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Advanced microscopy techniques applied to the in-vitro study of α -syn aggregation and toxicity mechanism

Doctor of Philosophy (Ph.D.) Thesis

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*Dedicated to my father who took life in his hands,
my mother, from whom I learned love,
and my brothers for their self-sacrifice to compensate my absence for my
parents.*

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Abstract

In this work, I applied advanced microscopy techniques to study the molecular mechanisms of α -synuclein (α -syn), the amyloidogenic protein responsible for Parkinson's disease.

The toxicity of α -syn is likely related to its interaction with the neuronal membrane. It is known that α -syn is present in both the cytosolic and extracellular space, and it has been demonstrated that both components have a role in neurodegenerative processes. To study the effect α -syn on lipid membranes, I used two complex lipid mixtures, preparing lipid bilayers that modeled the external and internal leaflets of the neuronal membrane. Exploiting this original approach, I investigated how α -syn may act on membrane structure and stability when it presents in the cytosol or the extracellular space. We have compared the action of α -syn in monomeric form and at different concentrations (1 nM, 40 nM, and 200 nM). I used atomic force microscopy (AFM) in different acquisition modes, and I complemented the results with QCM-D, and vesicle leakage experiments.

In a second work, I characterized amyloid aggregates formed by α -syn, applying a correlative technique based on the coupling between AFM and stimulated emission depletion (STED) microscopy. In particular, I focused on the definition of the eventual influence that the presence of fluorescent dye molecules can have on the aggregation process of the peptide. I demonstrated that the use of a site-specific labeling method is fundamental to avoid modification of the aggregation process.

List of Abbreviations

α -syn	Alpha-synuclein
A β	Amyloid β
PD	Parkinson's disease
DLB	Dementia with Lewy Bodies
AFM	Atomic Force Microscopy
TIRF	Total Internal Reflection Fluorescence Microscopy
SPR	Surface Plasmon Resonance
QCM-D	Quartz Crystal Microbalance with Dissipation monitoring
Chol	Cholesterol
E. coli	Escherichia coli
Hsp70	70 kilodalton heat shock protein
NAC	Non-amyloid Core
POK2	Polo-like Kinase2
A30P	Ala30-Pro
CW	Continuous wave
DLS	Dynamic Light Scattering
DMSO	Dimethylsulfoxide
DOPC	1,2-dioleoyl-sn-glycero-phosphocholine
DOPE	1,2-diolyeil-phosphatidylethanolamine
EDTA	ethylenediaminetetraacetic acid
TCEP	tris(2-carboxyethyl) phosphine

PSPD	Positions Sensitive Photodiode Detector
EM	Electron Microscope
PBS	Phosphate Buffered Saline
ER	Endoplasmic Reticulum
CD	Circular Dichroism
CR	Congo red
ThT	Thioflavin T
FD	Force-Distance
Fb	Breakthrough Force
SE	Stimulated Emission
FLIM	Fluorescence Lifetime Imaging
GM1	Monosialotetrahexosylganglioside
HCl	Hydrochloric acid
SiO ₂	Silicon Oxide
UV	Ultraviolet
DMF	Dimethylformamide
HFIP	Hexafluoroisopropanol
LUV	Large Unilamellar Vesicle
SUV	Small Unilamellar Vesicle
MLV	Multilamellar Vesicle
GUV	Giant Unilamellar Vesicle
CaCl ₂	Calcium Chloride
NaCl	Sodium Chloride

NA	Numerical Aperture
ROC	Radius of Curvature
NFT	Neurofibrillary Tangles
NMR	Nuclear Magnetic Resonance
PC	Phosphatidylcholine
PD	Parkinson disease
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
GM1	Ganglioside
Chol	Cholesterol
TES	2-[tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid
POPS	1-palmitoyl-2-oleoyl-SN-glycero-3-phospho-L-serine
SLB	Supported Lipid Bilayer
SVL	Supported Vesicles Layer
mEXT	External membrane
mINT	Internal membrane
SM	Sphingomyelin
QI	Quantitative Imaging
T _m	Transition temperature
SPM	Scanning Probe Microscopy
UHV	Ultra-high Vacuum
SR	Super-resolution
STED	Stimulated Emission Depletion

CLEM	Confocal Laser Scanning Microscope
STM	Scanning Tunnelling Microscope
FCS	Fluorescence Correlation Spectroscopy

Introduction

Misfolding can result in the aggregation of soluble proteins into intractable amyloid fibrils. Regardless of the sequence or the structure of the functional states of the proteins, amyloids possess a unique and universal cross- β quaternary structure that are about 10 nm in diameter and rich in β -sheets. This phenomenon is associated with a range of increasingly common human disorders, including Alzheimer's and Parkinson's diseases, type II diabetes, and several systemic amyloidosis. It has been evidenced that the toxicity of amyloidogenic proteins is likely related to their interactions with asymmetric neuronal membrane, characterized by the mutually disruptive structural perturbations. Membrane surfaces promote the conversion of amyloid-forming proteins into toxic aggregates, and amyloidogenic proteins, in turn, compromise the structural integrity of the cell membrane. Nowadays, the exact mechanism by which the toxic aggregates cause damage to the cell is still an open question. New biophysical approaches are fundamental to reveal new insights into the unraveled mechanism that drive this kind of aggregation.

In this context, several bulk measurements, including UV circular dichroism (CD), Fourier transform infrared spectroscopy, and Thioflavin T (ThT) have been done to characterize the process of aggregation in-vitro as a function of time. However, high heterogeneity and multi-pathways behavior of the aggregation process limits the application of bulk measurements in understanding specific parameters related to specific properties of each aggregate present in solution. Single molecule microscopy techniques, including atomic force microscopy (AFM), electron microscopy (EM), and super-resolution optical microscopy techniques (SR) such as stimulated emission depletion (STED) microscopy have been extensively utilized in the last

decade in the study of molecular mechanism associated with misfolded protein diseases at the nanoscale.

During my PhD, I developed my research career on two main issues: the first issue was focused on the interaction of monomeric α -synuclein (α -syn), the amyloidogenic protein responsible for Parkinson's disease, with model lipid membranes that mimic the outer and inner cellular leaflets, with the aim of investigating how α -syn may act differently when it presents in the cytosol or the extracellular space. The second issue was the characterization of the aggregation process of amyloidogenic α -syn by advanced correlative techniques, in particular correlative AFM–STED measurements, to the in vitro study of amyloid aggregates formation starting from monomeric peptides. On one side, label-free AFM as a primary technique in the study of amyloid fibrils and aggregates in vitro enables the visualization of molecular dynamics, including the nucleation rates of fibrillogenesis, as well as mapping the progression of variations in morphologies, and the development of structural hierarchy, happening near physiological conditions. On the other side, optical microscopes based on fluorescence as a mechanism of contrast can access the local distribution of specific molecular species. The coupling between AFM and super-resolved fluorescence microscopy solves the resolution mismatch between AFM and conventional fluorescence optical microscopy.

This thesis is organized in the following way: the first chapter introduces the protein misfolding and related diseases, introducing α -syn as an amyloidogenic peptide. In chapter 2, a brief introduction on cell membrane, lipid compositions and model membranes are provided. Chapter 3 gives an overview of the used microscopy techniques; Specific attention is focused on the stimulated emission depletion (STED) and atomic force microscopy (AFM). A brief historical view on correlative microscopy is presented and the progress of correlative AFM-STED microscopy

techniques in biological research is summarized as well. In Chapter 4, I examined the interaction of α -syn with supported lipid bilayers that mimic the inner and outer neuronal membrane leaflets. In Chapter 5, focusing on the results obtained by means of correlative STED-AFM recordings on α -syn aggregates have been reported. Chapter 6 is a short overview on a preliminary, but promising, study. I characterized insulin fibrils aggregated in vitro from a solution of partially labeled monomers. I verified that just a part of the fibrils is fluorescent after fibrillation. I applied transmission electron microscopy (TEM) on amyloid fibrils samples, trying to disclose the structural diversity between fluorescent and non-fluorescent aggregates.

Chapter1

Protein misfolding and related diseases

1.1 Protein folding and protein misfolding

The balance between proper protein folding and protein misfolding defines how functionally active proteins are produced within the cell. Historically, studies of protein folding began in the 1950s when several hypotheses, including ribosomal modifications, thermodynamic principles, and additional cellular components were raised to identify how proteins fold into their conformations ¹. In the late 1950s and early 1960s, Christian Anfinsen and his colleagues were among the first who proposed a theory to understand how proteins fold, a theory that would remain generally accepted for two decades ²⁻⁵. Anfinsen studied the enzyme Ribonuclease A, known as RNase, a small protein of about 100 amino acids (a.a) responsible for cleaving single-stranded RNA. He proposed that the protein's amino acid sequence was sufficient information for the correct folding of the protein to kinetically find a stable configuration with the lowest possible free energy. Anfinsen was awarded the Noble prize in chemistry for his theory. The self-renaturation phenomenon was also described for other proteins in vitro, and it became general knowledge that the proteins spontaneously fold into their conformations without any cellular help. Anfinsen's theory was challenged, in the late 1970s and early 1980s, when bacteria with recombinant genes were used to manufacture certain proteins on a mass scale. Instead of forming properly folded proteins, bacterial methods formed aggregates of misfolded proteins, having the same amino acid sequence but drastically differing structures ⁶⁻⁸ (Fig 1.1).

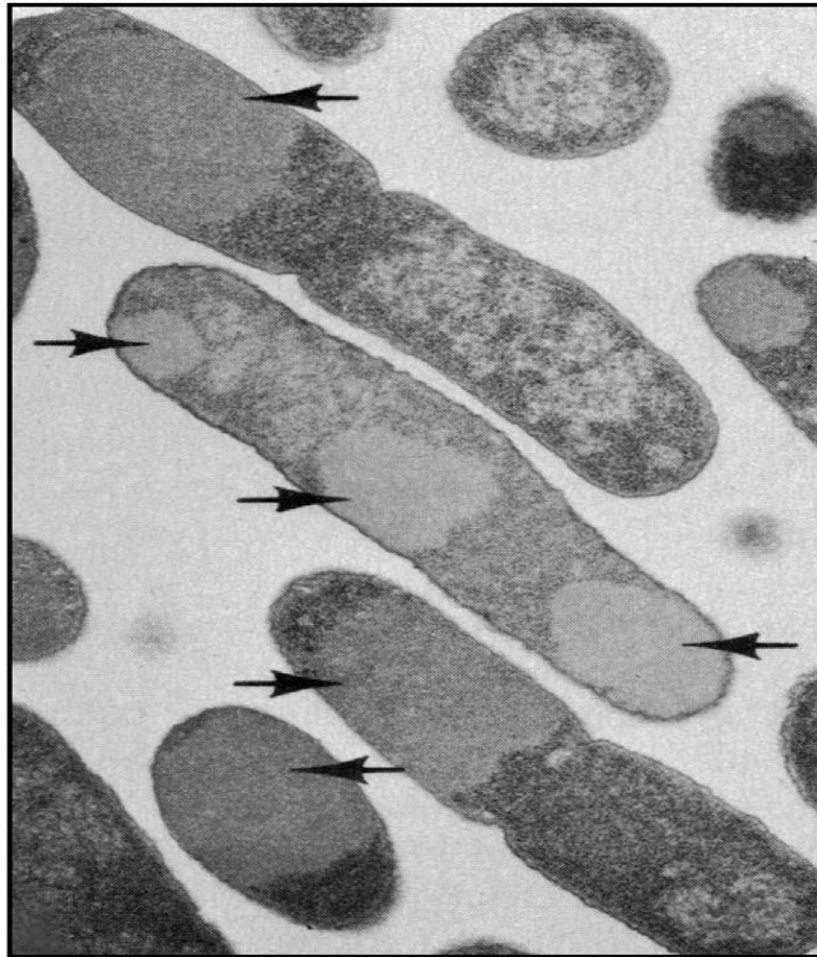


Figure 1.1 | Representing transmission electron micrograph of inclusion bodies (arrowed) formation in *E.coli* as an evidence of protein misfolding in vivo (Image from ⁹).

Also, several other discoveries, including the trafficking of proteins across membranes, raised questions about spontaneously protein folding's theory. In the late 1980s, researchers discovered a protein that assisted the refolding process of proteins that had crossed into the mitochondria of yeast mutant ¹⁰. The discovery of these proteins and other organisms demonstrated: although all the information about protein folding resides within the primary structure, many large proteins which undergo formation of tertiary or quaternary structures may require additional help to fold properly. Chaperons are proteins that stabilize partially or

improperly folded polypeptides, facilitating correct folding by providing proper microenvironments, where interactions between a protein and its surroundings favor certain conformations. They have a large chamber in their center, where unfolded protein chains are shielded from other unfolded proteins to prevent aggregation. Several types of molecular chaperons are found in organisms ranging from bacteria to humans. Two major families of chaperons, including Hsp70 family, and the chaperonins are both well studied⁹. Once a protein is synthesized co-translationally on the ribosome, a combination of local and non-local interactions forms the secondary and tertiary structures. The primary motifs of protein and the secondary structures are α -helices and β -sheets, formed because of hydrogen bond interactions between amide hydrogens and carbonyl oxygens. In addition to α -helices and β -sheets, turns, loops, and other structures can form due to the hydrogen bonds. Tertiary structure is determined primarily by the interactions of the amino acid sidechains which contribute to hydrogen bonding, disulfide bridges, and ionic bonding. Tertiary structure is also dependent on the orientation of sidechains, inward and outward, based on their hydrophobicity. These processes happen in a pathway that minimizes the resulting free energy of the protein consistent with Anfinsen's theory. Fig 1.2 summarizes a selection of the most important of these different states from a mechanistic and biological perspective.

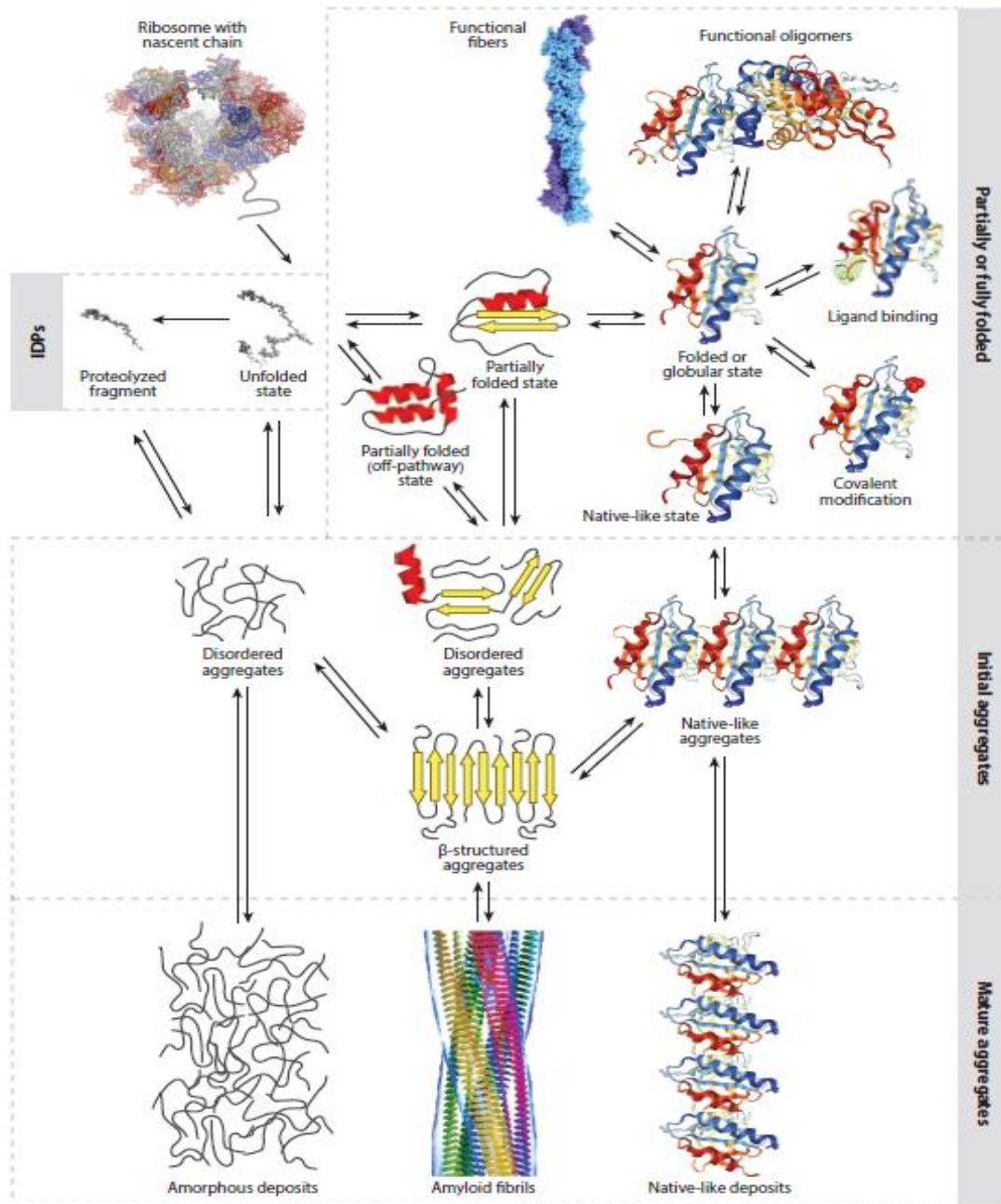


Figure 1.2 | Pathways of amyloid formation. This includes the formation of non-amyloidogenic oligomers without cross- β -sheet core, which give rise to the larger cross- β -sheet containing oligomers and proceed further to fibrils. Reference from ¹¹.

