effects were observed.

4.6.4 Artifact treatment

Despite the ability to reach high spatial resolution, the surface topography acquired with an AFM may sometimes not correspond to the real surface features, due to the existence of typical artifacts. Vibrations from the floor or acoustic sources can create periodic structures in an image, up to few $\mu$m high. This can be easily prevented by placing the microscope in a quiet room on a dampening support. Piezo actuators must of course be calibrated correctly in order to avoid inaccuracies, and the probe must be placed at the correct angle. Moreover the tip must be sharp enough to resolve the sample’s features, so broken or contaminated tips must be changed to avoid errors. Thermal drift in the piezoelectric scanner can cause drift in the image, especially if at high magnification. This artifact usually occurs only at the beginning of a scan, and can therefore be easily identified.

Several artifacts can be corrected via image processing, and the software Gwyddion was used for this purpose during the project. Tilt and bow background effects are caused by the non linearity of the piezo response, that creates a huge planar background. A line by line leveling easily corrects this artifact, creating dark bands in proximity of elevated points in the topography that can be subsequently excluded.

Only a few major AFM artifacts require quantitative estimation and reconstruction to be solved. The height lowering effect is due to the elastic deformation of studied objects. Specific knowledge of the sample stiffness is required to estimate it, but it is not an issue with most materials, including metal and crystals. Moreover special imaging methods can help reducing or eliminating it, for example using tapping mode to reduce the force exerted by the tip on the sample.

The profile broadening effect is due to the tip-sample convolution. Two main cases can be considered: when the tip radius of curvature $R$ is much less than the feature radius of curvature $r$ ($R \ll r$) and when the tip radius is approximately equal to the feature radius ($R \approx r$).

When $R \ll r$ the effect is due to the non null cone angle of the scanning tip, an example shown in Fig. 4.5 demonstrate the artifact with a series of spheres as features on a surface. Approximating the tip as a cone, from geometric considerations it easily follows that the
4.6 Atomic Force Microscopy

Figure 4.5: Schematics of the studied object and conical tip in case \( R \ll r \).

Object lateral width is:

\[
r_c = r \left( \cos \theta + \sqrt{\cos^2 \theta + \left( 1 + \sin \theta \cdot \frac{\sin \theta}{\cos^2 \theta} - 1 \right) + \tan^2 \theta} \right) \quad (4.6)
\]

where \( \theta \) is the cone half-angle. In this case, the object is broadened by \( 2(r_c - r) \) while its height remains the same \( (2r) \).

In the case \( R \approx r \) the finite radius of curvature of the tip must be known with precision to correct the artifact. Using the same example from above in this case the tip moving across the surface can be approximated by a sphere of radius \( R \) moving along spheres of radius \( r \). In other words the tip describes arcs of radius \( R + r \). Elementary calculations show that the lateral dimension is:

\[
r_c = 2\sqrt{Rr} \quad (4.7)
\]

and the relative height of the object is:

\[
H = r \left[ 1 - \sqrt{1 - \frac{r_c^2}{(R + r)^2}} \right] \quad (4.8)
\]

If the minimum distance between features is \( d < 2(R + r) \) then the tip passing between them will penetrate as deep as

\[
\Delta H = r \left[ 1 - \sqrt{1 - \frac{(d/2)^2}{(R + r)^2}} \right] \quad (4.9)
\]
In this case the object broadening is \( r_c - d/2 \); moreover, the tip finite size does not allow it to penetrate into narrow cavities on the sample surface resulting in their depth and width decrease.

In this project, image treatment has been performed with softwares Gwyddion and ImageJ. Profile broadening effect has been evaluated considering the tips' specifics and the typical size of the analysed features. Since the nominal radius of curvature of the tip (2 nm) is one order of magnitude smaller than the size of gold nanoparticles (15 nm) the error that it induces can be neglected. On the other hand the nominal aperture angle (25°) of the tips has been considered in order to achieve a better estimation of particles sizes.

### 4.7 Dynamic Light Scattering

Dynamic Light Scattering (DLS) is a technique that can be used to determine the size distribution profile of small particles in suspension. DLS measures Brownian motion and relates it to the size of the particles. Brownian motion of a particle suspended in a liquid is due to collisions with the solvent molecules that surround them; due to inertia, the larger the particle the slower the Brownian motion will be. The Stokes-Einstein equation for the hydrodynamic diameter is:

\[
 d(H) = \frac{k_B T}{3\pi \eta D}
\]  

(4.10)

where \( d(H) \) is the hydrodynamic diameter, \( k_B \) the Boltzmann constant, \( T \) the absolute temperature of the fluid, \( \eta \) the viscosity and \( D \) the translational diffusion coefficient.

It is evident that DLS requires an accurate measurement of temperature, also because the viscosity of a liquid is related to it. Temperature must also be constant during the measurement, in order to avoid convection currents that would cause non-random movements and bias the measurement. By measuring the translational diffusion coefficient \( D \) this technique estimates the diameter of a sphere that has the same \( D \) as the particle. This parameter is affected not only by the particle size but also its shape, any surface structure on it and also the ionic strength of the medium. In this project DLS has been used to determine the size of fibers fragments, which are not spherical but rod-like. Small changes in the length of a rod will directly affect its hydrodynamic diameter, whereas changes in the rod's diameter will hardly affect the diffusion speed. All fibers have roughly the same diameter, meaning the measure will have a good sensitivity.

DLS estimates the speed at which particles are diffusing by measuring the rate at which the intensity of the scattered light fluctuates when illuminated by a stable source, like a laser. Light scattered by the sample at a certain angle will form a speckle pattern, with dark spaces where the phase additions of the scattered light are mutually destructive and cancel bright blobs where the light scattered from the particles arrives with the same phase. Since the system is undergoing Brownian motion the pattern will change at every instant, and the rate at which these intensity fluctuations occur will depend on the diffusion coefficient and therefore on particles size.

Speckle patterns can be analysed via correlation, comparing signals acquired at different times. Since Brownian motion is a random process correlation will decrease over time, and will be null after a certain amount of time. Therefore, the time at which the correlation starts to significantly decay is an indication of the mean size of the sample. The steeper the line, the more monodisperse the sample is.
4.7 Dynamic Light Scattering

The correlation function at a particular wavevector \( q \)

\[
G^2(q; \tau) = \frac{< I(t) \cdot I(t + \tau) >}{< I(t) >^2}
\]  

(4.11)

where \( \tau \) is the correlator time delay, in steps of the sampling time of the apparatus. For a large number of monodisperse particles in Brownian motion, the correlation function is an exponential decaying function of \( \tau \)

\[
G^2(q; \tau) = 1 + B \cdot \exp \left( -2D \left( \frac{4\pi n}{\lambda_0 \sin \theta} \right)^2 \tau \right)
\]  

(4.12)

with \( n \) the refractive index of the medium, \( \lambda_0 \) the laser wavelength and \( \theta \) the scattering angle, the parameter \( B \) a correction factor that depends on the geometry and alignment of the laser beam in the light scattering setup. For polydisperse samples, the equation can be generalized as:

\[
G^2(q; \tau) = 1 + B \cdot g_1(\tau)^2
\]  

(4.13)

where \( g_1(\tau) \) is the sum of all the exponential decays contained in the correlation function.

Size can be inferred from the correlation function fitting it with a multiple exponential. The size distribution obtained is a plot of the relative intensity of light scattered by particles of various sizes, and is therefore known as an intensity size distribution. From this distribution one can infer information on the fraction of particles in a certain size range, remembering that the scattering intensity is proportional to \( d^6 \) in Rayleigh approximation.