In some circumstances, regardless of the sequence or the structure of the functional states of the soluble proteins, partially unfolded proteins and protein folding intermediates escape the quality-control activities of the chaperons and degradative pathways. Protein misfolding does

occur so that the soluble protein can convert into nonfunctional and potentially damaging protein aggregates ¹². Proteins, that are not properly folded, often have exposed hydrophobic residues on their surface, which are normally buried in the native state, that render having β -sheet structure, a process that is often accompanied by an increase in compactness and size. There are different types of protein aggregates, including amorphous aggregates characterizing the initial oligomers, and formation of amyloid fibrils, representing a remarkable manifestation of the multiplicity of pathways existing in protein aggregation and of the structures and morphologies that can be generated ¹³. Table 1.1 lists the proteins and the disorders that have been identified to be associated with the formation of amyloid fibrils or other types of aggregates, respectively.

Disease	Aggregating protein or peptide
<i>Neurodegenerative diseases</i>	
Alzheimer's disease	Amyloid β peptide
Spongiform encephalopathies	Prion protein or fragments thereof
Parkinson's disease	α -Synuclein
Dementia with Lewy bodies	α -Synuclein
Frontotemporal dementia with Parkinsonism	Tau
Amyotrophic lateral sclerosis	Superoxide dismutase 1
Huntington's disease	Huntington with polyQ expansion
Spinocerebellar ataxias	Ataxins with polyQ expansion
Spinocerebellar ataxia 17	TATA box-binding protein with polyQ expansion
Spinal and bulbar muscular atrophy	Androgen receptor with polyQ expansion
Hereditary dentatorubral-pallidoluysian atrophy	Atrophin-1 with polyQ expansion
Familial British dementia	ABri
Familial Danish dementia	ADan
<i>Nonneuropathic systemic amyloidoses</i>	
AL amyloidosis	Immunoglobulin light chains or fragments
AA amyloidosis	Fragments of serum amyloid A protein
Familial Mediterranean fever	Fragments of serum amyloid A protein
Senile systemic amyloidosis	Wild type transthyretin
Familial amyloidotic polyneuropathy	Mutants of transthyretin
Hemodialysis-related amyloidosis	β 2-microglobulin
ApoAI amyloidosis	N-terminal fragment of apolipoprotein AI
ApoAII amyloidosis	N-terminal fragment of apolipoprotein AII
ApoAIV amyloidosis	N-terminal fragment of apolipoprotein AIV
Finnish hereditary amyloidosis	Fragments of gelsolin mutants
Lysozyme amyloidosis	Mutants of lysozyme
Fibrinogen amyloidosis	Variants of fibrinogen α -chain
Icelandic hereditary cerebral amyloid angiopathy	Mutant of cystatin C

<i>Nonneuropathic localized diseases</i>	
Type II diabetes	Amylin, also called islet amyloid polypeptide (IAPP)
Medullary carcinoma of the thyroid	Calcitonin
Atrial amyloidosis	Atrial natriuretic factor
Hereditary cerebral haemorrhage with amyloidosis	Mutants of amyloid β peptide
Pituitary prolactinoma	Prolactin
Injection-localized amyloidosis	Insulin
Aortic medial amyloidosis	Medin
Hereditary lattice corneal dystrophy	Mainly C-terminal fragments of kerato-epithelin
Corneal amyloidosis associated with trichiasis	Lactoferrin
Cataract	γ -Crystallins
Calcifying epithelial odontogenic tumors	Unknown
Pulmonary alveolar proteinosis	Lung surfactant protein C
Inclusion-body myositis	Amyloid β peptide
Cutaneous lichen amyloidosis	Keratins

Table 1.1 | human diseases associated with formation of extracellular amyloid deposits or intracellular inclusions with amyloid-like characteristics (Image modified from ¹¹).

1.2 Amyloidosis: Amyloid-related disease

Amyloidosis are a group of protein misfolding associated with number of inherited and inflammatory disorders, characterized by deposition of predominantly extracellular fibrillar proteins, displaying a specific tinctorial affinity for Congo red and Thioflavin T (ThT), result in tissue damage and function compromise ^{14,15}. Experimental evidence indicate that the aggregates accumulate almost entirely in the intra-or extracellular compartment where the protein normally resides. The word “amylin” is a Greek word means starch ¹⁶. Matthias Schleiden, a German botanist, was the first to use the term “amyloid” in botany when applying this technique to analyze plant preparations, for starch, referring to “starch-like” to refer to “a normal amylaceous component in plants” ¹⁷. One of his ideas was the use the iodine starch test to study chemical and anatomical composition of a plant cell. Originally described in 1814 by Jean Jacques Colin and Henri François Gaultier de Claubry, this test is based on detection of a blue stain produced in the reaction of starch with iodine in the presence of sulfuric acid. A German pathologist, Rudolf

Virchow, applied it in the medical literature in 1854 ¹⁶. He used the word “amyloid” to describe some deposits in nervous system which showed the same color reaction with iodine and sulfuric acid, i.e., a change from brown to blue, typical to starch. Only five years later, it was discovered that those deposits were mainly proteinaceous.

Amyloid is not a single entity. There are more than 20 different proteins that can aggregate to form fibrils, and the mechanism of fibrils formation of each of these proteins is different ¹⁸. These fibrils are highly ordered and unbranched with a high degree of β -sheet structures, which resist to degradation of proteolytic enzymes, thereby causing cytotoxicity and diseases. Amyloidosis can be classified as systemic or localized, depending on whether they affect tissues throughout the body or only in a limited area. Neurodegenerative disease including Alzheimer’s disease and Parkinson’s disease, in which such aggregates are in the brain, to type II diabetes, in which deposits form in the pancreas, to systemic conditions, in which deposits can be found in multiple organs, including the liver and the heart.

1.3 The structure of amyloid aggregates

A common feature of almost all protein conformational diseases is the formation of amyloid aggregates caused by destabilization of the α -helical structure and the simultaneous formation of a β -sheet ^{14,19,20}. These β -sheets are formed between alternating peptide strands. Linkages between these strands result from hydrogen bonding between their aligned pleated structures. Hydrogen bonds between parallel β -strands make the system particularly stable. Such β -linkages with a pleated strand between from one molecule being inserted into a pleated sheet of the next lead to hydrogen bond formation between molecules. The prerequisites for β -linkage formation are the presence of a donor peptide sequence that can adapt a pleated structure and a β

sheet that can act as an acceptor for the extra strand²¹⁻²³. Structural studies of amyloids started in the 1930s using X-ray diffraction. In 1935, William Thomas Astbury and Sylvia Dickinson described a typical X-ray diffraction pattern, later referred to as “cross- β ” structure. From X-ray diffraction measurements, it is possible to determine inter-sheets and inter-strands distances, which are 10-11 Å and 4.7 Å, respectively^{24,25} (Fig 1.3).

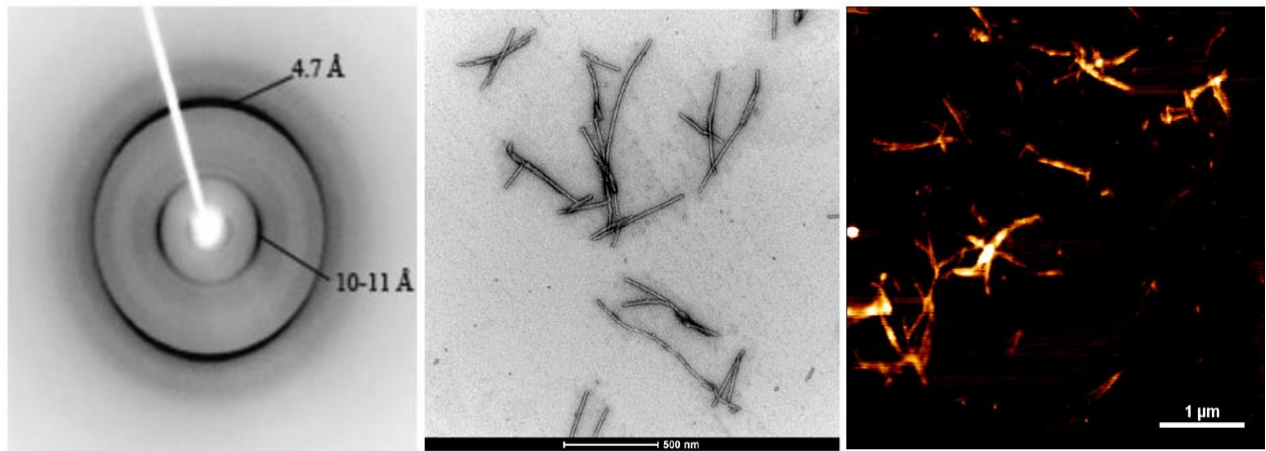


Figure 1.3 | (from left to right) Typical X-ray fibril diffraction pattern, showing the 4.7 Å meridional and 10-11 Å equatorial reflections that define the basic cross β structure²¹. Transmission Electron Microscopy of α -syn fibrils formed in vitro from synthetic peptide dissolved in water. AFM image of α -syn fibrils. Z range in AFM image: 20.9 nm.

A typical cross- β super-secondary structure, in parallel or anti-parallel way, models the core structure of each filaments whose strands are perpendicular to fibril axis. A typical fibril consists of two to seven proto-filaments unbranched, twisted like a rope around a hollow cavity, with an average diameter of 7.5–10 nm determined through AFM and electron microscopy (EM) measurements²⁶. In addition, advances in micro-crystallographic techniques have confirmed that many peptides, regardless of their sequence, adopt the characteristic cross- β pattern in the fibril state²⁷. The spine of the amyloid fibril contains dry steric zipper’s structure formed from self-complementary sequences²⁸, suggesting that amyloid diseases are similar not only on the fibril level but also on the molecular level²⁹.

The kinetics of fibrillization can be investigated with Thioflavin-T (ThT) assay, Congo red (CR), or their derivatives, which are thought to form ordered arrays along the lengths of the fibrils that give rise to specific spectral responses^{30,31}. ThT associates with cross- β structure of amyloid fibrils because of the rotational immobilization of the central C–C bond which connects the benzothiazole and aniline rings³², changing its absorption/emission spectrum from 385 nm (free dye absorption)/ 445 nm (free dye absorption) to 450 nm/ 482 nm. Since this variation only depends on the aggregated state and not on the interaction with monomeric or dimeric peptides, fluorescence signal at the new emission wavelength is a direct measurement of fibril formation. Amyloid peptides aggregate *in vitro* following a nucleation-polymerization process³³ (Fig 1.4), starting from a solution of soluble monomeric. In this initial condition, the aggregation process is not thermodynamically favored, a lag phase is observed, and the protein must overcome a significant energy barrier.

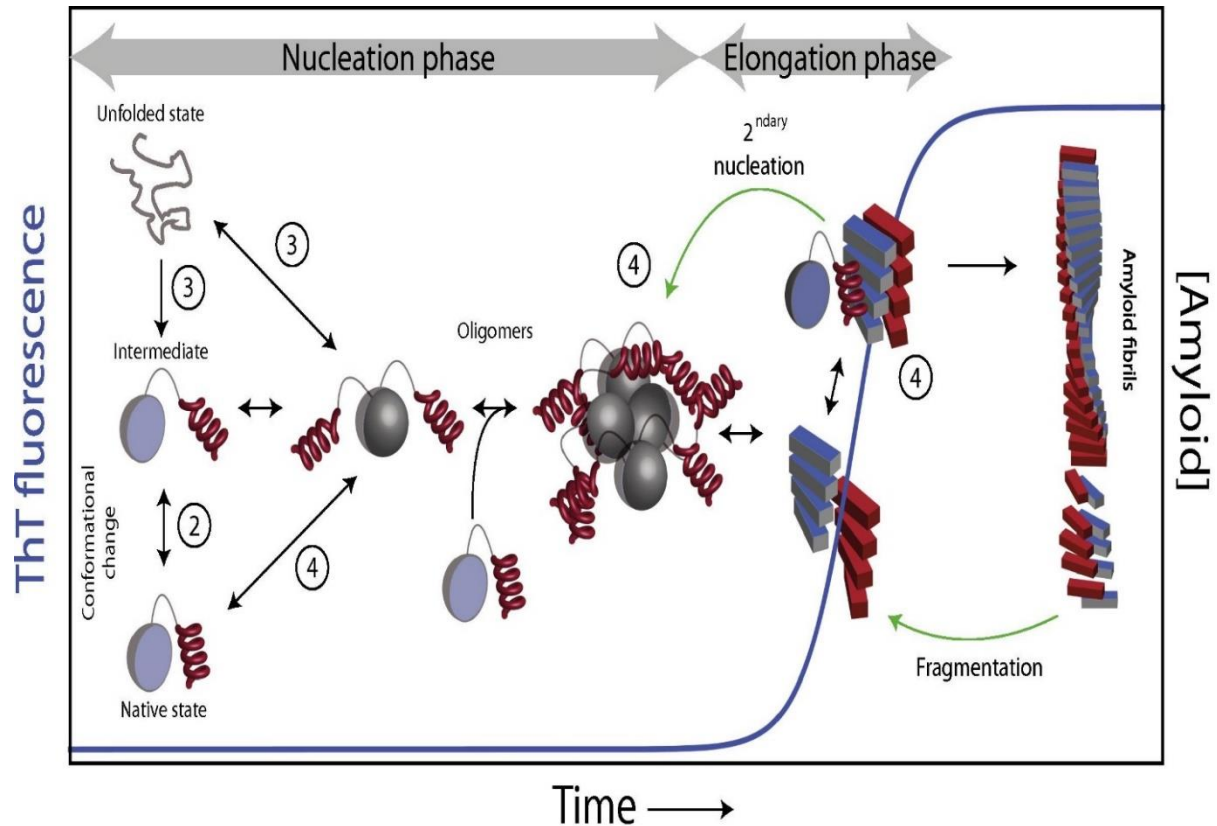


Figure 1.4 | Depiction of the nucleation polymerization pathway for amyloid fibrillogenesis (adapted from reference ³⁴).

At the onset of the fibrillation process, early aggregates are distinguished as an oligomeric protein with a diameter of 2.5–5.0 nm, as confirmed by AFM. After the generation of a critical oligomeric nucleus, protofibrils, displaying the characteristic amyloid structure, start forming. The formation of amyloid fibrils is not the only pathogenic form of amyloid deposition; these species follow different possible pathways. They frequently associate with each other into bead-like chains or small annular rings, acting as precursors for the formation of extremely organized amyloids such as longer protofilaments and eventually generating mature fibrils. Kinetics is extremely fast during fibril elongation phase since the presence of fibrillar templates promotes the quick incorporation of amyloidogenic monomers. This cooperative phase is followed by a plateau, which indicates that all the material in solution displays a fibrillar form. Amyloid aggregates corresponding to each phase of the kinetics can be characterized using different techniques, including Atomic Force Microscopy ^{22,35,36}.

1.4 Parkinson's disease (PD) and related proteins

Parkinson's disease is the second most common neurodegenerative disorder characterized by the loss of dopaminergic neurons in the brain cells ³⁷. The major symptoms include muscle rigidity, tremor at rest, abnormal speech, and gait ³⁸. The symptoms can be relieved, but the disease itself cannot be completely cured. In the Parkinson's disease, the affected neurons are mostly located in the substantia nigra ³⁹. Presynaptic terminals release dopamine, a neurotransmitter that signals the part of the brain that controls movement and coordination, from compartments known as synaptic vesicles. Although the exact cause has not well understood yet, it has been observed

that there is a close link between Parkinson's disease and a protein called alpha-synuclein (α -syn)

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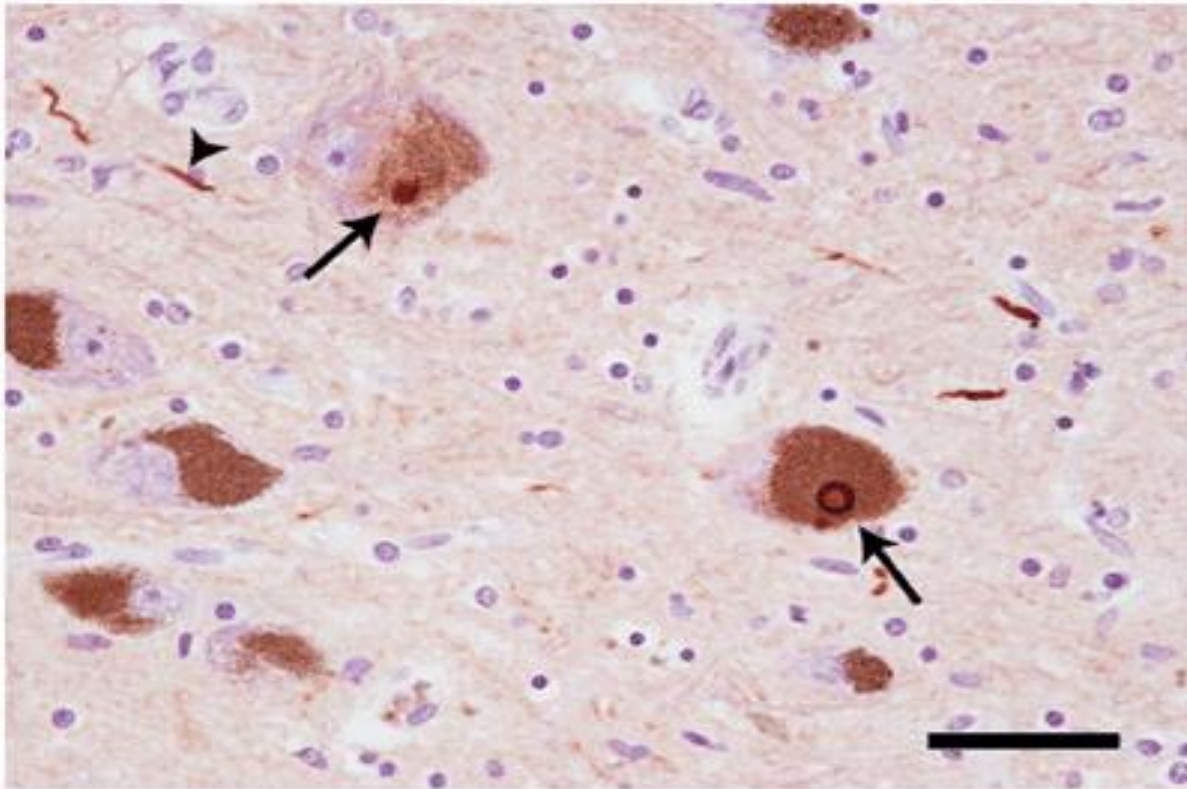


Figure 1.5 | the image represents Lewy bodies and Lewy neurites, stained with a polyclonal antibody directed against α -synuclein amino acids 126–135, in substantia nigra of a PD patient (20 $\times$ magnification). The arrows point toward Lewy bodies, and the arrowhead points toward a Lewy neurite. Scale bar: 50 μ m. Photo: Leire Almandoz Gil, Uppsala University.

“Synucleinopathies” is a very broad term, associated with neurodegenerative diseases where α -syn plays a key role, have no known cure but incorporate diseases such as Lewy bodies dementia, multiple system atrophies, and Parkinson's disease ⁴¹. Although many of these diseases are associated with different genetic lesions, the hallmark sign that confirms the disease, formation of Lewy bodies and Lewy Neurites, is common to all the synucleopathies ³⁹ (Fig 1.5). The name synuclein points at the fact that antibodies for protein label both synapses and nuclei. α -syn is a small protein that is abundant in the human brain, found mainly at the tips of nerve cells (neurons)

in specialized structures called presynaptic terminals and interacts with phospholipids and proteins⁴². Although its function has not been elucidated completely, it is thought to be involved in different processes⁴³, including synaptic vesicles trafficking, chaperone activity for neurotransmitter release⁴⁴, and it has been proposed to promote dilation of the exocytotic fusion pore⁴⁵. Aggregation of α -syn into cross- β fibrillar structures is related to neurodegenerative disorders⁴⁶. Through hydrophobic interactions and covalent modifications, including truncations, nitration, and phosphorylation, the monomers can aggregate and form dimers which is stabilized by cross- β structures. α -syn can also form ring-like oligomers structures in vitro which can interact with the membrane, allowing abnormal calcium influx leading to cell death⁴⁷. These misfolded structures are toxic and affect the function of mitochondria and the endoplasmic reticulum causing neurodegeneration⁴⁸.

1.4.1 Alpha-synuclein (α -syn) structure

The human α -syn protein is a sequence of 140 amino acids that are subdivided into three partially overlapping regions: N-terminal lipid-binding region, non-amyloid β component, and carboxyl C-terminal⁴⁹ (Fig 1.6). α -syn can be present as an α -helix structure in association with phospholipids (lipid-binding region) or an unfolded conformation in the cytosol, suggesting that it plays specific roles in different cellular locations based on its dynamic structure⁵⁰. The N terminus is a positively charged domain, including residues 1-60, and it is rich in lysine. It also contains a consensus sequence of KTKEGV, imperfectly repeated throughout the sequence, enabling the formation of an amphipathic helix upon interaction with lipid membranes⁵¹⁻⁵³. It has been object of debate whether the main physiological soluble form of α -syn is a tetramer, stabilized by KTKEGV repeat motifs^{54,55}, or a monomer⁵⁶, but there is consensus on the involvement of the

monomer in the early steps of pathological states. Both random coil and α -helix conformations of the α -syn depend on membrane curvature ⁵⁷. In addition, in the random coil conformation, a dynamic equilibrium has been shown to occur between a helix formed by the whole segment and a short helix, rigidly bound to the membrane and comprising only the first 25 residues.

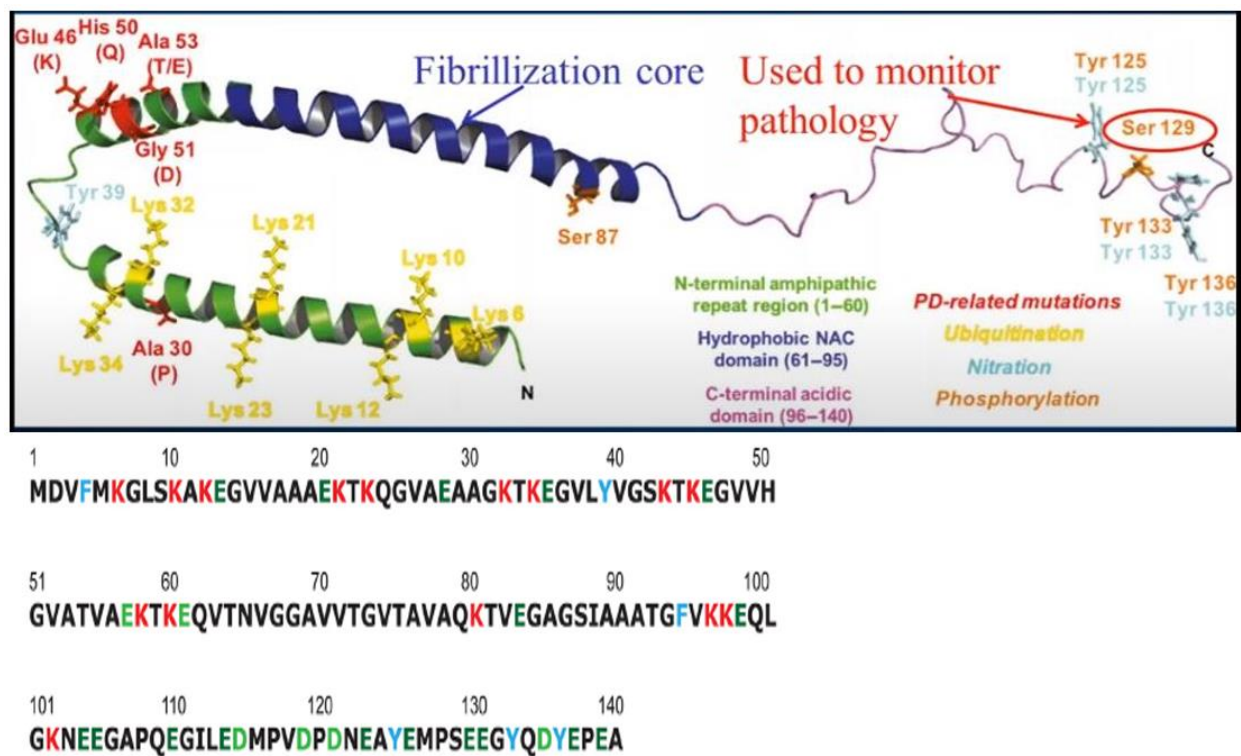


Figure 1.6 | The primary and secondary structure of α -synuclein. (Image modified from ⁵⁸).

The rest of the N-terminal region in an unstructured conformation weakly interacts with the membrane ⁵⁹. Pathologic mutations of α -syn (A30P, E46K, and A53T) associated with the early development of PD are all located in the N-terminal region, suggesting a central role of α -syn-membrane interaction in Parkinson's disease ⁶⁰. The central NAC domain (originally identified as the non-A β component of Alzheimer's amyloid plaques) is a hydrophobic domain composed of residues 61-95, and it is involved in protein aggregation with the formation of cross- β structure ^{61,62}. The hydrophobicity is what allows the protein to self-associate and aggregate into

cross β structures. It is also found that deletions or mutations within this motif would decrease oligomerization and fibrogenesis in vitro. The rest of the protein residues is in the disordered C terminus. This domain is highly acidic and negatively charged. The C-terminal domain of α -syn is present in a random coil structure due to its low hydrophobicity and high net negative charge ⁶³. One important residue to point out in this domain is serine129 which can be phosphorylated by POK2 (polo-like kinase2). Studies have shown that antibodies against α -syn phosphorylated at serine129 are indicators of Lewy pathologies ⁶⁴. Phosphorylation alters the hydrophobicity and further increases the chances of aggregation. This may be a causal event for the deposition of α -syn, given the fact that 90% of α -syn deposited in Lewy bodies consists of phosphorylated serine. Although not fully proven, it is suggested that the C terminus is involved in regulating nuclear localization of the protein and its interactions with other proteins, small molecules, and metal ions ^{43,65}.

1.4.2 α -syn cytotoxicity

Many in vitro and in vivo experimental models mimicking α -syn pathology such as oligomerization, toxicity and more recently neuronal propagation have been generated over the years. In particular, cellular models have been crucial for deep comprehension of the pathogenic process of the disease and are beneficial for screening of molecules capable of modulating α -syn toxicity. The ability of α -syn to bind phospholipid membranes and fatty acids has been well documented and is presumably necessary for the protein's function in regulating synaptic vesicle trafficking ^{51,66,67}. The toxicity of α -syn is likely related to its interaction with the asymmetric neuronal membrane. Within this scenario, pathological states may result from alterations of protein folding as well as imbalance of cellular lipid homeostasis, the two factors being closely

interconnected. The increased propensity of α -syn to undergo aggregation in the presence of membranes versus in solution could be because the two-dimensional surface of the lipid bilayer increases the probability of molecular interactions needed for oligomerization^{68,69}. It has also been observed that α -syn exhibits an enhanced propensity to form high molecular weight aggregates in the presence of polyunsaturated fatty acids^{70,71}. Membrane-induced α -syn aggregation may play a role in neurodegeneration by triggering membrane thinning, a process that could result in increased ion permeability^{49,53,72,73}. It has been shown that α -syn interacts more favourably with negatively charged lipids, and the key role of electrostatics has been demonstrated by modulating the interaction with different ionic strengths or competing charges⁷⁴. At low lipid/protein ratios, negatively charged membranes enhance the aggregation rate of α -syn by favouring protein recruitment at the membrane surface, providing nucleation sites. Increasing the lipid/protein ratio usually inhibits α -syn aggregation due to a reduction of the amount of free monomer available for aggregation, as the protein is mainly bound to the membrane in alpha-helical state⁷⁵. However, some cases in which aggregation is enhanced at high lipid/protein ratios have also been reported⁵², and it has been suggested that aggregates can form on the membrane and then dissociate from the membrane surface⁷⁶. I will describe more in detail the interplay between amyloidogenic α -syn and cell membrane in chapter 4.

Chapter 2

Cell membrane

The biological plasma membrane is a flexible, semi-permeable membrane that surrounds all cells, forming a barrier between the intracellular fluid inside the cell and extracellular fluid outside the cell ⁷⁷ (Fig 2.1). It is a complex structure involved in important biological processes, including bidirectional transport of molecules and cell-cell communication. The main structure of the plasma membrane is the lipid bilayer, a thin (5-10 nm) membrane made of a double layer of amphiphilic lipid molecules, with the polar parts pointing toward the aqueous medium and the hydrophobic moieties facing each other. Proteins, and carbohydrates, in a ratio which can be vary significantly different among different cell types, are other important components of the membrane ⁷⁸.

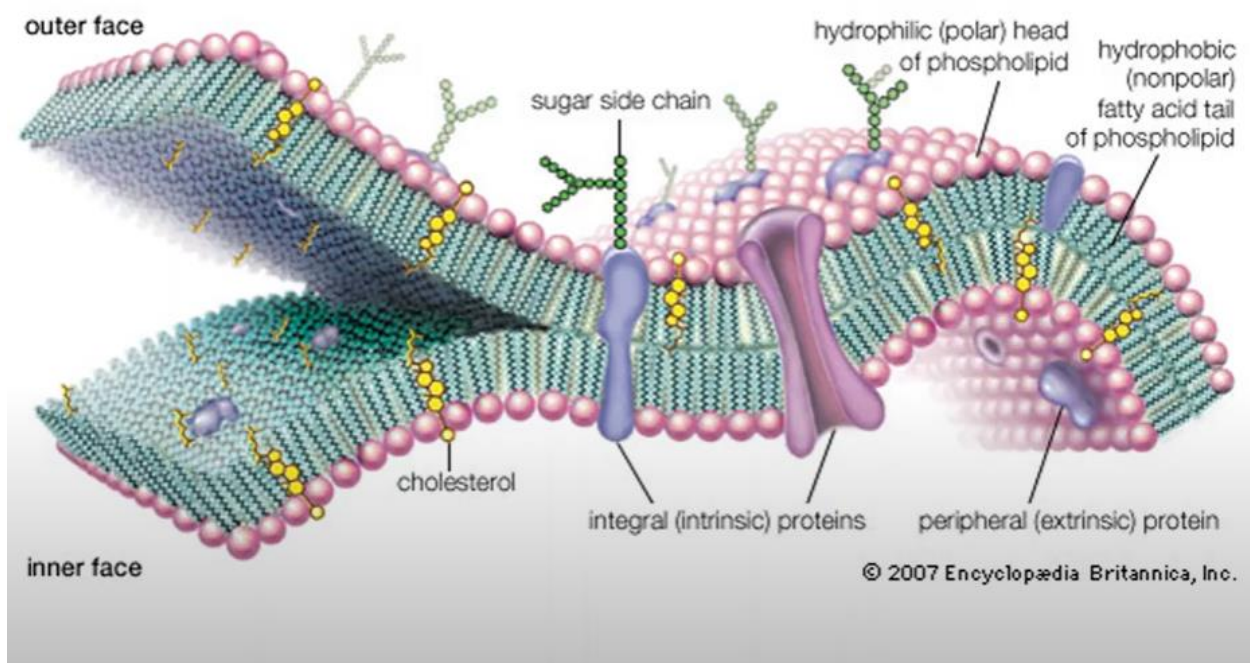


Figure 2.1 | Schematic representation of plasma membrane (from *Encyclopædia Britannica, Inc.* 2007).

Membrane proteins can be classed as peripheral, or integral. Peripheral proteins sit on the inner or outer surface of the plasma membrane, attached to the polar heads of the phospholipids. Integral proteins span the whole plasma membrane and are firmly anchored between the fatty acid tails arranged in an α -helix. They are key to another property of the plasma membrane, its permeability. The transport of ions and large uncharged polar molecules is facilitated by integral proteins such as selective ion channel proteins, carrier proteins, and receptor proteins which can be passive or active, depending on whether they require cellular energy. This selective permeability allows the plasma membrane to regulate what enters and exits the cell. Moreover, the fluidity of the membrane is vital for providing structural support to the cell and permitting movement. Membrane fluidity is what allows the insertion of membrane proteins within the lipid bilayer. In 1972, Singer and Nicolson suggested the fluid mosaic model of the membrane structure based on thermodynamic principles of organization of the membrane lipids and proteins ⁷⁹. The main drawback of this model was that it did not consider an asymmetry and lateral mobility of proteins or lipids within the membrane matrix. However, further works overcame the drawbacks of the fluid mosaic model and led to the introduction of phase-separated lipid domains, with a mean size between 10 - 200 nm, enriched in sphingolipids and sterols, called lipid rafts ⁸⁰⁻⁸². Lipid rafts are strongly related to the high heterogeneity of membrane lipids, which can coexist in different lipid phases, ordered and disordered liquid phases known as L_o and L_d , respectively. They differ in composition and physical properties from the surrounding regions and are thought to have an important role in many cellular processes, such as cell signaling and protein trafficking ⁸³⁻⁸⁶ (Fig 2.2).