A Malvern Instruments® Zetasizer Nano S has been used in this project, with the detector at 173° from the laser source. Data have been analysed with the instrument software. Since fiber fragments are quite small (10-100 nm) in order to maximise the amount of scattering from the sample the measurement position has been set in the center of the cuvette. This also helps reducing the laser flare effect on the cuvette’s walls.
4.8 FDTD simulations

Finite Difference Time Domain (FDTD) is a numerical analysis technique used for modelling electrodynamics. It allows to find approximate solutions to the system defined by Maxwell’s electrodynamic equations. Being a finite difference method it is grid based and discretizes the equation using central-difference approximations to space and time partial derivatives. Since it uses a time difference approach, nonlinear material properties can be simulated with ease, and a wide range of wavelengths (i.e. frequencies) can be covered in one simulation.

At any point in space, the updated value of the electric field in time depends on the stored value (the value at the previous instant) of the field and the numerical curl of the local distribution of the magnetic field in space. Magnetic field is computed in the same way. Iteration of these steps on both fields results in a marching-in-time process that can be repeated until a steady state (within a desired approximation) electromagnetic field behaviour is reached. The best scheme to describe the problem is the Yee lattice (proposed by K. Yee in 1966): vector components of the electrical and magnetic field are disposed on rectangular unit cells of a Cartesian computational grid so that each electrical component is located midway between a pair of magnetic components, and vice versa (see Fig. 4.10). A leapfrog scheme can be used to march in time, so that the electric field is updated midway during each time-step between successive magnetic field updates, and vice versa. This approach avoids to solve simultaneous equations, but requires an upper bound on the time-step to ensure numerical stability. The need for thousands of time steps to reach completion was a serious issue in the '60s, but thanks to the exponential development in processors capacity nowadays this method is the backbone of many current FDTD softwares.

FDTD computations have been widely used in the last couple of decades, going from ~10 papers per year with the descriptor 'finite difference time domain' in 1980 to over 2500 in 2014. That is no wonder, since FDTD is fully explicit and accurate, and since its errors are well known and can therefore be minimized. It is a systematic approach that
4.8 FDTD simulations

Figure 4.10: Illustration of a standard Cartesian Yee cell used for FDTD. Electric field components form the edges of the cube, while magnetic field components form the normals to the faces of the cube. A three-dimensional space lattice consists of a multiplicity of such Yee cells.

allows to treat nonlinear and impulsive behaviours naturally, reducing complex structures to a problem of mesh generation. Moreover it is a numerical method that works really well with parallel-processing computer architectures.

In this project the software FDTD Solutions from Lumerical Computational Solutions Inc. has been used. The software offers a large number of options and plugins, allowing the creation of targets of virtually any shape, size and material. The supported boundary conditions and the possible sources allow the simulations of periodic, time evolving or multi-layered systems.

In order to simulate infinite chains of gold nanoparticles, a finite computational volume (or box) was created. One gold particle was placed at the center of such volume, and periodic boundary conditions were set on two opposite box sides in order to simulate the infinite chain. On the other sides PML (Perfectly Matched Layer, an ideal, perfectly absorbant material) boundary conditions were set, in order to avoid any reflections of the incident electromagnetic wave. The distance between periodic bounded sides was equal to the desired interparticle spacing along the chain (~ 40 nm), while the other two dimensions were set to be larger than half a wavelength of the incident field (> 500 nm) in order to avoid any interference. The final simulation volume resembled a squared plank, with two dimensions equal and one much smaller (see Fig. 4.11). The source was created as an unpolarized electromagnetic wave normally incident to the chain, in order to simulate the conditions in the spectrophotometer. Finally, the mesh grid was set with a 0.5 nm step. This value was chosen after several trials: larger steps introduced errors in the simulation due the small distance between particles, and smaller steps gave no better results while increasing considerably the computation time. Since it was impossible to simulate the air/glass/air system created by the glass substrate in the spectrophotometer due to the thickness of the substrate (10^5 nm), an effective refractive index of 1.265 was set in the simulation volume. This value is the average between the refractive index of air and glass (1.53), since the fibers are at the interface between two media. This simplifying approximation works well, and the simulated spectra for non interacting gold nanoparticles did show no disagreement with the experimental ones.

Extinction cross section for a single particle in an infinite chain was calculated using
the special far-field estimator. Scattering cross section were estimated with a total field-scattered field approach, where the incoming electromagnetic wave is subtracted outside an inner box domain in order to calculate the scattered field.

Figure 4.11: Chain simulation from different angles, using the software FDTD solutions.
Chapter 5

Results and discussion

During this project, biotemplated gold nanochains self-assembly has been studied at multiple preparation steps. Amyloid fibers growth has been monitored, trying different protocols in order to optimize the process and obtain fibers most suitable for metallization. Fibers have then been deposited on silicon and glass substrates suitable for AFM characterization, previously treated with piranha solution.

After deposition, fibers have been metallized with citrate-capped gold nanoparticles. The metallization process has been investigated, in order to achieve a uniform and reproducible attachment on the fibers. Different batches of gold nanoparticles synthesized with the Turkевич method have been tried, and the trial has been extended to commercial batches. Deposited gold nanoparticles have subsequently been enhanced in order to increase their size and reduce interparticle spacing. The structure and optical response of these structures have been studied, focusing on the dipole coupling interaction between particles.

The coupling interaction in 1 dimensional nanoparticle array has been modelled using the basic dipole-dipole approach and a more advanced FDTD method, and the results have been compared to the experimental data.

5.1 Fibers formation

Monomeric HEWL forms amyloid fibers via thermal denaturation if incubated at high (>37°C) temperature in acid environment for extended periods of time. Previous studies [15] [14] have shown that the best protocol is to incubate a \( \approx 1 \mu M \) HEWL solution at pH 2.0 and 60° for several days. Higher pH slows down the fibrilization rate while higher temperatures favour the formation of spherical aggregates instead of amyloid fibrils.

The process also depends on HEWL starting concentration, both fiber nucleation and growth being considerably slowed down if the concentration is too low. If it is too high the protein begins to nucleate too easily and the quantity of non-fibrilized HEWL left in solution is not high enough to let the fibers grow, resulting in fast fibrilization but a considerably shorter average fibers length. Several other factors can be important in the fibrilization, starting with the purity of the peptide.

The process is in the end quite sensitive, there have been numerous report of batch to batch variability both within and between laboratories [65]. This is compensated by the low cost and ease of use of the setup, that allows also the production of several batches at the
same time. Since the focus of this thesis is more about fibers metallization instead of the precise kinetic of the fibrilization, no problem occurred in producing fibers for metallization.

HEWL amyloid samples have been analysed via Congo Red dye, UV-Vis spectroscopy, atomic force microscopy and dynamic light scattering in order to investigate the best way to study samples’ properties, first of all fiber density and length distribution. Direct measurements via AFM have proven the most immediate and reliable way to investigate a batch of fibers, but the practicality of UV-Vis spectroscopy has been proven very useful.

5.1.1 Congo Red evaluation

The use of Congo Red dye is a very common method to detect amyloid in biological samples. In this project it was used as a first test to verify the presence of amyloid structures in solution.