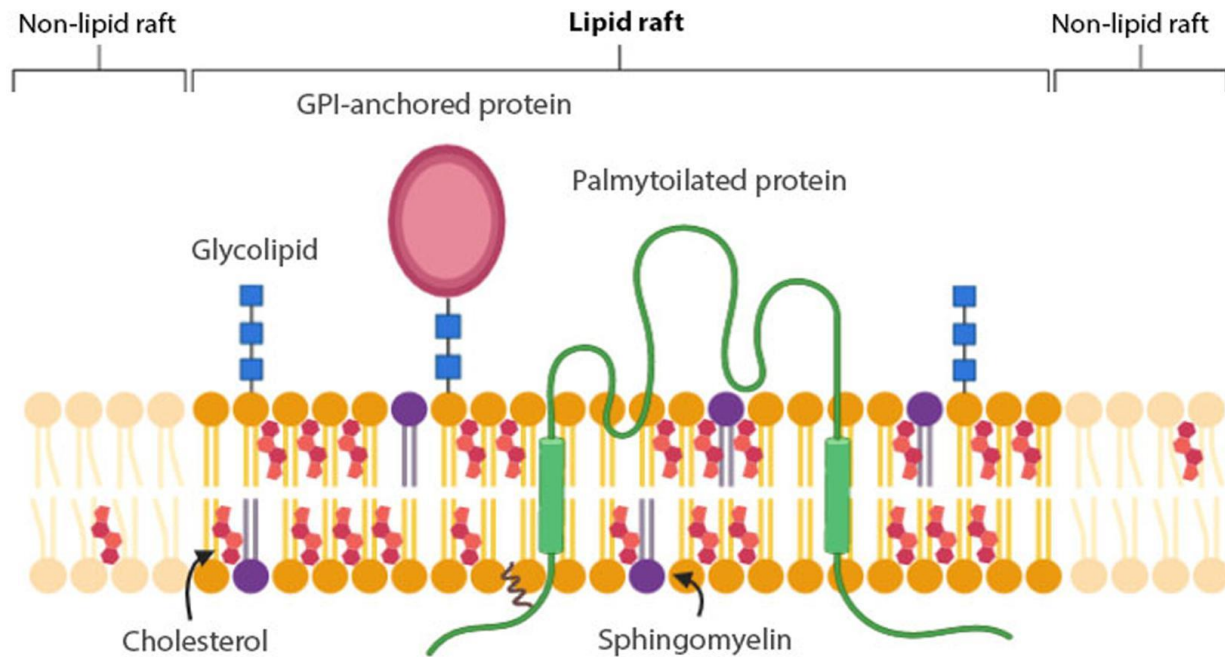


Figure 2.2 | Membrane raft structure. Lipid rafts are composed of cholesterol, saturated phospholipids, and sphingolipids, such as glycolipids and sphingomyelin (SM). GPI-anchored proteins and lipidated – especially palmitoylated- proteins have a higher affinity for lipid rafts than non-lipid rafts (Image from ⁸⁷)

Although the concept of lipid rafts was proposed more than twenty years ago ^{83,88,89}, a direct observation of these structures in the plasma membrane is still missing, likely due to the challenging technological issues imposed by their small size ⁸¹, and to their possible transient nature. Indirect evidences of the presence of distinct lipid phase domains were derived from single particle tracking experiments ⁹⁰⁻⁹³ and from the evidence of lipid rafts resistance to non-ionic detergents such as Triton X-100 or BRE98, when used at low temperatures, provided the hypothesis that the lipid rafts can be extracted from the plasma membrane ^{89,94-96}. When such a detergent is added to the cells, the fluid membrane will dissolve while the lipid rafts may remain intact and could be extracted (Fig 2.3). However, the validity of the detergent resistant methodology of membranes still is an open question due to complex behavior of the lipids and proteins within the membrane. Multi-component model systems can thus be used to reproduce raft behavior in a scale which is suitable for most of *in-vitro* investigation techniques.

Simplified model membranes with lipid compositions that mimic the cell membrane have shown that there is partition of different lipid species. The lipids in the ordered domains have a reduced mobility. Ordered domains are thicker and stiffer compared to the disordered lipid phase. Work in this direction has been performed using atomic force microscopy ^{97,98} and a combination of AFM and fluorescent correlation spectroscopy ⁹⁹.

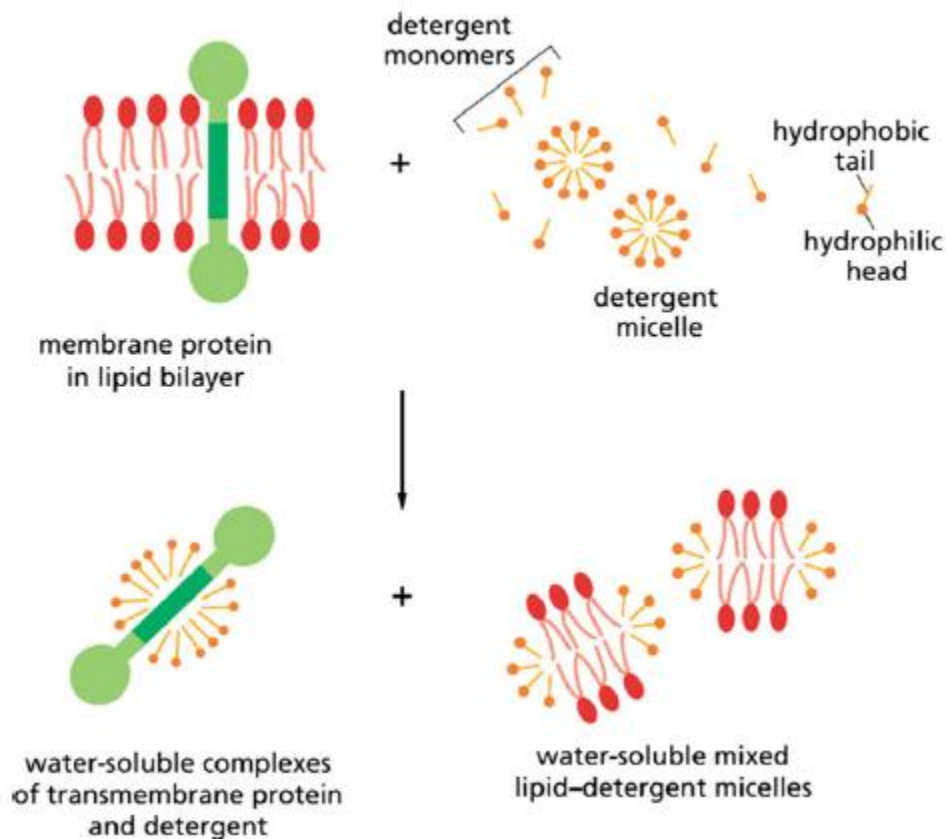


Figure 2.3 | A mild detergent such as Triton X-100 can solubilize membrane proteins. The detergent molecules (gold) are shown as monomers and micelles, the form in which detergent molecules tend to aggregate in water. The detergent disrupts the lipid bilayer and brings the proteins into solution as protein–detergent complexes. As illustrated, the phospholipids in the membrane are also solubilized by the detergents, forming lipid detergent micelles. (Image from ⁷⁷)

2.1 Membrane lipids

Membrane lipids are amphipathic molecules, i.e., they consist of a hydrophilic (“water-loving”) head and a hydrophobic (“water-fearing”) tail. They diffuse freely in cell membrane and form a bilayer framework structure with their hydrophobic nonpolar tails buried in the interior and their hydrophilic heads exposed to water (Fig 2.4).

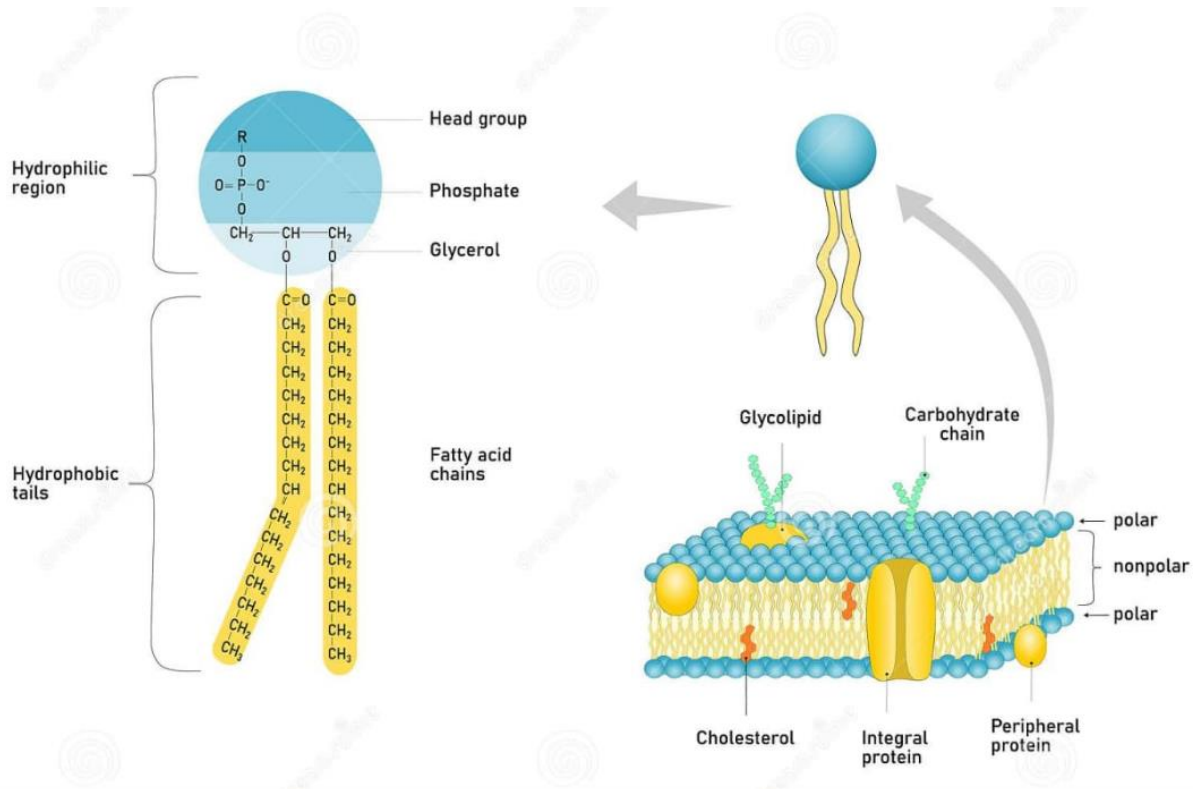


Figure 2.4 | The typical structure of a phospholipid molecule consists of a phosphate-containing hydrophilic head group linked to a pair of hydrophobic tails. Due to their amphipathic nature, lipid molecules spontaneously form lipid bilayers, that are at the base of biological membranes. (Image from dreamstime.com)

Membrane lipids can be divided into three groups based on their chemical structure: phospholipids, ceramide-based sphingolipids, and sterols⁸⁸ (Fig 2.5). The major structural lipids in plasma membrane are glycerophospholipids. The glycerophospholipids are composed of two fatty acids, esterified with two carbon atoms of glycerol while third carbon of glycerol is bound to the

phosphate group. The phosphate group is negatively charged at neutral PH. The phospholipids such as glycerophospholipids and some sphingolipids consist of phosphate aid joined through phosphodiester linkage with the two saturated or *cis*-unsaturated fatty acids, typically containing 16-18 carbons. A polar head, which may be choline, ethanolamine, serine, or inositol, is also bound to glycerol through a phospho-ester bond. Sphingolipids represents the other class of structural lipids, acting as recognition sites in plasma membrane. They consist in a ceramide backbone (a sphingosine linked to a fatty acid), which may bind different headgroups. Sphingolipids are divided into three classes based on their polar head groups as: sphingomyelin (SM), neutral or uncharged glycosphingolipids, and ganglioside. The main sphingolipids in mammal membranes are sphingomyelin (SM), a phosphosphingolipid composed by ceramide and phosphocholine. Since they consist of phosphatidylcholine (PC) or phosphatidylethanolamine (PE) as their head group, sphingomyelins are classified as phospholipids. Sphingomyelins lack net charge on their head group. Glycosphingolipids, also known as neutral glycolipids, are characterized by a mono- or oligosaccharide headgroup, which is linked to sphingosine through a glycosidic bond. Gangliosides are the sphingolipids that contain complex oligosaccharides as their polar head groups and one or more sialic acids, such as N-acetylneuraminic acid. The sialic acid gives negative charge to gangliosides at the neutral PH. Glycerophospholipids and sphingolipids are amphiphilic molecules, consisting both of a hydrophobic part (diacylglycerol / ceramide) and a hydrophilic one (polar headgroup).

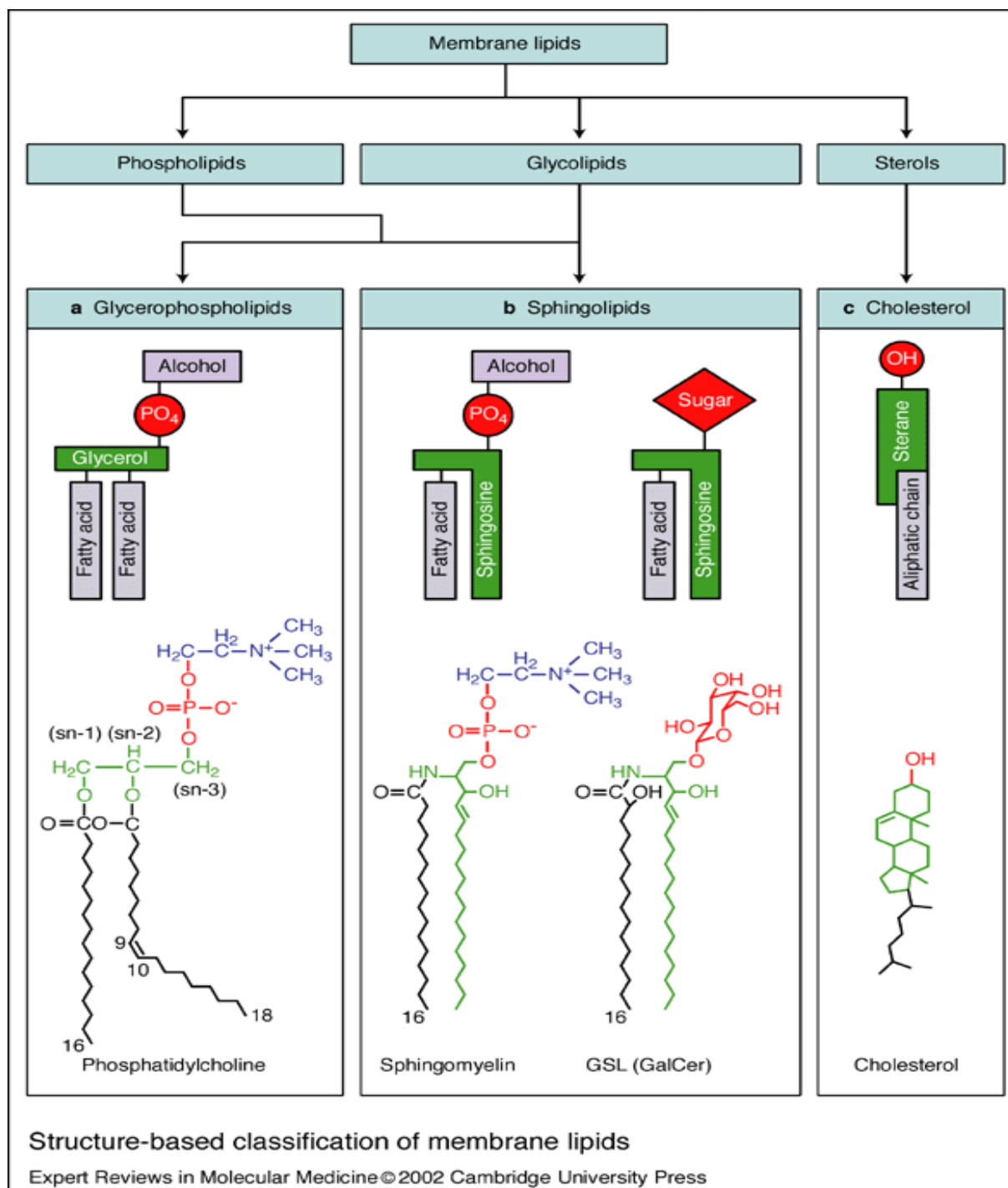


Figure 2.5 | Four major plasma membrane lipids.

The last class of the membrane lipids are sterols. Cholesterol consists of four linked hydrocarbon rings, forming the steroid portion of the molecule and a small polar part (an OH group), generally considered as a non-polar lipid in mammal plasma membranes. According to the umbrella

model ^{100,101}, each cholesterol molecule orients itself in the lipid bilayer so that its single hydroxyl group is close to the polar head group of a neighboring phospholipid molecule. This hydroxyl group forms a hydrogen bond with the oxygen of the ester bond between the glycerol backbone and a fatty acid. The hydrophobic steroid rings and hydrocarbon chain portions of the cholesterol molecule are found embedded in the hydrophobic portion of the cell membrane with the phospholipid fatty acid tails.

2.2 Membrane fluidity

The fluidity of the plasma membrane is dependent on two factors: the composition of lipids in the bilayer and the amount of cholesterol present. Due to constantly diffusion of specific lipids from one mono layer to the other, lipids are not uniformly distributed between the two membrane leaflets ^{88,102,103}: phosphatidylcholine (PC), which accounts for ~ 40–50% of the total phospholipids found in membranes, is mainly located in the outer leaflet, as well as sphingolipids, while phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol are predominantly found in the inner leaflet and comprise only a small percentage of the overall membrane composition in a cell. Cholesterol resides for more than 70% in the inner leaflet ¹⁰⁴. Nevertheless, its presence and distribution in the outer leaflet is fundamental in determining lipid lateral organization (Fig 2.6). Membrane fluidity is determined by the relative movement of phospholipid molecules within the membrane. The relative movement of the molecules within the membrane is a property of the strength of the intermolecular interactions between the lipid molecules, characterized by a phase transition temperature (T_m). As the temperature is increased, the kinetic energy of the lipids increases. As a result, the intermolecular bonds, holding the lipids, can no longer maintain a packed and well-ordered structure. The collapse of the well-defined structure leads to a less ordered and more fluid membrane. A membrane with strong intermolecular interactions will have a higher transition temperature than a membrane with weaker ones. The strength of intermolecular bonds and therefore the fluidity depends on the length and the degree of unsaturation

(the number of cis double bonds) in fatty acid chains, as well as concentration of cholesterol ^{100,105} (Fig 2.6). Longer fatty acids can form more London dispersion bonds than shorter ones. Therefore, the presence of longer fatty acids decreases the fluidity and increases T_m . Saturated fatty acids create a well-structured arrangement of hydrocarbons parallel to each other. The straight hydrocarbon chains can form stronger intermolecular bonds with the nearby fatty acids, favoring rigidity and raising T_m . Lipids, containing long saturated fatty acid chains, tend to organize in a tightly packed lamellar configuration, called liquid-ordered phase (L_o), characterized by a high molecular order and a reduced lateral mobility. A cis double bond in the hydrocarbon chain creates a kink (bend) in the chain that interferes with the well-ordered structure, favoring membrane fluidity and decreasing the transition temperature.

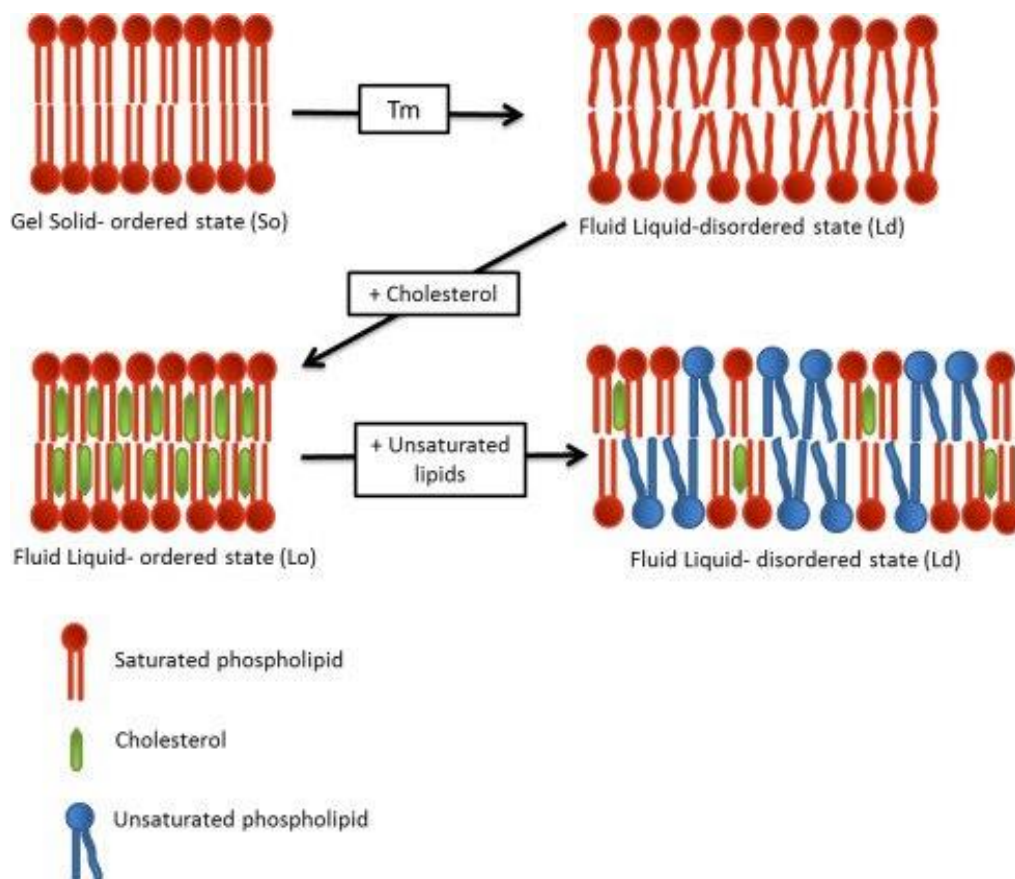


Figure 2.6 | Different lipid phases of glycerophospholipids and sphingolipids in presence and absence of cholesterol. Addition of cholesterol induces a transition from S_o phase and L_d phase into L_o phase. (Image from ¹⁰⁶)

Most of the glycerophospholipids containing unsaturated fatty acid chains, characterized by the presence of double bond in the hydrocarbon chains, exist in liquid disordered phase (L_d) at room temperature, characterized by high lateral mobility. Phospholipids can form a solid-ordered or gel phase (S_o) only below a specific transition temperature (T_m) and depending on cholesterol content of the considered mixtures, which, in presence of unsaturated chains, is significantly lower than the physiological one and is not enough to trigger the full transition into liquid ordered phase L_o . Cholesterol content interferes with the regular interactions of the fatty acids. However, cholesterol seems to form complexes with glycosphingolipids in the form of lipid rafts. In fact, its rigid structure imposes a conformational order between unsaturated phospholipids, affecting in a minor way their lateral mobility, resulting in rigidity and resistance of the membrane to the transition phase. On the other hand, cholesterol insertion between fully saturated chains strongly increases bilayer fluidity, without significantly perturbing the conformational order.

2.3 Model membranes

Extracting meaningful data from experiments on whole cells in their native physiological condition is challenging due to the complexity of cell membranes. Model membranes are multicomponent artificial lipid bilayers for mimicking the cellular membrane compositions to gain insight into the properties and functions of different components, physiological, and pathological phenomena happening at the nanoscale at cell interface using surface characterization techniques^{107–109}. In an aqueous environment, the amphipathic character of lipids causes them to self-assemble into micelles, liposome vesicles, and planar bilayers, depending on their molecular shape and chemical composition (Fig 2.7). For example, lysolipids which have one acyl chain per headgroup form micelles, whereas phospholipids with two acyl chains form planar bilayers or

liposome vesicles. The most well-known and common model membrane systems to mimic the behavior of these assemblies are lipid monolayers, lipid vesicles, and supported lipid bilayers (SLBs). In this section, I describe in detail the lipid vesicles and supported lipid bilayers used in this work.

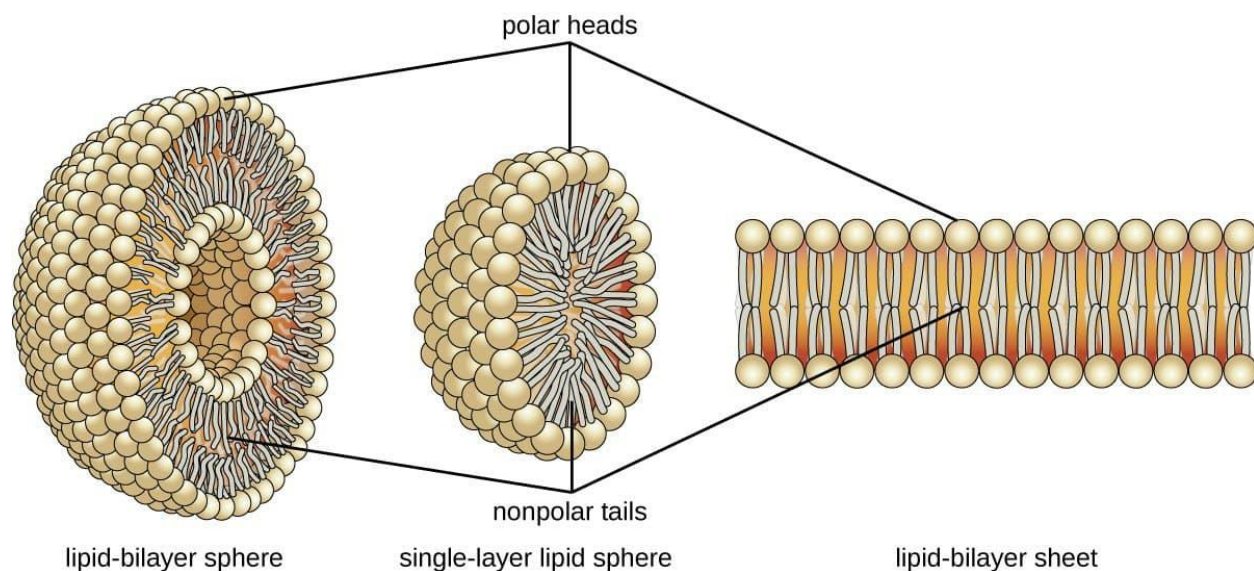


Figure 2.7 | Phospholipids tend to arrange themselves in aqueous solution forming liposomes, micelles, or lipid bilayer sheets. (Modification of work by Mariana Ruiz Villarreal)

2.3.1 Lipid vesicles

A mixture of different phospholipids dissolved in suspended in water, to attain an energetically favorable structure, arrange themselves in a double layer of phospholipids. The hydrophobic effect is the major driving force in membrane formation. The interaction of the hydrocarbon tails release water molecules from the non-polar regions, which is energetically favorable. Since the hydrophobic edges of the membrane bilayer are in contact with water molecules, the bilayer sheet is unstable. Such unfavorable interactions are totally avoided when the bilayer sheets fold on themselves to form closed sealed lipid vesicles, or liposomes. Lipid vesicles or liposomes are spherical lipid bilayers with an internal aqueous compartment. Typically,

lipid vesicles (liposomes) are multilamellar (multilayer) until the application of external mechanical forces (e.g., sonication or extrusion), facilitating the formation of unilamellar vesicles. These vesicles, according to their sizes, are classified into different sizes, ranging from SUVs (small unilamellar vesicles, 20-50 nm in diameter), LUVs (large unilamellar vesicles, 100-500 nm in diameter), and GUVs (giant unilamellar vesicles, 10-100 μm in diameter) ^{110,111}. GUVs are closest in size to actual cells, allowing the use of optical microscopy techniques such as fluorescence or confocal microscopy as well as the micromanipulation of individual vesicles ^{112,113}. SUVs and LUVs are generally prepared through extrusion or sonication of a solution containing multilamellar vesicles (MLVs), which spontaneously form upon hydration of dried lipid films (as described in materials and method section). These model systems can be stable thermodynamically for prolonged periods of time (up to several months), depending on the composition and the size of lipid vesicles as well as on the environmental parameters (temperature, pH, ionic strength, presence of external molecules and ions).

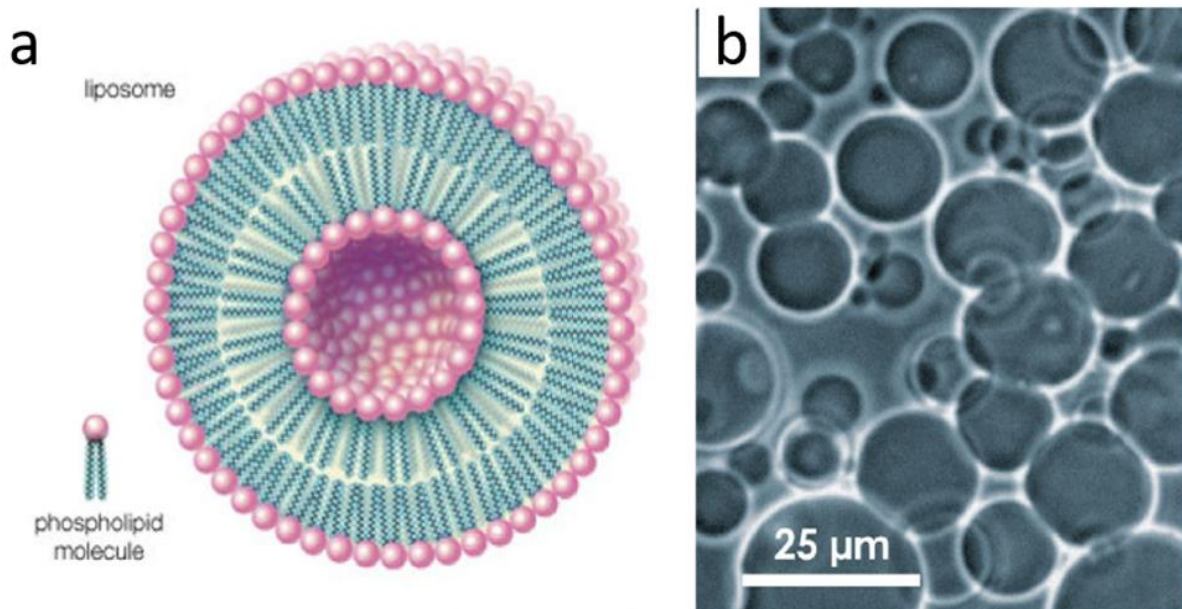


Figure 2.8 | (a) Schematic representation of the liposome [“Liposome,” *Encyclopædia Britannica, Inc.* 2012.], (b) a micrograph of giant unilamellar vesicles GUV [http://www.nanion.de/images/stories/images/p44_1_GUV.jpg].

2.3.2 Supported lipid bilayers (SLBs)

Supported lipid bilayers (SLBs) are stable model membranes, consisting of a flat double layer of phospho/sphingolipids supported on a solid surface such as mica, glass, or silicon oxide wafers, covered with aqueous solution and separated from the substrate by a very thin (around 1 nm) hydration layer^{109,114,114,115}. This model membrane, for the first time, was suggested by Tamm and McConnell in early 1980s¹¹⁶ and has been widely utilized as a model for fundamental studies of phase behavior, the molecular organization of biological membranes, and the molecular interactions of proteins and drugs with cell membranes^{117,118} as well as for developing biosensors^{119–121}. This method is useful for the formation of asymmetric bilayers, and high stability makes it possible to carry out long term experiments. Planar geometry covered with the bilayer is widely used in a large number of studies, involving surface characterization techniques such as ellipsometry, surface plasmon resonance (SPR), QCM-D, total internal reflection fluorescence microscopy (TIRF), and AFM. Also, the SLB is an interesting system for studying cell adhesion on an artificial substrate due to the structural similarity with the cell membrane. During the past decades, many modifications of the basic supported bilayer system have been developed to expand the application area. Among them, one can name polymer-cushioned lipid bilayer, hybrid bilayer, tethered bilayer, suspended lipid bilayer, supported vesicle layer (Fig. 2.9).

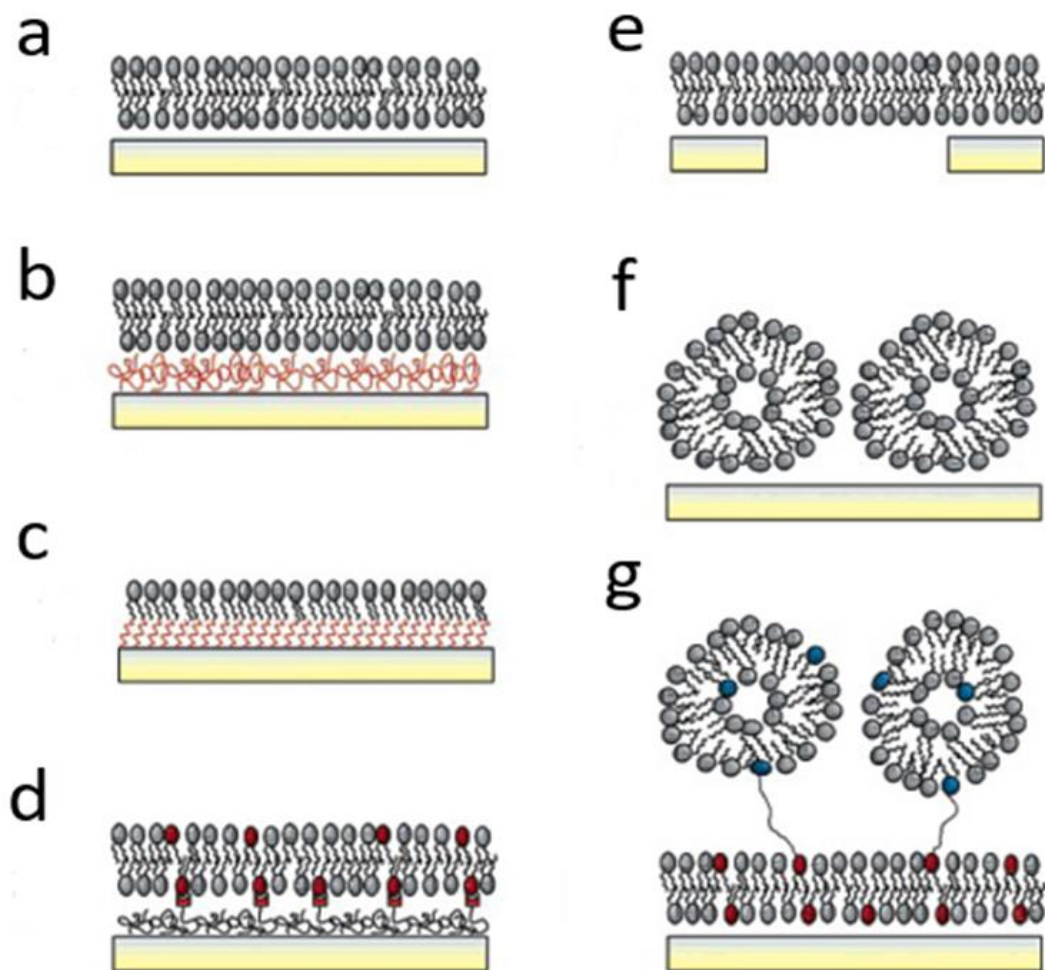


Figure 2.9 | Surface-confined membrane models: (a) solid-supported lipid bilayer; (b) polymer-cushioned lipid bilayer; (c) hybrid bilayer, consisting of a self-assembled monolayer (e.g., thiols on Au or silanes on glass or silica) and a lipid monolayer; (d) tethered lipid bilayer; (e) freely suspended lipid bilayer; (f-g) supported vesicular layers [<https://doi.org/10.1021/la052687c>].

The simplest, effective, and most widely used way of SLBs formation is vesicle deposition: being deposited onto hydrophilic surface, lipid vesicles rupture and fuse with each other, forming lipid bilayer^{114,122}. This method is used in the present work and described in detail in Chapter 4.