Different concentrations of Congo Red have been tested, and the optimal result is accordingly to the spectroscopic assay protocol described in [66]. The ratio 1:1 between 7 mg/mL CR solution and HEWL solution was proven optimal to visualize the peak that shows presence of \( \beta \)-sheet rich structures. The dyed sample was diluted 200 times, and its absorption spectrum was acquired. Congo Red solution spectrum was acquired as well, as a reference.

Dyed sample and CR solution spectra were normalized on the Congo Red absorption peak at 500 nm, then a mathematical subtraction was performed between the two spectra. As expected a maximal spectral difference at 540 nm was observed, which indicates presence of amyloid fibers (see Fig. 5.2).

![Congo Red solution and dyed samples absorption spectra. Samples with different HEWL concentration were measured after 3 days of incubation.](image)

This assay is widely used in medicine to determine the presence of fibers in biological samples \textit{in vitro}. These measurements confirm the formation of amyloid structures in HEWL solution within the conditions described above, in perfect accordance with the
5.1 Fibers formation

Figure 5.2: Subtracted sample spectra. A maximal spectral difference between dyed fibers and CR spectrum can be seen at 540 nm, demonstrating the formation of amyloid fibers. The sharp peak around 290 nm is typical of the lysozyme spectrum.

literature. This protocol is not best suited to monitor the kinetic of the fiber growth, because it was not possible to find a correlation between the 540 nm peak height and the fibrillization of the protein. That is mostly because the CR concentration is critical and even little differences can vary the peak's height. Moreover, the CR solution cannot be stored for long and has to be prepared every time. Congo Red dyed fibers precipitate in the liquid, requiring mixing before the acquisition of spectra. In the end, Congo Red dye is in the end an excellent way to determine the presence of amyloid in samples, but not to quantify the fibrillization itself. For this reason the turbidity measurements proved to be a more efficient (and non-invasive) method to monitor fiber growth.

5.1.2 Fiber growth

In literature HEWL concentrations between 5 and 20 mg/mL are suggested for the preparation amyloid fibers. In this project, concentrations of 5, 10 and 15 mg/mL were used, and 10 or 15 mg/mL were shown to be the optimal concentrations, since 5 mg/mL samples show less fibrilization after equal incubation time (see Fig. 5.3, 5.4).

The observed fibrillization concur with literature. The process' kinetics appeared to start with a lag phase during which no structures are seen, followed by a rapid growth right after the appearance of the first fibers. During the lag phase, which lasted usually around 48 hours, the entropically unfavourable process of initial association occurred. In this phase nothing appeared to change in the incubated sample, which appeared to be clear to the naked eye and showed no differences in the absorbance spectrum, while AFM measurements showed occasionally short, monodispersed fibers.

Once the aggregation process begins and a critical nucleus is formed, the aggregation proceeds rapidly into amyloid fibrils as long as native lysozyme is present in the solution,
then stops as soon as there is no more native HEWL left. Insoluble amyloid fibers tended
to aggregate in plaques visible also to the naked eye, the size of which grows with time. The length of the fibers increased with the duration of heat treatment, and AFM characterizations showed that the surface coverage increased as well. 192 hours appeared to be optimal to achieve a high density of fibers. Fiber growth became much slower for longer times, and is replaced by a phase of fiber breakdown, rearrangement and aggregation that leaded to the formation of complex scaffolds. The study of these objects, even if interesting, is beyond the aim of this thesis.

Fully grown fibers were 3-6 nanometers wide, and several µm long (see Fig. 5.5). Bigger fibers could appear, probably as a result of coiling of two or more fibers into one structure. Despite the relative homogeneity in fibers width, fibers length can vary widely in a sample. As seen in Fig. 5.3 and 5.4 even a long-incubated sample contains a broadened length distribution of fibers, spanning from several µm down to ∼ 50 nm.

5.1.3 Material dependence

Fibrilization appears to be dependant also on the adhesion of the material in which the HEWL solution is stored during incubation. Glass and plastic tubes have been both tested as containers for incubation, giving slightly different results. While in plastic fibrilization was visible around 96 hours, in glass the process was slightly faster and becomes usually noticeable within 48-72 hours. Lysozyme aggregation appeared to start more easily in vicinity of the surface of the tube in which the solution is stored. Growth in glass tubes vouches for this hypothesis: fibrilization was faster and big structures (naked eye visible) appears within 24 hours, apparently growing and then detaching from the glass surface. In plastic the lag time was longer and the fibers growth slower, while structures formed as well in the solution they seem to be smaller and less concentrated on the tube’s walls.

Absorbance of incubated samples was measured every 24 h for several days. Apart from the typical lysozyme absorbance peak around 290 nm, a secondary feature appeared in the 310-360 nm range (Fig. 5.8). This feature was not expected, and there is no evidence in prior literature. The intensity of this feature arose with incubation time and it is similar using both glass and plastic tubes. The height of the lysozyme peak, which is proportional only to protein concentration, increased as well. In a simple approximation the protein
Figure 5.6: AFM characterization of 10 mg/mL HEWL solution at different incubation times.
5.1 Fibers formation

Figure 5.7: AFM characterization of 10 mg/mL HEWL solution at different incubation times.

peak can be modelled as a superposition of two Gaussian functions, and the unexpected feature as a third, broadened, Gaussian function. Fitting the absorbance spectra in such fashion models the feature as a Gaussian of centroid in the 325-330 nm range and $\sigma \sim 70$ nm, with height that increases with incubation time.

The best option to evaluate the 330 nm peak would be to first fit the 290 nm protein peak with a double Gaussian before starting the incubation and then use the fit results as fixed parameters to fit the third peak at different incubation times (see Fig. 5.9). However, given the variability of the data and the presence of fluctuations among different measurements (probably due to temperature differences in the glass and quartz cuvettes, since the samples needed to be extracted from the thermal bath to be measured), this approach introduces significant errors in the final results. This could be avoided simply by increasing the data significance by repeating the experiment several times.

A simpler way to interpret this preliminary data is to plot the absorbance of the spectra at 330 nm (see Fig. 5.10), that is close to the centroid and in a first approximation not affected by the tail of the protein peak. The trend appears to be regular, especially in the
Chapter 5: Results and discussion

Figure 5.8: Lysozyme absorbance spectra at different incubation times in glass tubes.

Figure 5.9: Gaussian fit interpretation of the absorbance spectra of lysozyme. Peaks 1 and 2 form the lysozyme protein peak, while peak 3 is the unexpected feature.

glass incubated samples. Samples incubated in plastic tubes show more fluctuations and an overall higher absorbance than when incubated in glass vials at the same concentration. In order to measure the absorbance of plastic incubated samples the solution needed to be removed from the tube and placed in a quartz cuvette, thus disturbing considerably the system (and possibly breaking fibers). On the other hand glass tubes could be easily used in the spectrophotometer, avoiding any contamination of the samples. For this reason the measurements of glass incubated samples are more reliable, but the trend appears anyway
5.1 Fibers formation

Figure 5.10: Lysozyme absorbance at 330 nm at different incubation time and different incubation conditions.

similar in both cases.

In a first approximation the trend can be considered linear, and one can consider the dependence of the angular coefficient $m$ with material and HEWL concentration. The approximation works well for glass incubated samples, while for plastic incubated ones errors are higher and correlation worse (see Tab. 5.1). As mentioned before plastic incubated samples have a strong bias, that has most probably affected the linearity of the trend. For glass incubated samples the height of the 330 nm peak appears then to be directly proportional to incubation time and, from a first inspection also to HEWL concentration. Further measurements are needed in order to investigate better this phenomenon, increasing the significance of the data and estimating closely the dependence upon the system parameters. Surprisingly there is no direct evidence of the lag time and the fast nucleation phase that are well-known in literature.

Practically speaking this phenomenon has been proven useful to monitor the growth of HEWL amyloid fibers, also with seeded growth, as explained below.

<table>
<thead>
<tr>
<th>Material/Concentration</th>
<th>$m$ [h$^{-1}$]</th>
<th>$\sigma_{%}$</th>
<th>$\rho$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass 5 mg/mL</td>
<td>$(6.5 \pm 0.2) \cdot 10^{-4}$</td>
<td>3.4 %</td>
<td>0.992</td>
</tr>
<tr>
<td>Glass 10 mg/mL</td>
<td>$(10.2 \pm 0.5) \cdot 10^{-4}$</td>
<td>4.5 %</td>
<td>0.986</td>
</tr>
<tr>
<td>Glass 15 mg/mL</td>
<td>$(13.7 \pm 0.7) \cdot 10^{-4}$</td>
<td>4.8 %</td>
<td>0.984</td>
</tr>
<tr>
<td>Plastic 5 mg/mL</td>
<td>$(7.3 \pm 0.7) \cdot 10^{-4}$</td>
<td>9.9 %</td>
<td>0.935</td>
</tr>
<tr>
<td>Plastic 10 mg/mL</td>
<td>$(17.6 \pm 0.7) \cdot 10^{-4}$</td>
<td>4.2 %</td>
<td>0.988</td>
</tr>
<tr>
<td>Plastic 15 mg/mL</td>
<td>$(19.0 \pm 2.0) \cdot 10^{-4}$</td>
<td>10.4 %</td>
<td>0.929</td>
</tr>
</tbody>
</table>

Table 5.1: Angular coefficients and correlations for linear fit in Fig. 5.10.
5.1.4 Seeded growth

Thermal denaturation provides a broad distribution of fiber lengths, going from few nm up to several µm. We want to obtain long, metallized nanowires, but length based separation is hard to achieve and there is no way to change the starting parameters to narrow down the length distribution. The problem was bypassed by seeding the solution with a a little amount of amyloid fragments (see 5.1.5) to start immediately the growth and skip the lag phase (see Fig. 5.11). The seeds grown before native HEWL started to aggregate, (partially) preventing the formation of short fibers. The resulting length distribution was narrower and the average length was considerably higher.

![Nucleation Polymerization Model of Aggregation](image)

Figure 5.11: Schematic for the nucleation-polymerization process for amyloid fibers formation. The regular process is represented by the solid line, while seeded growth is described by the dotted one. From [66].

1, 10 and 100 µL of fragments suspension (obtained by sonicating a 10 mg/mL sample incubated for 168 h) were tested as seeds in a 10 mg/mL HEWL solution. In a parallel experiment, 10 µL were tested also in 5 and 15 mg/mL HEWL solutions.

Analysing the absorption spectra of the samples as in 5.1.3 shows that the growth with seeds is faster, and slows down after few hours. Absorbance at 330 nm increases rapidly in the first hours and keeps increasing thereafter, but at a slower pace. The trend is no longer linear, proving that the process is indeed accelerated by the presence of fibers fragments. After 4 h of incubation with 100 µL of seeds, the absorbance at 330 nm is about the same as after 24 h in the same conditions and concentration without fragments, and after 24 h is comparable with 120 h. The absorbance increase slows down after some time probably because after the rapid starting phase the fibers behave as for the normal growth. The rapidity of the initial phase appears to be dependant on the concentration of seeds, as with 100 µL is significantly higher, while the trend is quite similar for 10 and 1 µL.

In the end the seeded growth allowed a way faster formation of long fibers while corroborating the hypothesis that the 330 nm broadened absorption peak is strictly related to the stage of HEWL amyloidization.
5.1 Fibers formation

Figure 5.12: Seeded growth characterized with AFM. 15 mg/mL HEWL with 10 µL seeds, in plastic tube, after 6 h and 96 h.

Figure 5.13: Seeded growth characterized with AFM. 15 mg/mL HEWL with 10 µL seeds, in plastic tube, after 96 h.
5.1.5 Fiber sonication

The best way to create the short fibers needed for the seeded growth was to break the long ones in fragments via sonication. Fibers longer than few µm are more fragile and break into long fragments even if the suspension is just stirred. Shorter fibers (<1 µm) are more rigid and harder to break.

Amyloid suspension treated with an ultrasonic bath showed no fiber breakdown at any frequency, even at the maximum power. For this reason an ultrasonic horn directly immersed in the suspension was used, and it was proven effective, breaking fibers with a power output of ~ 5 W at 15 kHz frequency. Fibers broke immediately after the beginning of the process, as shown in Fig. 5.15, eventually fragmenting down into 10-100 nm long fragments (Fig. 5.16).

AFM characterizations suggested that most fibers completely break down after 30 s of sonication, then further application induced no major effect on the amyloids suspension. Fragments were short enough to make possible to run a particle analysis on the AFM images in order to evaluate the maximum Feret diameter. Length distribution obtained in such fashion is shown in Fig. 5.17. Most of the fragments were 20-40 nm long after 30 s of sonication, and the fraction sharply increases in that range after 60 s.

Spectroscopy showed an overall decrease of absorbance in the visible range (turbidity decrease) with sonication. Measures at 330 nm show a negative trend with sonication until 25-30 s, then absorbance staid almost constant. This observation concurs with the considerations from AFM characterizations. Assuming that the absorbance at 330 nm is related to the fibrilization stage this evidence may suggest that the sonication process partially 'reverses' the fibrilization by breaking the structures. In other words, the organization of the protein in fibers could be (one of) the reason of the unexpected absorption peak.

DLS measurements confirmed that the disappearance of long (> 1 µm) fibers in the
5.1 Fibers formation

Suspension increases with sonication, and that the fragment average length was between 10-100 nm (see Fig. 5.19). A shift of the intensity peak from 80 nm down to 40 nm that goes with sonication time confirmed that bigger fragments gradually break into smaller...
ones. Estimating the length distribution from the DLS measurements (see Fig. 5.20) gave results compatible with the one from AFM analysis, confirming the relatively narrow length distribution of the fragments. Since DLS analysis is highly sensitive to the particle size distribution’s polydispersity (and fibers and fragments are all but equal in length) the use of a less distribution dependent technique (as AFM) is preferable.

![Figure 5.17: Fiber length distributions from AFM image analysis.](image)

In order to obtain 'seeds' for the seeded growth sonication was better combined with centrifugation: keeping only the supernatant after every sonication step removed almost every large object left from the suspension.

Due the small volume of the sample, the temperature of the liquid rise quite fast because of the heat generated by acoustic cavitation. In order to avoid peptide denaturation it is
5.2 Fiber metallization

Figure 5.19: DLS measurement at different sonication time (10 mg/mL HEWL sample for 96 h incubation).

Figure 5.20: Fibers length distribution estimated via DLS at different sonication time (10 mg/mL HEWL sample for 96 h incubation).

better to sonicate the sample for 5-10 s and then wait until the suspension cools down before using the horn again.