Chapter 3

Experimental techniques

In this Chapter, I describe the techniques used in this work. My research activity is based on the application of advanced microscopies to biological systems. The first part of the chapter introduces the modern applications of microscopy in biology. In the following, I will focus on the specific techniques that I employed in my work. I describe the scanning probe principles, with a specific overview on atomic force microscopy (AFM). I introduce optical fluorescence techniques, with a brief introduction to confocal microscopy. Specific attention is focused on the reference of super-resolution microscopy, in particular stimulated emission depletion (STED). Finally, I present a brief overview on correlative microscopy and summarize the progress of correlative AFM-STED microscopy techniques in biological research.

3.1 Microscopy techniques for biological applications

Thanks to the German mechanic Carl Zeiss, the first reliable microscopes were produced in the late 19th century although the field of optical microscopy dates to at least the 17th-century. Optical microscopy is perfectly suited for biological sample investigation since it can work in all the environments, also in physiological conditions, exploiting all its capabilities. It has provided a fundamental contribution to the development of cell biology. The main limit of optical microscopy is related to its resolution. The diffraction of light limits the resolution of optical microscopy. In 1873, Ernst Abbe, first described the diffraction limited resolution of a microscope as the ability to distinguish two objects separated by distance $d = \lambda/2n \sin \theta$, where λ is the wavelength of light,

and the numerical aperture (NA) of the objective lens is defined as the refractive index of the imaging medium (n) multiplied by the sine of the aperture angle θ , $NA = n \sin \theta$ ¹²³. Nowadays, most oil objectives have a maximum NA of 1.25, meaning that the maximum resolution of a light microscope is limited to around 250 nm that is far from the scale at which molecular processes occur and not compatible with the distance between molecules in a crowded environment such as the cell.

In the twentieth century, new techniques able to reach and overcome resolutions at the nanometer scale enriched the microscopy world.

Electron microscopy ¹²⁴, born in 1933, has made significant contributions in various fields of science while remaining limited to the study of biological samples in a specific environment, i.e., in vacuum, a condition that is far from the physiological environment. The scenario changed in 1986, the year in which the first AFM was presented ¹²⁵.

AFM is a whole set of tools capable of doing a comprehensive study of bio-mechanisms, such as the self-assembly of molecules ¹²⁶, or bio-samples properties, such as the morphological and mechanical properties of cells and tissues ^{127,128}. The combination of measuring forces with a high spatial resolution of biological samples without labeling and working near-physiological conditions makes AFM unique ¹²⁹. Despite this wide range of demonstrated abilities, this technique alone does not allow the specific identification of individual elements of a heterogeneous sample due to the lack of chemical specificity. This limitation could be restrictive in the study of crowded molecular environments, such as cells or bio-membrane. Furthermore, the AFM is a surface technique that cannot access the inner region of a three-dimensional sample, such as a cell.

Fluorescent light microscopy is an advanced part of light microscopy that enables the recognition of specific component/components inside a heterogeneous sample with very high

specificity¹³⁰. The photophysical process in a conventional fluorescence microscope is best described by Jablonski diagrams (Fig 3.1). In this technique, a fluorophore is first excited by an excitation light pulse, and then it decays non-radiatively towards a lower metastable state. From this state, spontaneous emission, or fluorescence occurs. The fluorophore again goes back to the ground state, emitting a photon with the same energy of the gap between the two states (spontaneous emission).

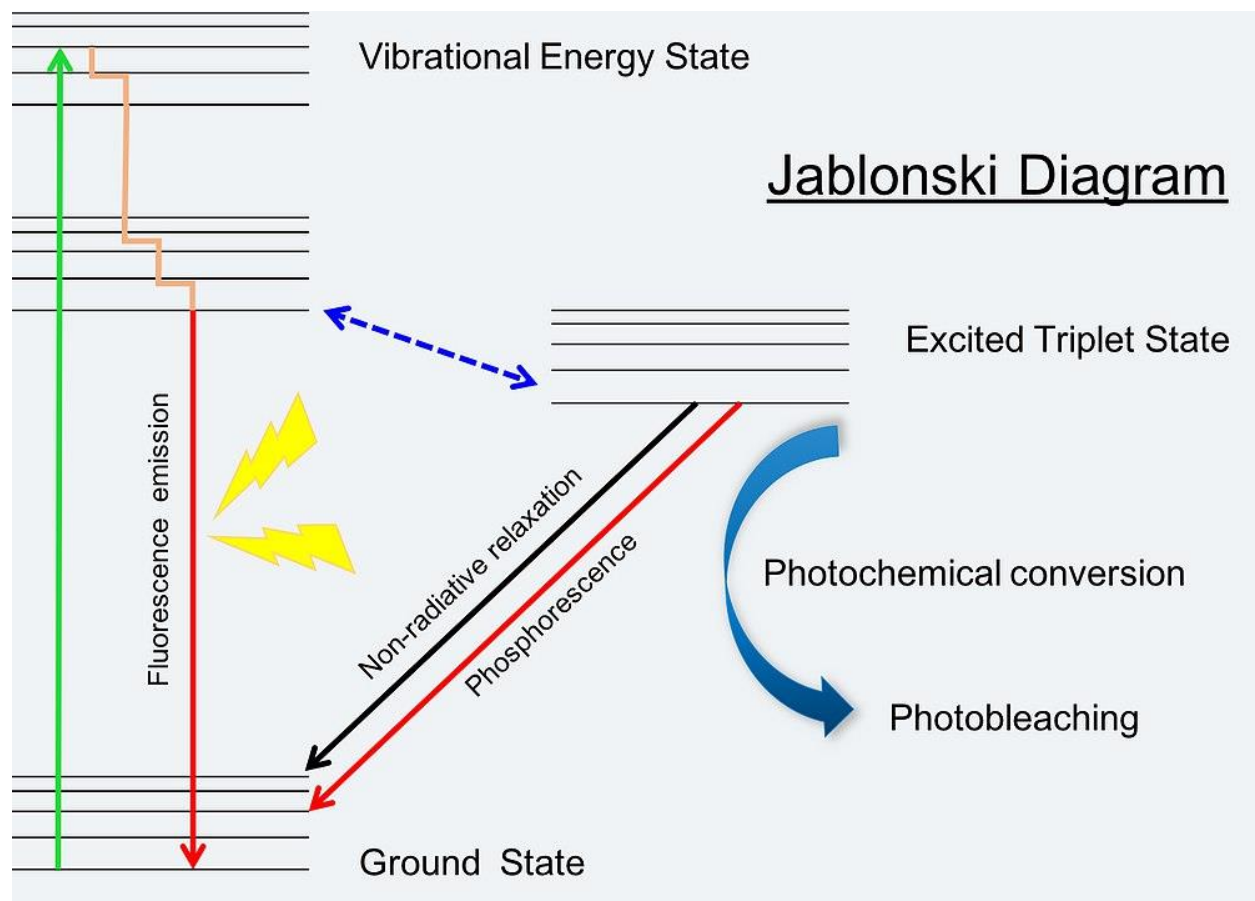


Figure 3.1 | Representative Jablonski energy diagram, describing fluorescence emission in a conventional fluorescence microscope. (Image from [www. aicjanelia.org](http://www.aicjanelia.org))

Fluorescence microscopy resolution is dictated by the diffraction limit, as all the techniques based on the use of light and optics. However, starting from the nineties, different approaches to overcome the resolution limit have been presented. Super-resolution microscopy was suggested in

1994 by Hell and Wichmann that introduced the concept of Stimulated Emission Depletion (STED) microscopy ¹³¹. Super-resolution microscopes resolve details of a biological sample as small as 20 to 30 nanometers, which accounts for an approximately 10-fold improvement in resolution over traditional fluorescence microscopy.

Correlative microscopy is an approach that integrates different imaging modalities in one instrument to provide information that one or the other microscope alone cannot. The complicated relationship between structure and function of biological specimens can be visualized by exploiting correlative information. With this motivation, the first STED-AFM coupled system took shape in 2012 ¹³². Sub-diffraction level of morphological features can be achieved by combination of AFM with a super-resolution microscopy system such as STED, generating a nanoscopic tool with very high versatility. Furthermore, the two techniques can also integrate different information combining them in different ways. The AFM topography, e.g., can be coupled with spectroscopic optical data, the fluorescence imaging can be boosted by the coupling with high-resolution mechanical data.

3.2 Scanning Probe Microscopy

Scanning probe microscopies (SPMs) are the family of instruments used for investigating the topography of surfaces down to atomic resolution. Their working principal is based on the interactions between a sharp probe tip and sample surface due to small separation (10-100 Å) between scanning probe tip and sample surface (Fig 2.2). In these techniques, the sharp probe scans across the surface of the sample, and the image of the surface is obtained by mechanically moving the probe in a raster scan path. The history of SPM starts in 1982 with the invention of the first member of the family, the scanning tunneling microscope (STM) by Gerd Binnig and Heinrich

Rohrer, led to winning the Nobel Prize in 1986 ¹²⁵. This technique is based on the quantum phenomenon of the tunnel effect in which electrons can penetrate potential energy barriers. When the sharp conductive tip gets close enough to the sample (typically 10 Å far), the electrons start tunneling through a barrier gap, and a tunneling current can be established.

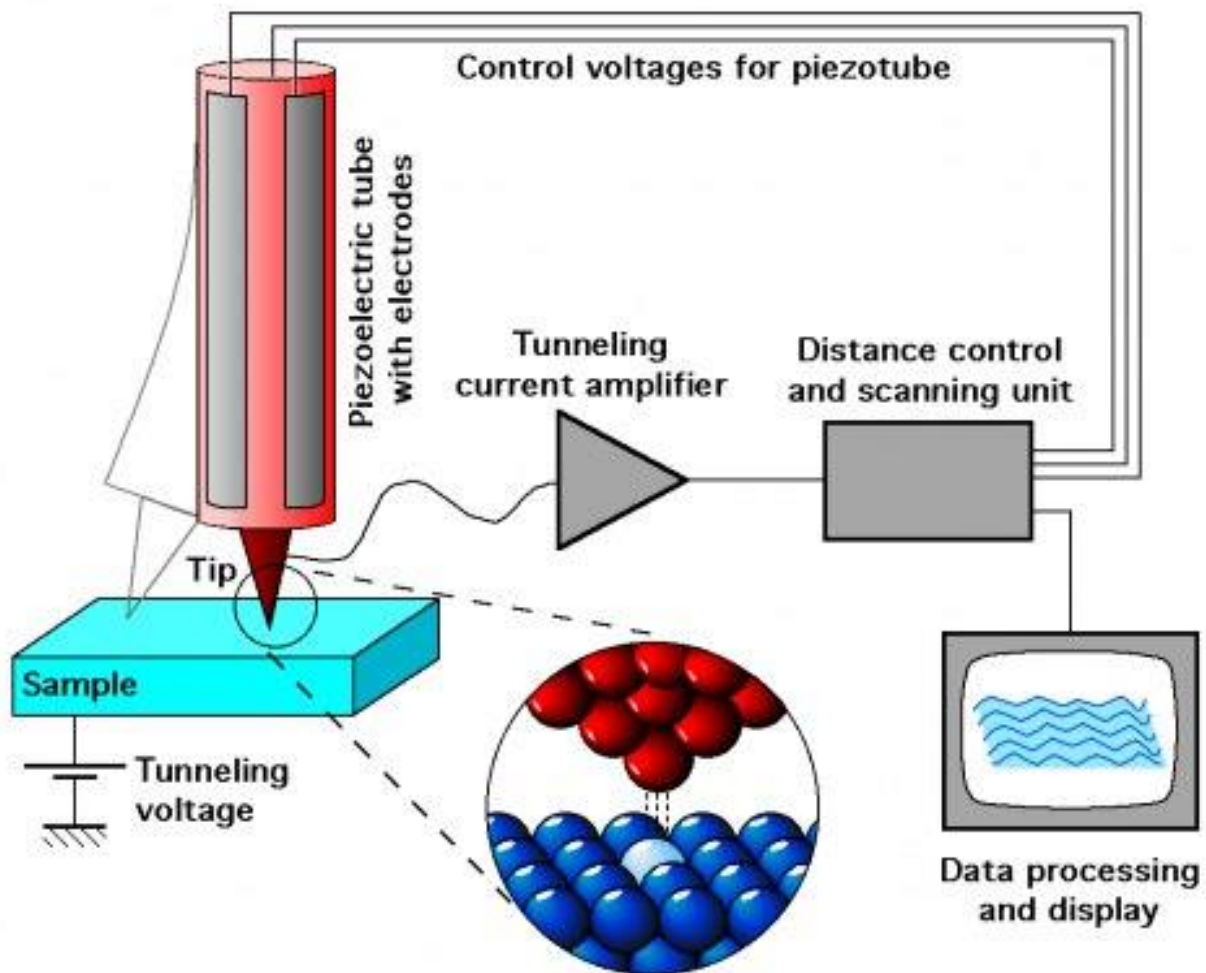


Figure 3.2 | Schematic representation of a scanning probe microscope. the signal used for image reconstruction, which in this case is the tunnelling current in constant height mode or the piezo displacement in constant current mode. (Figure from Michel Schmid, TU Wien)

The tunneling current is directly proportional to the applied voltage between tip and sample and depends exponentially on the gap distance which is the key for the high sensitivity of the STM. The lateral and vertical nanometric movements of the tip is performed by a piezoelectric system

and an electronic feedback control. While scanning the surface, the tip is raised and lowered to keep the tunneling current constant to across the surface, reproducing the surface topography. Although STM exhibits unprecedented lateral and vertical resolution, its application is only limited to imaging of conductive or semiconducting surfaces. AFM was born as natural design to extend STM capabilities to non-conducting materials.

3.3 Atomic force microscopy (AFM)

AFM, born in 1986, in the class of set of scanning probe microscopes, provides a 3D profile of the surface on a nanoscale. The heart of the AFM is in the cantilever/tip assembly that refers to as the AFM probe to measure forces between a sharp tip and surface. The tip, with a typical radius of curvature (ROC) of 10–30 nm, is placed on the end of the cantilever, acting as a spring. This cantilever is about 100–200 μm in length, and the tip scans over the sample surface recording the small force between the tip and the surface. The amount of force between the tip and sample can be described by Hook's law, $F = -k \cdot x$, in which k is spring constant of the cantilever, and x is its vertical deflection. The elastic constant, k , depends on cantilever geometry: short, thick cantilevers present high k values, while long, thin cantilevers are softer. Reference k values are generally reported for commercial probes, but, if we are interested in knowing exactly the applied force, it is advisable to calibrate each cantilever before measuring¹³³. After calibration, force can be easily derived from the measured deflection. The motion of the probe across the surface is provided by piezo-electric based actuators and is controlled using a feedback loop to maintain constant the interaction between the probe and the sample. Commercial AFMs use laser beams to detect the position of the cantilever, and the deflection of the cantilever is measured by a beam bounce method¹³⁴. The basic principle of measurement is that a laser beam bounces off back of

the cantilever (Fig 2.3). As soon as cantilever gets bent by the change in topography, the laser spot will also be reflected on different positions sensitive photodiode detector (PSPD). As the tip is scanned over the sample, the detector measures the bending of the cantilever to monitor both vertical and lateral motions of the cantilever. Then a feedback loop maintains a certain position of the laser on the photodiode by adjusting the cantilever position by applying a voltage, and then voltage to the piezo. The necessary movements of the piezo are monitored for each pixel in the image used to create a topography map.

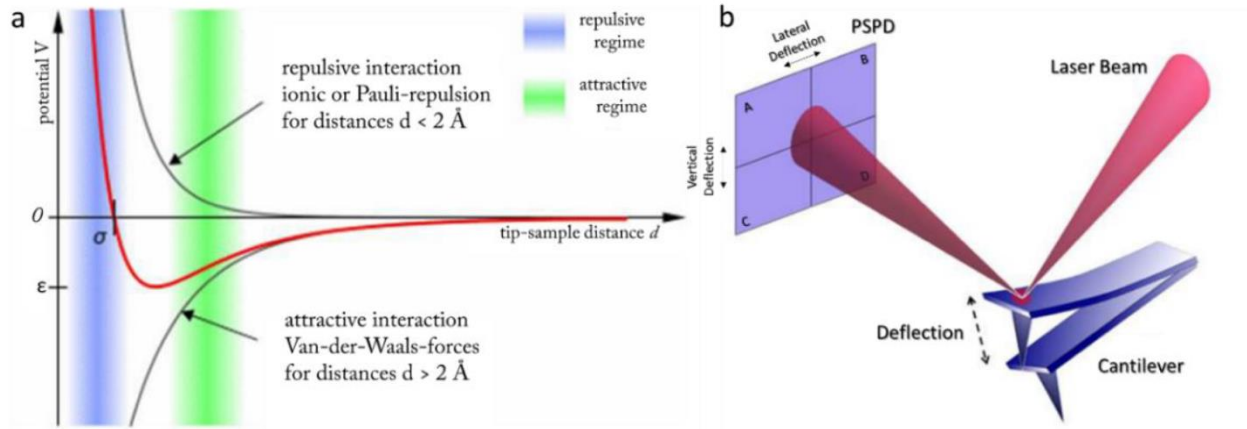


Figure 3.3 | (a) The Lennard-Jones potential (red curve) describes standard tip-sample interaction forces at small distances. (b) The basic working principle of an AFM, which consists of a laser reflecting off the back of a cantilever into a position-sensitive quadrant photodiode. Cantilever deflection results in a shift in laser spot position on PSPD. AFM main signal (vertical deflection) is calculated from $(A+B)-(C+D)$, while frictional torsion (lateral deflection) is $(A+C)-(B+D)$. (Image from <http://www.uni-leipzig.de>)

Depending on the tip-sample distance, we get into different regimes of forces between the tip and the sample. Tip-sample force is composed of the Van der Waals force and the electrostatic and magnetic forces as well as the chemical. The Lennard-Jones potential is a good approximation of the energy of the interaction between probe and sample

$$U_{LJ} = 4\epsilon \left[\left(\frac{\sigma_0}{r} \right)^{12} - \left(\frac{\sigma_0}{r} \right)^6 \right] \quad (3.1)$$

Where σ_0 is the zero potential distance, r the tip-sample distance, and ε the potential well's depth. The dominant interactions at short tip-sample distances in the AFM are Van der Waals interactions, due to the overlapping of electron clouds and to the repulsion between atomic nuclei. However, long-range interactions (i.e., capillary, electrostatic, magnetic) are significant further away from the surface up to 100 nm. There are three primary modes in AFM that can be operated to obtain images: contact mode, intermittent contact (tapping mode AFM), and non-contact mode, which exploit different interaction force regimes (Fig 3.4).