5.2 Fiber metallization

Fibers obtained with incubation times from 144 to 192 h are the most suitable for metallization, because they are fully grown and do not start yet to break nor aggregate. Nanoparticles obtained with the Turkevich method were deposited on the fibers, and an commercial particle suspension from Sigma Aldrich was tested as well. Particles arranged on fibers with an average regular spacing, caused by the electrostatic repulsion between the
negatively charged NPs rather than a periodic distribution of positively charged residues on the fiber. The spacing appeared to be more regular on long fibers, but there might be small fluctuations between samples.

Figure 5.21: Fibers from 10 mg/mL HEWL solution incubated for 168 h and metallized with 15 nm diameter gold nanoparticles, deposited on silicon.

Fibers were deposited on both glass and silicon samples, and metallized with the same
5.2 Fiber metallization

protocol. Both silicon and glass substrates were suitable for AFM characterization, but
glass also made it possible to investigate the optical properties of the metallized fibers using
a spectrophotometer. It is known that the surface charge of silicon oxide surfaces highly
depends on the preparation of the surface itself (native, thermally grown, glass...) and
not only on the cleaning protocol [67], and that may influence the deposition of charged
particles. A difference in interparticle spacing between glass and silicon could in fact be
observed in the AFM images.

For metallization purposes no remarkable difference was observed between fibers grown
in glass or plastic tubes, while the nanoparticle batch could be a critical parameter. The
batch purchased from Sigma Aldrich was not suitable for deposition because of the surfac-
tant present in the solution, introduced to reduce long-term particle aggregation. Cent-
rifugation partially solved the problem because it removed the particle aggregates and
homogenized the suspension, but home made batches prepared by citrate reduction worked
better. Also, the presence of surfactant might alter the gold enhancement protocol, that
also used a surfactant (CTAB).

The regular interparticle spacing on the fibers can be evaluated via the Radial Distribution
Function (RDF), that describes how particles density varies as a function of distance
from a reference particle. RDF could be computed via the calculation of the value of the
autocorrelation via the Fourier transform function of ImageJ, but since it does not assume
periodic boundary conditions it can be used only on large images (>2 µm²). As shown in
Fig. 5.22, the autocorrelation function is 0 for small distances (the distance between the
centers of two 15 nm diameter particles cannot be less than 15 nm) and is maximum for
an interparticle distance of 45-50 nm. At large distances the RD function oscillates, going
asymptotically at 1 (no correlation). No secondary and tertiary peaks (which represent
the second and third order neighbours in the chain) are visible in the plot. Those peaks
were usually visible for single samples but disappear in the average plot because interpar-
ticle spacing was not widely constant and might slightly change between samples, an effect
probably caused by small differences in the preparation (citrate concentration, incubation
time, fiber batch, etc.) or in the surface. A general difference emerges anyway between
silicon and glass samples: on silicon substrates the average interparticle distance was 37 ± 5
nm, while on glass it was 41 ± 5 nm. A pulse function can be used to fit the RDF in order
to determine the position of the first probability peak, but further measurements (at a
higher resolution and precision) are needed in order to better estimate the interparticle
distance.

AFM characterization of fibers metallized with one of the nanoparticle batches synthe-
sized with Turkевич method showed that only larger fibers were successfully metallized,
while thinner ones were stripped off the substrate (see Fig. 5.23). This may be imputed
to an accidental excess of citrate in the synthesis, which could lead to an higher nanopar-
ticle surface charge implying an a larger repulsion force between the negatively charged
nanoparticles and the substrate. This repulsion might prove high enough to win over the
attractive force between fibers and substrate and detach them. Fragments of fibers appear
to be left on the sample, probably corresponding to positively charged residues like lysine
(Lys) and arginine (Arg) attached to the surface. Thicker (∼ 6 nm) fibers did not break
probably because they screen better the electrostatic particles-substrate repulsion.

Metallization was tested on both purified and unpurified batch of fibers. Purification
appears to be critical because fiber fragments often aggregated and caused disordered
agglomeration of particles on them. This might lead to a shift in the optical response of
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Figure 5.22: Autocorrelation function for deposited nanoparticles on silicon and glass (average on several samples).

Figure 5.23: Deposition of 15 nm gold nanoparticles with an excess of citrate may lead to partial detachment of the fibers from the surface (15 mg/mL incubated for 168h, on silicon).

the assembly due to dipole coupling on the fragment aggregates and not on the fibers (see Fig. 5.24). Both dialysis and centrifugation were used in order to remove fiber fragments from solution; centrifugation was in the end proven more efficient and less time consuming. Also, before metallization some samples were heated to 50° for 30’ in an attempt to better fixate the fibers on the surface. Heating the sample on a hot plate dehydrates and slightly
denatures the protein fibers, improving attachment.

After 1’ deposition the samples were rinsed with DI water or 1mM citrate buffer, and then their absorbance were measured. The results are shown in Fig. 5.24, 5.25.

![Absorbance of unpurified fibers metallized with different protocols.](image)

Figure 5.24: Absorbance of unpurified fibers metallized with different protocols.

![Absorbance of fibers metallized with different protocols. Fibers have been previously centrifuged in order to remove small fragments.](image)

Figure 5.25: Absorbance of fibers metallized with different protocols. Fibers have been previously centrifuged in order to remove small fragments.

For unpurified fibers, heating before metallization improved the formation of fragments agglomerates, since the plasmon peak in these samples is more redshifted (about 40 nm) than in the not heated ones. For unpurified fiber samples, the plasmon coupling was anyway always evident, thus demonstrating the importance of purification. For purified fiber samples heating had no striking effect. The plasmon peak of purified fibers samples is around 520 nm, as expected for 15 nm non-interacting gold nanoparticles.

Citrate rinsing seems to improve the density of particles deposited on the samples.
compared to DI water rinsing, but appears to increase the number of particle deposited on the bare surfaces as well. Also, citrate left on the sample could interfere with the gold enhancement process, so DI water rinsed samples were used to proceed to the next step.

5.2.1 Gold enhancement

Metallized fibers were enhanced with a CTAB, HAuCl₄ and ascorbic acid solution that allowed the deposited nanoparticles to grow in size. Particle positions are fixed, so nanoparticles grown close to each other, although they did not appear to fuse together. Enhancement was tested both on silicon and glass substrates. Different protocols were used as well, varying the salt concentration in the enhancement solution to study the possible impact of ionic strength on the process. The solution is specifically designed to deposit gold only on gold surfaces, in order to avoid the creation of a gold coating both on substrate and fibers. During the process the glass samples noticeably changed colour, going from a pale shade of red to an intense red colour, then shifting to purple for longer enhancement times. This colour shift is a first hint of the dipole coupling interaction that arise when growing particles come close to each others. A similar behaviour could be observed on the silicon samples, looking closely in reflection.

![Image](image.png)

Figure 5.26: 15 nm particles on glass, 60° enhancement.

Particles grown in all three spatial directions both on silicon and glass, as shown in 5.27, 5.28, 5.30, 5.31. No striking differences were seen between growth on the substrate plane (x, y) and in the vertical direction z (even if in average growth appears to be slightly enhanced on the plane), but the effect could vary substantially from particle to particle. All the data was obtained assuming the 5 x 5 μm² AFM images as valid statistical reference for the whole sample.

Particle size on the (x, y) plane was investigated using the particle analysis tool from
5.2 Fiber metallization

ImageJ. Once particles area distribution was calculated, diameter distribution could be obtained assuming sphere-like particles. The size measurement is anyway subject to tip convolution, caused by the finite curvature of the scanning tip. This effect is a systematic error and can be therefore eliminated, but its estimation is subject to error itself. Height distributions ($z$ direction) were instead calculated directly from the characterizations and were not affected by any systematic error, since the tip (nominal radius 2 nm) was relatively small compared to the size (> 15 nm) of these features. For these reasons height measurements are more reliable in order to determine the particles true size.

![Figure 5.27: Gold nanoparticles size distribution after different enhancement time, on silicon substrate.](image1)

Size increase appeared to be faster in the first 30' minutes of incubation, and after
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Figure 5.29: Average particle diameters on silicon and glass substrate for different enhancement times. Errorbars represent standard deviation from the average.

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<th>Silicon $D$</th>
<th>Silicon SD</th>
<th>Glass $D$</th>
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<td>15.7</td>
<td>1.3</td>
</tr>
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<td>3.6</td>
<td>21.3</td>
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<td>4.7</td>
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</tbody>
</table>

Table 5.2: Average particle diameters $D$ and standard deviation from average SD on silicon and glass substrate for different enhancement times, as shown in Fig. 5.29.

that the radius distribution seemed to stabilize, with most of the particles having a radius between 25-30 nm (Fig. 5.27, 5.28). Size distribution widened significantly with the duration of the process, showing the effect of the enhancement, as shown in Fig. 5.29. In other words particle growth was not uniform and after more than 40’ minutes treatment two particles on the sample might have their size differ by a factor 2.

Particles height increased as well with enhancement time, in a fashion similar to the one described above for the $x$ and $y$ directions. The height distributions in Fig. 5.30 and 5.31 are broad, and resemble the distribution of sizes in the substrate plane. Neglecting the peak at 0 nm that represents the substrate surface it is evident that particle height increased with enhancement time. The local maximum in the distribution before the descending slope represents the height of the largest part of the particles. Before enhancement it is of course close to 15 nm. At longer times the local maximum is around 25-35 nm, in accordance with the trend observed in the $(x,y)$ plane. According to this data, gold nanoparticles grown almost equally (on average) in every direction, showing no particular trend making them diverge from the starting spheric shape.
5.2 Fiber metallization

After 60’ of enhancement the size distribution widens consistently on the glass substrates, while on silicon almost no particles grew beyond 50 nm. This difference may be related to a surface effect but more probably to some differences in the deposition / metallization process, and requires further investigation. Salt concentration did not appear to influence in any significant way the enhancement process.

AFM characterization also shown that the differences in size on the same sample are correlated to the particle position: particles grew more when they are isolated from each other. The most isolated particles grew up to 50-60 nm diameter, and scarcely metallized fibers displayed big particles as well. Particles that were arranged periodically on fibers grew less and slightly more evenly. Closely packed particles offer more gold surface per unit
of volume than isolated ones, so it is possible that with less surface available gold deposit faster because the gold concentration nearby the surface of a single particle depletes slower. AFM showed the presence of circular structures on some samples deposited on glass (see Fig. 5.32), most probably due to drying effects. A droplet of solution that redeposit as the surface is dried might be the cause of these areas without deposited fibers. Few isolated gold particles appear to bind on the bare surface inside these droplets prints, and these particles grew significantly more than the ones on fibers.

**Figure 5.32:** Metallized fibers on glass substrate after 120’ of enhancement, particular of a microbubble effect on fiber disposition.

The average interparticle spacing on fibers on glass substrate (center to center) was $\sim 50-55\,\text{nm}$, so 15 nm diameter particles edges were about 40 nm from each other. After more than 30’ enhancement the diameter of particles periodically attached on fibers increased up to 20-30 nm, so distance from gold surfaces decreased to 25-35 nm. On silicon the behaviour is similar, with slightly smaller interparticle distances.

Observing the height profile of metallized fibers at different enhancement times (see Fig. 5.34) allows a better understanding of how the enhancement process influences the particles relative positions.

Before enhancement peaks in the height profile are evenly spaced and with the same height, apart from a few outlier particles that are slightly bigger than 15 nm. After 5’ there are no remarkable changes, but after 20’ some differences appear. Particles seem to grow close to each others in pairs, alternating a wide edge-edge spacing with a smaller one. This trend is confirmed at further enhancement times, and after 120’ the gap between pairs is small enough to be barely resolved by the microscope.

Height of the particles on fibers increased with enhancement time, and size as well, but some particles appeared to not grow much or at all. This difference was probably due to orientation of the crystalline planes of the particles, since the enhancement process
Figure 5.33: AFM characterization of the gold enhancement process on silicon substrate at different time.
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Figure 5.34: Height profile of metallized fibers, before and after 0', 5', 20', 120' enhancement, on glass surface.

has a lower deposition rate on the \{100\} direction [64]. Particles with the \{100\} direction exposed would thus grow less. Moreover, particles in an array could not grow freely in every direction, since they had at least two other particles that set a limit to the growth in their directions. This might be one of the reasons both for why isolated particles grew more and why particles on fibers appear to grow in couples.

The optical response of enhanced fibers is shown in Fig. 5.35 and 5.36. The overall absorbance increases with enhancement time, and two peaks can be seen. The first peak is probably caused by single nanoparticles plasmon peak and transverse modes, while the second one is due to the longitudinal plasmonic coupling modes along the chain.
5.2 Fiber metallization

Figure 5.35: Absorbance of metallized fiber samples after different enhancement times, with precursor solution prepared in DI water.

Figure 5.36: Absorbance of metallized fiber samples after different enhancement times, with precursor solution prepared in a 5 mg/mL NaCl solution.

Of course this optical characterization, performed with a spectrophotometer, is performed on an ensemble of nanoparticles. For this reason a quantitative correlation between the plasmon resonance shift and the size and shape of the aggregate is difficult to obtain, especially for a broad distribution of aggregate morphologies. Because of the distributions in nanoparticle size, chain length, and geometry (i.e., straight, coiled, crossings, etc.) and the dynamic averaging over different chain orientations and conformations in the sample, the spectra mainly show these two broad plasmon resonances.

In accordance with previous studies, one resonance is centered at the same wavelength as a single particle, while the second one is shifted to longer wavelengths [68]. The amount of red-shifting was found in these studies to depend on interparticle separation and chain
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5.3 FDTD simulations

Because the spectrum comprising of two plasmon resonances resembles that of nanorods with different aspect ratios, calculations in several studies have modelled these extinction spectra to a first approximation as arising from straight linear chains with various lengths [69]. For this reason amyloid templated gold nanochains were approximated as infinite, straight chains of spherical gold nanoparticles. This approximation could of course be refined, considering the curvature of fibers and the not perfectly regular particle spacing, but is sufficient to understand the optical response shown in Fig. 5.35 and 5.36.

Finite Difference Time Domain (FDTD) simulations were used to model these infinite chains. This approach allows to solve Maxwell equations on a desired mesh grid and then compute far-field properties such as the extinction cross section. Linear chains were simulated starting from the data explained so far. As shown in Fig. 5.37 and 5.38 different interparticle spacings were considered, varying the particle diameter in order to see differences in the optical response. The spacing considered was in the range suggested from the experimental analysis. Simulations were set using an effective refractive index (metallized fibers stand between air and glass) and unpolarized, perpendicular incident light, as in the actual spectrophotometer. Extinction cross section was calculated, in order to compare simulations with the experimental data.

![FDTD simulations for infinite chains of gold nanoparticles of different diameter, with interparticle spacing fixed at 35 nm.](image)

Since simulated spectra only take into account one regular chain, plasmon resonance peaks are much narrower. As a general rule the interaction gets stronger as much the particles’ surfaces get closer to each other. As predicted the peak at 520 nm blueshifts due transverse modes in the chain, but since its shift is at best ~10 nm and its amplitude does not change considerably, it is hardly visible in the experimental ensemble measurement.