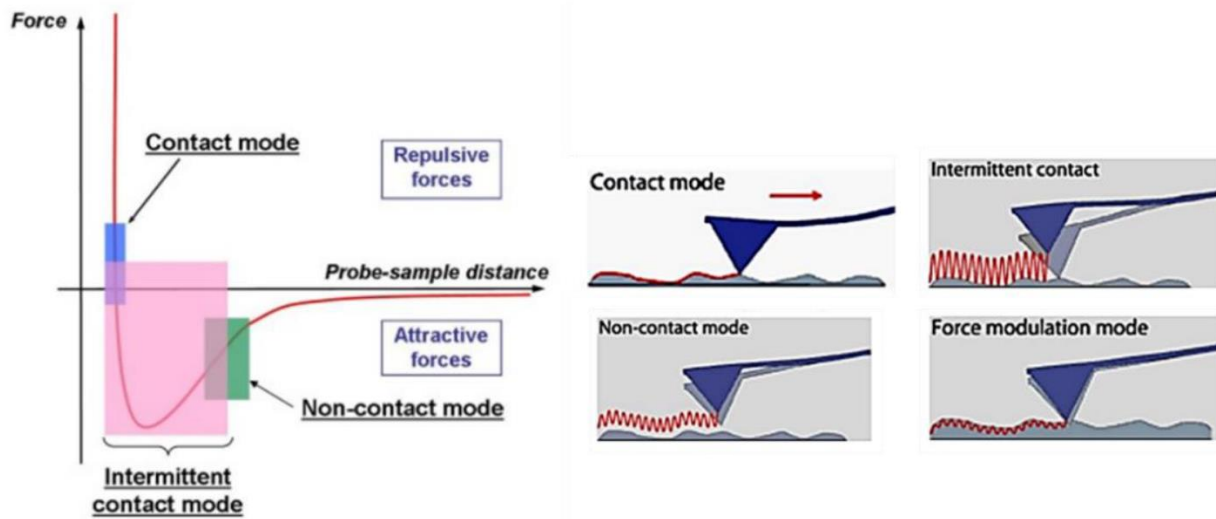


Figure 3.4 | Different AFM imaging modes (modified from AFM Handbook JPK Instruments NanoWizard, 2012)

3.3.1 AFM Imaging modes

Contact mode

In this mode, the cantilever is held at a distance less than 0.5 nm from the surface of the sample. Before starting, a force setpoint is chosen, generally low, to reduce the interaction with the sample. The laser bounces off the back of the cantilever and the probe drags across the sample

with its tip in contact with the sample surface. During contact with the surface, the probe predominately experiences repulsive Van der Waals forces, and that leads to the tip detection. In this repulsive Van der Waals regime, the spring constant of cantilever is less than the stiffness of the surface so that the cantilever bends, and the force on the tip is repulsive. In this mode, the cantilever deflection is the feedback parameter, i.e., the position of the laser on the photodetector is displayed as the photodiode voltage which is maintained constant during imaging. The piezo actuator provides the vertical displacement of the probe to maintain the deflection constant. The sample topography image is obtained by storing the signal sent by the feedback electronic in each point to move the scanner up and down to follow the surface morphology, i.e., to maintain the deflection constant. The advantages of this mode are fast scanning, high resolution. The main drawback is related to the presence of orthogonal and lateral stresses applied on the sample during scan. Because of this interaction, the tip can damage or displace the sample, and even the AFM probe can blur or collect contaminant on its surface. In this mode, because the tip is constantly touching the surface of the sample, it is usually done on samples that have relatively hard surfaces. That leads to one of the main disadvantages of contact mode AFM so that the tip can damage or deform the sample if the sample is too soft. In such cases, it is advantageous to work in tapping mode.

Intermittent contact mode

In intermittent contact mode, also known as tapping or AC mode, the cantilever is oscillated vertically close to its resonance frequency, with oscillation amplitudes typically hundreds kHz in air, and tens of kHz in liquid. During scanning, the probe lightly taps on the sample surface contacting the surface at the bottom swing of the cantilever vibration. In this case,

the feedback is based on the amplitude of oscillation which is maximum when the tip does not touch the sample and reduced when the tip interacts with the surface. Therefore, intermittent contact involves both repulsive and attractive regime. By maintaining a constant oscillation amplitude, a constant tip-sample interaction is maintained, and an image of the surface is acquired from the vertical signal of the feedback circuit, as in the case of contact mode. Therefore, the main benefit of tapping mode is the capability to minimize friction or shear forces. Intermittent contact mode allows high resolution imaging on samples that are easily damaged, or loosely held to a surface. This mode can be good for soft samples like glass polymers, or biological samples. However, it is more challenging to image in liquids, and sometimes slower scan speeds are needed.

Non-contact mode

In this mode, the tip never touches the surface, but it oscillates above the surface that can get further away up to 10 nm. Using a feedback loop to compensate changes in the amplitude of oscillation due to attractive Van der Waals forces, the surface topography can be measured. The advantage of this mode is that a very low force $10^{-12}N$ is exerted on the sample surface meaning that it could be scanned with extended lifetime, and the tip never damage the surface. However, this mode often has lower lateral resolution, slower scan speed to avoid contact with fluid layers, and the possibility of contaminant layer on surface which can interfere with the oscillation. Therefore, oftentimes this mode is not used unless conditions, e.g., in an ultra-high vacuum (UHV).

3.4 Force spectroscopy technique and related methods

The idea in force spectroscopy is to find out the amount of force by which the sample is being indented by the AFM probe tip. Generally, force spectroscopy is performed in contact mode to generate force curves for different materials.

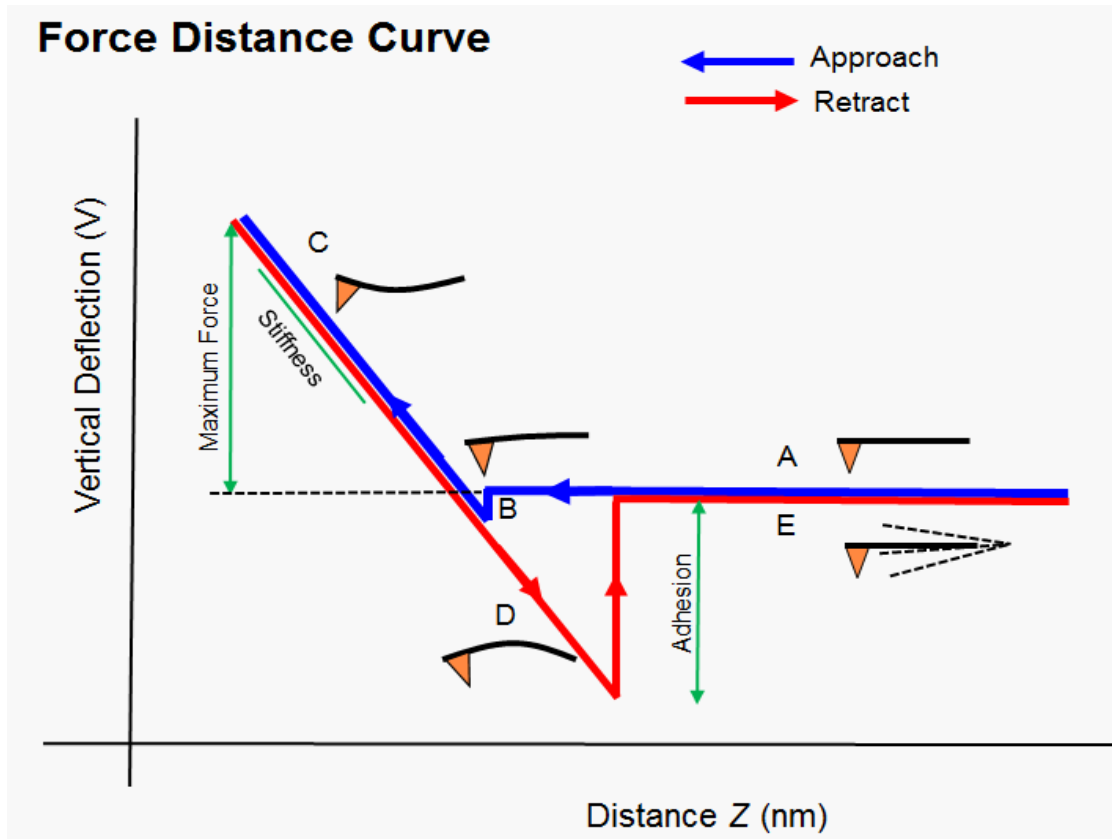


Figure 3.5 | Schematic representation of a force vs distance curve. Tip approaching (blue) and retracting (red) curves are represented. From: <http://www.jpk.com/>.

Force curves typically show the deflection of the cantilever as the probe is brought vertically toward and then away from the surface using the vertical motions of the piezo scanner^{135,136}. By considering the difference between the motion of the stage and the deflection of the cantilever, the actual distance through which the sample has been indented can be obtained. During an approach-retract cycle (Fig 2.5), the tip experiences different force fields. First, the tip starts from position A when it is far away and not in contact with the surface. Then eventually at position

B, it gets close enough to the surface where attractive Van der Waals forces take over, and it will kind of snap down into contact with the surface. After position B, depending on the relative stiffness of the cantilever with respect to the sample, the tip will start indenting on the sample causing the cantilever to be deflected until a certain force has been detected before turn-away points at position D. At position D, the tip pulls off so that the maximum adhesion occurs, and the tip reaches back to its original position. To obtain quantitative data from force-distance curves, the optical detection system of the cantilever needs to be calibrated under two technical steps. In the first step, the optical detection system is characterized by the deflection sensitivity to convert the voltage output into the metric cantilever deflection. This is done by calibration on a relatively hard substrate compared to the sample of interest to directly measure the movement of the stage. In this case, the relationship between piezo motion and displacement of the laser spot on the photodiode gives a conversion factor in nm/V of this displacement. The next step is to convert metric deflection into units of force. This step is done by measuring the spring constant of the cantilever using a thermal noise method. In this method, the thermal fluctuations in the deflection of the cantilever can be related to the spring constant using the equipartition theorem¹³³. Force curve analyses can be used to determine mechanical properties of the sample such as adhesion, elasticity, stiffness of the sample and rupture bond lengths.

3.4.1 Quantitative imaging mode

The acquisition of curves FD curves can be performed in different X-Y positions of the sample, possibly periodically spaced in a grid that covers a particular area¹³⁶. During the acquisition of a map of FD curves, the coordinates vary in the X-Y plane, but also in Z, the data points are collected in a tridimensional space. This method is usually designated as Force Volume.

Height information can be derived for each X-Y point, considering the movement performed by the piezo to reach the maximum force setpoint, as in conventional contact imaging mode. In this way, the topography of the corresponding area can be obtained. Furthermore, the information deduced from FD curves, describing local physical properties of the sample, can be remapped with a direct reference to the topography. This process can be done automatically during real-time measurement, but in most of the cases, it requires a post-processing. The main drawback of conventional Force Volume is that curve acquisition is extremely time-consuming. The JPK Quantitative Imaging (QI) mode attempts to obtain high-speed Force Volume. In QI mode, the X-Y position is maintained constant during each FD curve acquisition, allowing the lateral movements of the tip to be carried out without any interaction between the tip and the sample (Fig 3.6). This feature can be exploited to image the sample avoiding dragging artifacts. The force setpoint for image acquisition is maintained as low as possible, to avoid sample deformation. In this case, FD curves end just after the contact point between tip and sample and are extremely short; thus, high-resolution images (mainly 256x256) can be collected within a reasonable time (10-30 min) by moving the tip at a reasonable velocity (tens of $\mu\text{m/s}$). In this thesis, QI is employed to characterize the mechanical stability of model membranes (see Chapter 4) before and after the interaction with monomeric α -syn.

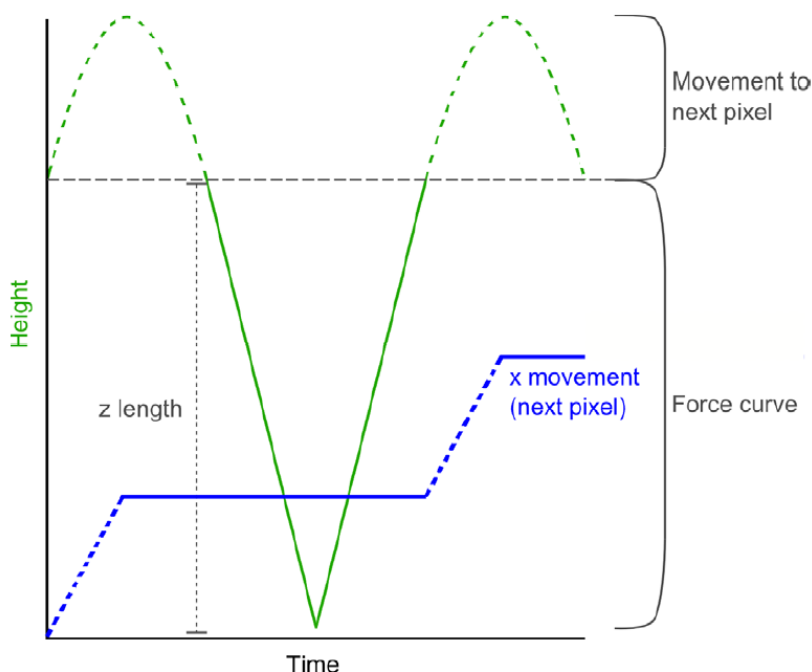


Figure 3.6 | The schematic view of the tip movement algorithm in QI mode. (Image from JPK Instruments AG. JPK Instrument Technical Report: QITM Mode -Quantitative Imaging with the NanoWizard ® 3 AFM.)

3.5 Confocal microscopy

Confocal microscopy is a powerful approach in many areas of current biological research interest for in-vivo corneal, retinal, and optic disk imaging. It provides three-dimensional images of a fluorescent specimen with better rejection of out-of-focus information, relying on upon enhancement of both lateral and axial resolution, overcoming the limitations of the traditional wide-field fluorescence microscopes. Conventional fluorescence microscopes use wide field illumination, in which the entire field of view, 3D space, is illuminated with light from a light source (Fig 3.7 a). As the illuminating light passes through a 3D specimen, the fluorescent molecules above and below the current plane of focus of the specimen undergo full excitation on every instant, leading to in- and out-of-focus light points contribution overlapping and having a blurred image¹³⁷.

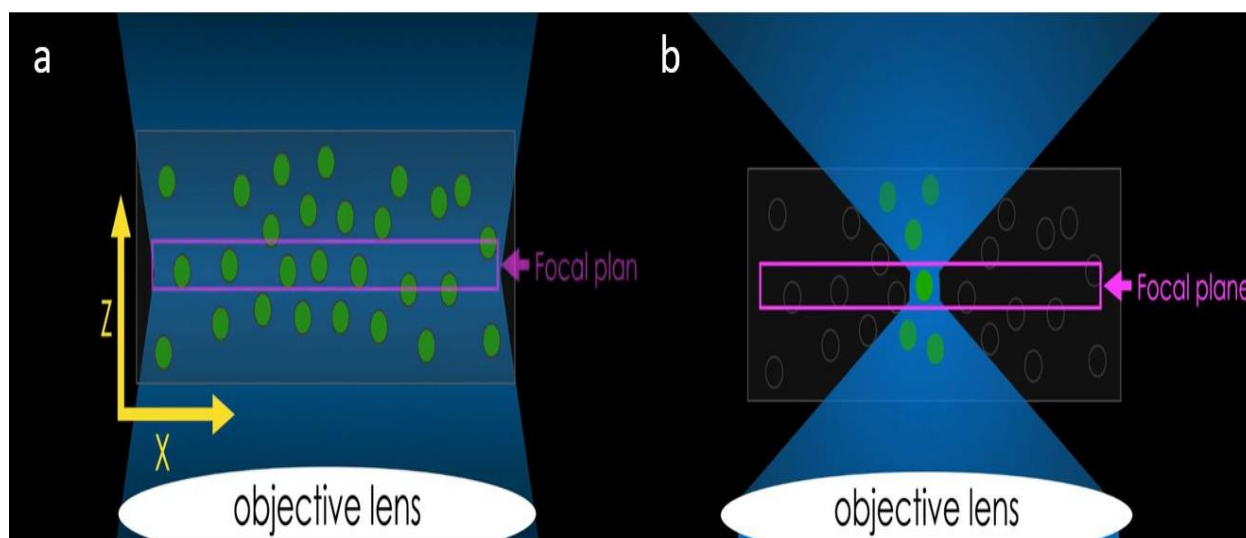


Figure 3.7 | Schematic illustration of (a) wide field illumination vs. (b) confocal illumination.