On the other hand contribution from longitudinal modes is massive, and the peak redshift can reach 300 nm. In experimental data the redshifted peak had its maximum between
5.3 FDTD simulations

Figure 5.38: FDTD simulations for infinite chains of gold nanoparticles of different diameter, with interparticle spacing fixed at 40 nm.

650-700 nm wavelength, so the major contribution appears to be from nanoparticles 30-35 nm wide which surfaces are 2-3 nm from each others. The broadness of the second peak reflects the broadened size distribution on glass surfaces.

Increasing enhancement, time one would expect the coupling peak to rise in amplitude and gradually shift towards longer wavelengths, but surprisingly the peak seemed to arise directly at 670 nm. This might be caused by an anisotropic particle growth, with closely spaced particles that tends to grow slightly more towards each others.

Although it is clear that there was plasmon coupling along the chains after the enhancement process, while there was none for fibers metallized with 15 nm particles. FDTD simulations for 15 nm particle chains at different spacings showed only small changes in the spectra, as expected: the rising of the second peak demonstrated the interaction between surface plasmons.

Nanoparticle dimers behaviour was simulated as well, varying particle size and spacing. For particles with sizes in the range suggested from AFM characterization, dimers showed resonances that did not shift much (as compared to infinite chains of same particle size and spacing). Plasmon redshift is considerable only when particles are really close to each other (≤ 1 nm) which is unlikely to happen, especially at low enhancement time (for example, Fig. 5.39).

Similar considerations can be made for short nanoparticle chains: keeping spacing and size constant while adding particles to a dimer indeed increases the redshift (even if not in regular steps) for every particle added, but the shift remains low unless the spacing is small or the chain long. Simulations confirm that chains with more that 8-9 particles have a behaviour similar to that of the infinite approximation, and short (4-7 particles) chains show similar optical responses, with less pronounced shift of the plasmon peak. Short chains contribution is then not negligible, but most of the deposited fibers were several µm long (or fragments).

Simulations were run also creating (infinite) chains with periodic boundary conditions using a 'base unit' with two instead of one particle. This allowed to create chains with
Figure 5.39: Extinction cross section of 30 nm diameter particle dimers at different distances.

periodicity but with spacing that is not constant between particles. In these chains particles go in couples, with dimers with low interparticle spacing separated by a larger distance. For sizes and spacings similar to the ones used before, these simulations showed results similar to that of the regularly spaced infinite chains in terms of plasmon redshift, in some cases with broadened resonance peaks, probably because of the hybridization of the modes.

Fibers curvature was not considered in these simulations, both because it implies a big step both in terms of simulation design and computational time (periodic boundary conditions on non-finite curves are more difficult to set) both because curvature radius of amyloid fibers was usually above 500 nm and often exceeded 1 µm, so fibers were almost straight on a short distance, with respect to a 40 nm spacing. Fiber deposition is anyway not uniform, and fibers were likely to cross each other. Chain crossing, T-shaped junctions and similar structures might influence plasmon interaction along the chains. This effect is most likely not to have a striking effect on the measurements showed in this thesis, because fibers coverage is not dense enough to induce crossings too often on a single chain. The effect could be anyway interesting to study (especially if one is interested in plasmon propagation), but exceeds the goals of this project.

Further analysis is needed in order to understand in more details the coupling, and a better tuning of the gold enhancement process. In order to obtain better statistics regarding interparticle spacing along fibers and the effect of enhancement, more AFM characterizations have to be performed, at slow speed and high resolution in order to reduce systematic errors and have better precision when analysing the image.

5.4 Dark-field single molecule spectroscopy

While sufficient to verify plasmon coupling, ensemble spectroscopic measurements are inadequate to investigate in depth the optical properties of metallized fibers. The relatively random disposition of fibers prevented the use of a polarizer in the spectrophotometer to verify the optical response with different polarization angles. This problem could be solved using a single-molecule spectroscopic microscope, that can illuminate only a small, selected
area of the sample.

A preliminary measurement was performed on a sample deposited on glass and enhanced for 120°, by adding a polarizer to a combined AFM-dark field microscope. Due to the device limitation it was not possible to select a single fiber in the scan area, but a qualitative result could anyway be observed. An AFM characterization of the scanned area can be seen in Fig. 5.40. No information beyond the chain disposition can be extracted from the picture, due the presence of large artifacts. This was caused by damage to the scanning tip and also the conductive coating deposited on the tip, which leads to a larger tip broadening effect.

![AFM characterization of the area investigated with dark-field spectroscopy.](image)

Figure 5.40: AFM characterization of the area investigated with dark-field spectroscopy. Image quality is bad because the integrated AFM is designed for feature recognition before spectroscopic analysis, not characterization, and the tip was contaminated or damaged at the beginning of the experiment. Arrows indicate polarization of the electric field.

Given chains orientation on the sample, a consistent difference in the scattering spectra was expected. As shown in Fig. 5.41 when the sample was illuminated by light polarized at 0° (electric field approximately parallel to the chain) a bright peak centered at 630 nm could be observed. On the other hand, with orthogonal polarization (90°) the scattering absorbance was less intense and peaked at 560 nm.

Variability of optical response with light polarization is another proof of plasmon coupling along enhanced nanoparticle chains. The measurement was performed a relatively disordered assembly of fibers, which means that the incident electric field at a given polarization angle would interact with different angles on the fibers, thus spoiling the observation of the chain response at precise polarization angles. Nonetheless, the difference in scattering between opposed polarizations was significant and is a good starting point for future analysis.

Scattering spectra shown in Fig. 5.40 are then not the result of 'pure' longitudinal and transverse modes but a combination of both, in different proportion. In the 0° measurement, longitudinal coupling is clearly dominant (as expected from AFM characterization) and the resonance is redshifted to 630 nm. The longitudinal plasmon resonance is probably slightly more redshifted, in accordance with the spectroscopic measurements.
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Figure 5.41: Scatterplot of the scattering optical response of the sample at different polarization angles. Some intensity peaks from the Hg(Ne) lamp spectra are visible. Scattering cross section at different polarization angles for chains with 40 nm spaced 37 nm diameter particles are plotted for comparison (longitudinal mode has been scaled down 5 times).

The spectrum acquired for a 90° polarization angle shows a non-gaussian shape typical of the scattering cross section of single particles. FDTD simulation proved that chain scattering cross section in case of transverse polarization depends on wavelength in a way similar to single particles, blueshifting as the interparticle distance decreases. The spectrum in Fig. 5.41 is instead redshifted with respect to single particle resonance (520 nm), but that is to be expected because the electric field is not perfectly perpendicular and transverse modes blueshift is about one order of magnitude lower than longitudinal modes redshift.

According to FDTD simulations this scattering at 0° polarization expected to be much brighter than observed, when compared to the measurement at 90° polarization. This is probably due that scattering cross section decreases rapidly as the polarization move away from 0° polarization, and the measurement at 90° is higher than expected due to the contribution from the longitudinal mode.
Chapter 6

Conclusions and outlook

In this project, biotemplated gold nanochains have been assembled with a bottom-up approach starting from molecular and colloidal building blocks. Amyloid fibers from hen egg white lysozyme have been synthesized and used as scaffolds to assemble 15 nm diameter gold nanoparticles via electrostatic interactions. Because of these interactions, particles assembled with regular spacing, and a subsequent enhancement treatment allowed to increase the size of the particles on fibers. The physical conformation and optical properties of the chains have been analysed, proving the coupling of plasmon excitation modes along the structures.

Amyloid fibers have been synthesized in vitro under conditions chosen on the basis of previous studies (thermal denaturation at 60°C in acidic aqueous solutions), varying enzyme concentration, container and incubation time in order to obtain fibers suitable for metallization. Results from previous studies have been confirmed, as fibrilization appeared to be visible after ~2 days of incubation. Concentration was proven to be a key parameter in the process, and in the end 10 mg/mL and incubation times between 144 and 192 hours proved to be the optimal conditions to produce straight, thin fibers and avoid excessive aggregation. Lower concentration resulted in a delayed fibrilization and a slower growth, while high concentration increased the percentage of larger fibers (caused by two or more fibrils coiling together). While incubating lysozyme for less than 5 days led to short fibers, longer incubation times (>9 days) induced aggregation in the suspension. The container material appeared to not influence the quality of fibers but only the growth rate: in glass containers fibrilization was easier than in plastic ones, and slightly faster. This is probably due to surface interactions, as in glass containers large aggregates spawned along the inner surface. AFM and spectroscopic measurements did not show striking differences between fiber samples from suspensions incubated in glass or plastic containers.

Fibers growth has been monitored via AFM, that provided information on the size and length of the structures, and with absorbance spectroscopy. Spectroscopic analysis showed the presence of a broad absorption peak at 330 nm that is not typical of native lysozyme absorbance pattern. This feature, which nature is not yet clear, appears to be proportional to the fibrilization rate in suspension. This hypothesis appeared to be corroborated by a seeded growth experiment, in which amyloid fragments were used as 'seeds' to accelerate the fibrilization rate, in which this feature increased faster as well. This result still needs confirmation but may prove cheaper, faster and direct way of monitoring amyloidization as compared to AFM imaging or chromogenic reactions.
Fibers have then successfully been metallized with citrate-capped gold nanoparticles, starting from prior experimental design [34]. 40 nm spaced gold nanochains were created, on fibers deposited on glass and silicon substrates. In order to achieve long, uniformly coated chains several issues had to be figured out. The presence of short fibers caused formation of disordered nanoparticles aggregates, biasing spectroscopic measurements. Centrifugating fibers batches proved useful to remove the largest fraction of these fragments, while proper dilution of the batch before deposition helped avoiding excessive fibers crossing. Nanoparticles source was proven to be an important factor as well, home made gold nanoparticles are preferable due the presence of surfactant in commercial batches. Residual citrate in batches after reduction could be a problem as well, since nanoparticle surface charge could get too high and charge repulsion with the substrate would cause the fibers to be stripped away from the surface.

Optical measurements showed that the 15 nm particle chains did not initially present any significant surface plasmon coupling. An enhancement protocol that allowed reduction and deposition of gold ions on gold surfaces was used to increase the deposited particles diameter and bring their surfaces closer to each others. Particles growth increased with time, and size distribution broadened as well. Closely packed particles appeared to grow less than isolated ones. Samples treated with this protocol led to the rise of a secondary, broad absorption peak at 650 nm. This absorption peak is caused by interactions of the electron oscillations in nanoparticles, i.e. coupling of single particles plasmon modes along the chains. FDTD simulations showed that 32-38 nm diameter particle chains with spacing in the 35-40 nm range (as the ones observed with AFM characterizations) have a bright absorption peak in the 630-670 nm range. Dipole-dipole interactions are the basic explanation for this phenomenon, but due the close distances between particles, higher order modes (quadrupoles, etc.) contribution is not negligible. Broadness in particle size distribution accounts for the broadness of the absorbance plasmon coupling peak, and made a quantitative analysis difficult. A preliminary experiment with dark-field single molecule spectroscopy was performed, analysing a small area of the sample. Optical response varied greatly with the orientation of the polarization of the incident light, as predicted by theory. This is an additional confirmation of the plasmonic nature of these structures, demonstrating the possibility to create nanochains with particular optical properties using amyloid fibers as templates.

In the end, HEWL amyloid fibers proved to be a robust and reliable scaffold for metallization via electrostatic interactions. Gold nanoparticle chains assembled on such templates are evenly spaced and, when properly enhanced, show peculiar optical response due to plasmon coupling along the chains. For this reason, metallized fibers may in the future find applications as waveguides at the nanoscale, maybe even allowing propagation for several micrometers if optimized for subradiant modes. Several studies have been performed over the possibility of guiding electromagnetic waves with a transverse confinement below the diffraction limit using near-field interactions, showing transport up to several µm. Biotemplating may be a good option for creating cheap, biocompatible and versatile guides, as self assembly may allow fabrication of chains in situ. Moreover, amyloid resilience grants a good reliability under a wide range of conditions, and particle size can be tuned to achieve the desired plasmon resonance wavelength. Amyloid fibers persistence length could also be an advantage, as sharp turns in waveguides often cause severe losses, while chains with low radius of curvature are preferable. For the same reason metallized fibers could find application in biosensing, both to transfer signal and as sensors if particles
on the chain are functionalized.

Concerning future developments, several options can be considered. Better fibers immobilization and alignment control would help greatly to investigate the physical properties of these structures. The first could be improved by functionalizing the substrate to achieve a higher adhesion force, for example crosslinking the fibers using glutaraldehyde or covalently bonding them to the surface via silanization. The latter is more difficult to achieve, even if an appropriate use of the nitrogen flow in some cases allowed deposition in a more ordered fashion. A solution may be to pattern the surface via lithography to create 'guides' that favour binding only in specific directions. Other biological scaffolds could be investigated as well, since a wide variety of proteins are able to form amyloid fibers. Albumin, lactalbumin and insulin, for example, are common proteins that form amyloid fibers with physical properties similar to that of lysozyme but with different moieties and isoelectric points. Amyloid generating protein could be genetically engineered as well. On the other hand completely different biological structures could be investigated, such as scaffolds made from DNA and viral capsids. These elongated structures have physical properties entirely different from amyloid fibers, are more fragile but have fixed lengths which can be genetically programmed.

Since it was proven in a previous work that interparticle spacing can be tuned controlling the surface charge of particles, it could be worth to investigate the effect of the enhancement process on differently spaced chains. In order to better measure the plasmon coupling along the chains, the gold enhancement process could be refined as well. A careful investigation on the effects of parameters such as concentration, ionic strength and temperature may allow the attainment of narrower distribution of particle sizes along the chains. On the nanomaterial side, the use of alloy particles could open entirely new avenues. Nanoparticles made of different metals could be tested, and since similar enhancement processes exist for reduction of other metals, an alloy coating could be created on already deposited particles. Core-shell particles could be used as well to combine optical properties and surface functionalization.

AFM has proven a great tool to investigate the structure of these chains, and the use of single molecule spectroscopy techniques would allow to characterize in depth the plasmon resonances. In order to avoid chain crossing fibers should be deposited in lower concentration, allowing also a better comparison between experimental data and simulation. With adequate setup samples deposited on silicon could be analysed as well in reflection.

Summarizing, the development of biotemplated metal nanoparticle chains may prove challenging but is a great way to explore a new path in biophysics and nanosciences and has the potential to create new opportunities in the nanofabrication of versatile plasmonic devices.
Bibliography