The solution to the problem is simplified when using confocal laser scanning microscope. The concept of confocal microscopy was originated, in the 1950s, by Marvel Minsky for studying neural networks in the living brain ¹³⁸. The key concept in confocal laser scanning microscopy lies in reducing the background fluorescent or out-of-focus fluorescent using two features:

1. Focused illumination: Illumination is focused to a small portion of the entire field of view within the specimen (Fig 3.7 b) through an illumination pinhole located in front of the excitation beam, limiting the entire excitation of fluorescent molecules in the sample, which reduces the background contributions.
2. Pinhole at the image plane: Most of the light emitted from regions above and below the focal plane is blocked from reaching the detector by means of a second pinhole in front of the detector.

Fig 3.8 shows a typical confocal laser scanning microscopy set-up. To acquire an image, the excitation light coming from the laser passes through a collimating lens, which forms a beam of light, and it is focused through the pinhole. The light is reflected by a dichroic mirror to the

objective lens which focuses the collimated beam of light into a point source of light that resides at the focal plane of the objective lens. The excitation light must be fully delivered to each point of the sample, acting like a pointillist painter using a brush.

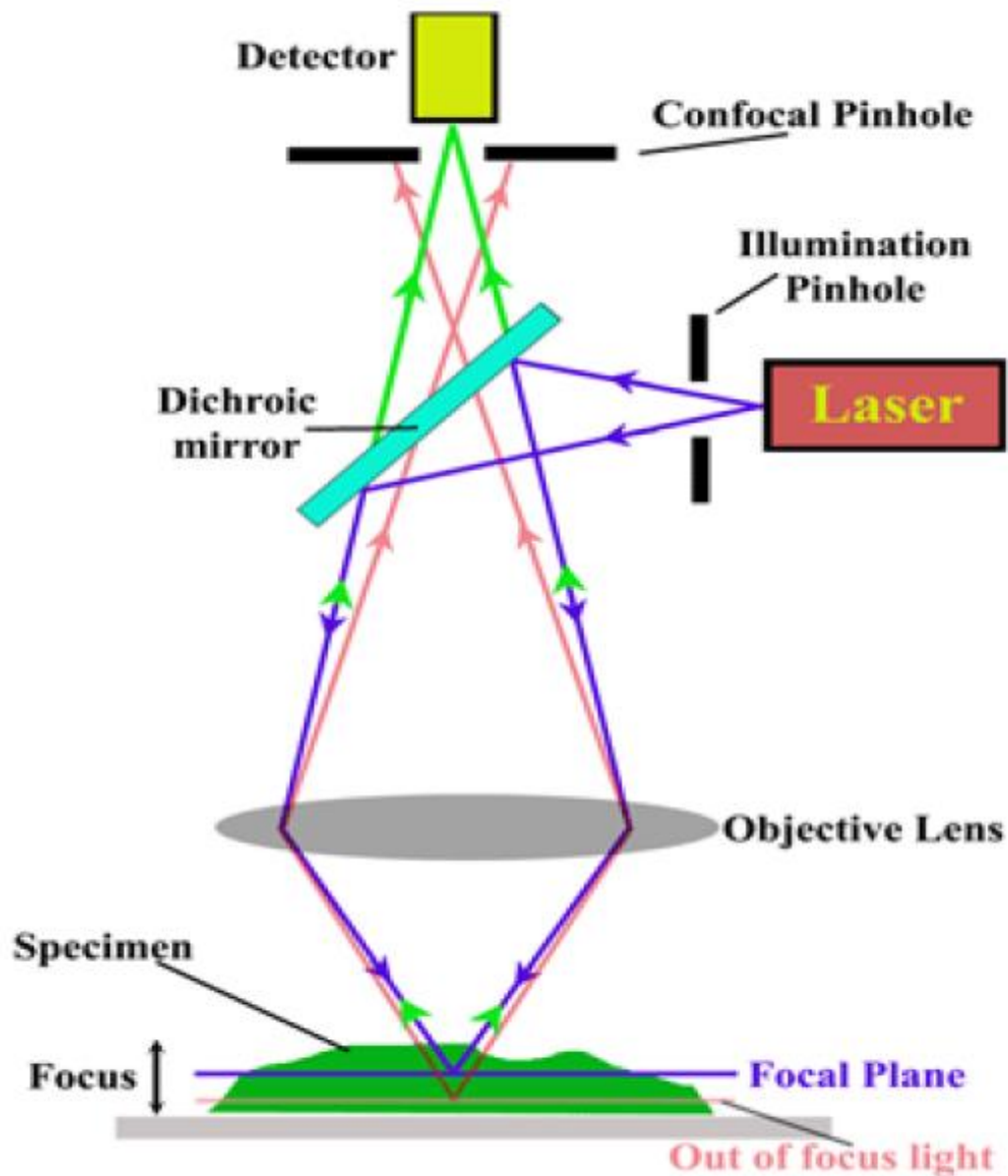


Figure 3.8 | Schematic illustrating the principle of confocal laser scanning microscopy (Image from ¹³⁹).

The emission signal, collected by the objective lens, passes through the dichroic mirror, and is focused by the tube lens. The second pinhole is positioned such that the focused emission signal can pass through the pinhole and can be detected through the photomultiplier tube or and hybrid detector. Therefore, the pinhole blocks most of the light emitted from out-of-focus fluorescence, preventing the light from reaching the detector. The focal point in the sample and the pinhole lies in conjugate planes; this optical arrangement is called ‘confocal’. Overall, the numerical aperture of the objective lens, the wavelength of the excitation/emission light, the pinhole size, the refractive index mismatch of components along the light path, and the overall alignment of the instrument determine the z-resolution and optical sectioning thickness of a laser scanning confocal microscope.

3.6 STED microscopy

3.6.1 Basic principles

The goal of STED microscopy is to reduce the effective fluorescence emission in the local periphery of the excitation volume using a ring-shaped depletion laser which is referred to as STED beam ^{140,141}. In this technique, a Gaussian excitation beam of a scanning microscope is coaligned with a doughnut-shaped beam across a specimen (Fig 3.9 b, c). Since the experiment is done at room temperature, there is a ground state (OFF state), and a singlet excited state (ON state) with some vibrational levels (Fig 3.9 a). During the fluorescence process, fluorophores absorb the energy of an excitation photon, and they are excited from OFF state to ON state. Once they are reached that state, immediately after and within a specific fluorescence lifetime (τ), which lasts several nanoseconds, the depletion beam is added. Then the fluorophores which are located at center of Gaussian beam, where is no STED beam, are de-excited via fluorescence spontaneous

emission, and those fluorophores located at the local periphery of the excitation volume are de-excited via stimulated emission (SE) with the wavelengths equivalent to the depletion beam which are significantly different than the spontaneous emission wavelength. Then, a filter can be used to block all depletion wavelengths but passes most of the fluorescence wavelengths preventing detection of those fluorophores that have undergone stimulated emission depletion. In the most typical STED microscopy implementation (Fig 3.9 b, c), when that donut beam appears on top of the excitation beam, it sets up an intensity profile which has almost no intensity at the center of the Gaussian beam, and away from there, it has higher values. At the center of the Gaussian beam where there is no STED beam, what happens is that an excitation photon is sent onto the specimen, and what will come out in that situation will be a fluorescent photon. Away from there, there is less available fluorescence because the depletion beam causes other ways of de-exciting the excited molecules. Regions where an intense depletion beam is shined suppress fluorescence emission. Fluorescence quantum efficiency at some locations in the plane of illumination function of x , and y is given by

$$\varphi(x, y) = \frac{k_f}{k_f + k_{NR} + k_{STED} \cdot \frac{I(x, y)}{I_0}} \quad (3.2)$$

What comes out from this equation is a certain decay rate due to all the possibilities that a fluorophore has to the OFF state. The depletion of the fluorescent signal depends on the number of stimulating photons. To efficiently minimize fluorophore's spontaneous emission and consequently maximize fluorescent quenching, all the stimulating photons must act shortly after the excitation event and within fluorophore's excited-state lifetime when fluorophores are in ON

state. This short temporal interval and the small cross-section of SE require a high flux of stimulating photons. In this case, the spatial resolution can be given by

$$d = \frac{\lambda}{2n \sin \theta \sqrt{1 + \frac{I(x,y)}{I_0}}} \quad (3.3)$$

where the sub-diffraction spatial resolution requires much higher intensities causing problems such as photobleaching and phototoxicity.

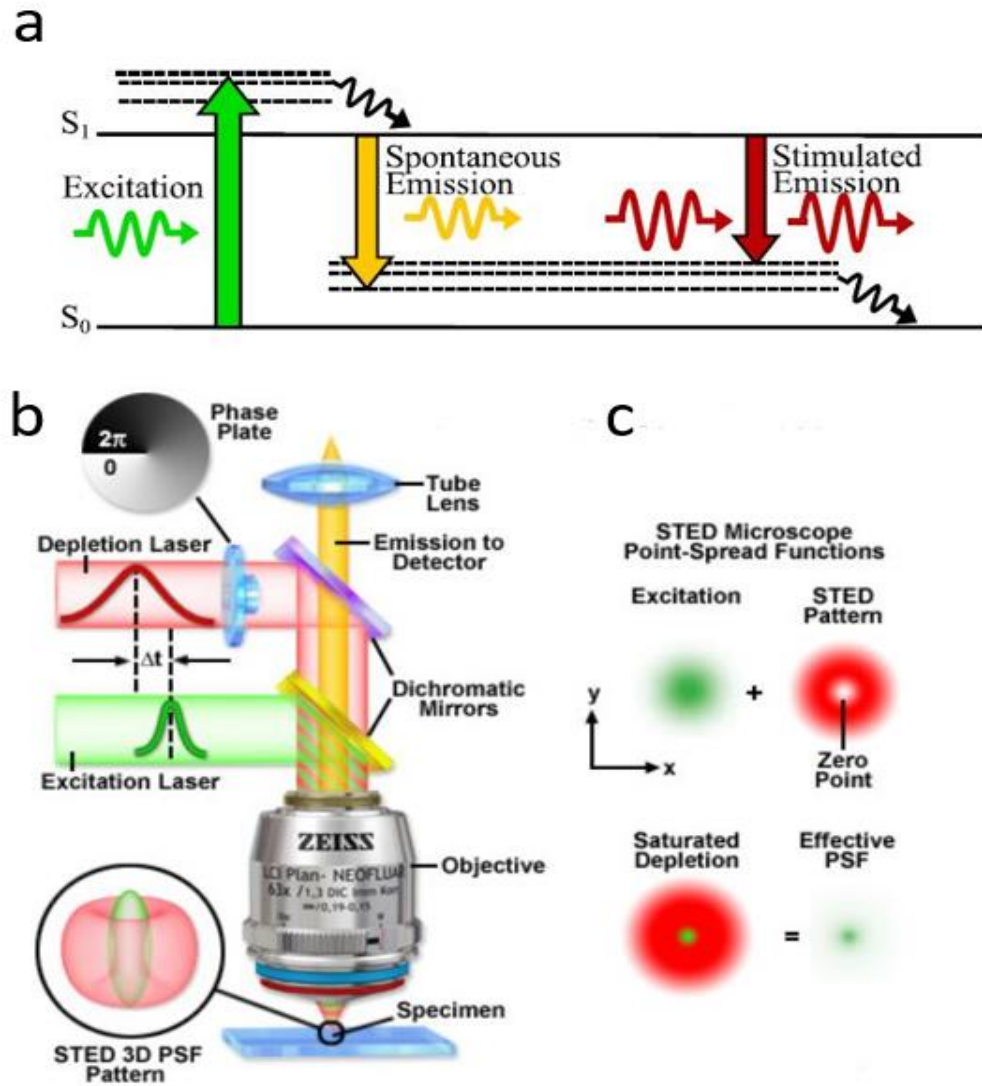


Figure 3.9 | (a) Jablonski energy diagram illustrating the photophysical processes of fluorophores, including excitation, spontaneous emission, and stimulated emission. (b) Schematic

configuration of STED system, showing the phase plate, excitation, and depletion lasers (red and green beams, respectively), dichromatic mirrors, tube lens, and objective. It uses (in addition to a fluorescence excitation laser) a second, ring-shaped laser to quench fluorescence. (c) Super-resolution PSF obtained by co-locating the donut-shaped STED beam and diffraction limited excitation beam (Image modified from <http://zeiss-campus.magnet.fsu.edu>).

3.7 Correlative AFM-optical fluorescence microscopy

3.7.1 Pioneering attempts

AFM and fluorescence light correlative microscopy is a platform based on the direct overlay of images acquired with AFM and light microscopy. The idea to couple the AFM with the optical microscopy rose right after the first application of the AFM on bio-samples¹⁴². Initially, the optical microscope was thought to be a tool for identifying a particular structure and choosing the area for the AFM inspection, particularly useful in cell imaging. This point was essential, since the positioning of the AFM probe on the object of interest by subsequent image acquisition was very time-consuming, mainly when the sample was randomly distributed on the substrate and not very dense. The second issue of this method was related to the high probability of tip contamination, or damage in the first preparation phase. Another fundamental idea was to enrich the AFM analysis with other important information from the same area, derived, e.g., by using fluorescence microscopy. Two different attempts to reach these goals were presented in 1992. Radmacher et al. introduced an AFM system with an integrated long-distance objective¹⁴³, able to image the sample in an upright configuration, also in fluorescence mode (Fig. 3.10 a). This solution was dictated by the configuration of the first AFMs that were generally sample scanning systems. The limitation of this setup was the poor magnification associated with the use of an objective with a working distance of several centimeters.

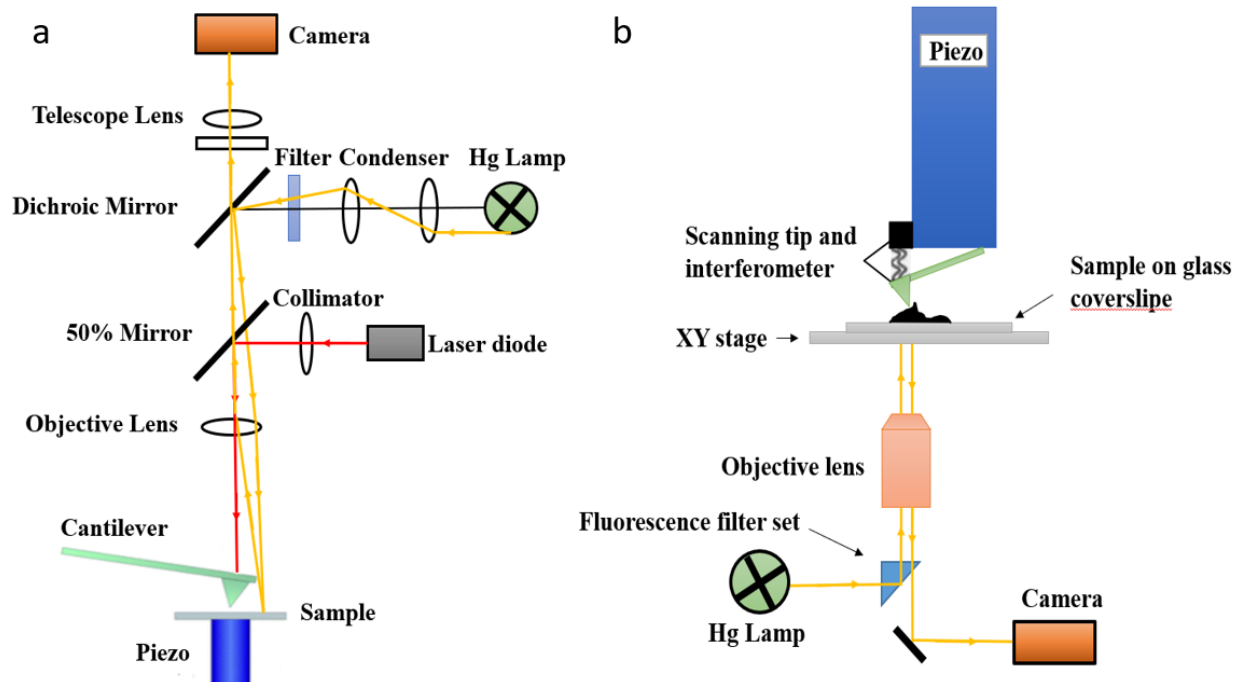


Figure 3.10 | The first integrated system proposed by Radmacher et al. was based on the use of a long-distance objective to focus the sample at a distance that was higher than the scanner thickness (a). The use of head-scanning AFM systems allowed the use of standard inverted optical microscopes (b). The solution here represented in b has been the most successful, especially in the study of bio-samples.

Just one year later, in 1993, the same group presented a new system. Taking advantage of the recent development of head-scanning AFM, a commercial inverted optical microscope was coupled with the AFM ¹⁴⁴. The lack of the piezo scanner between the scanner and the sample allowed this new solution that will be preferred for the most advanced application in the following. The objective approaches the transparent substrate without any steric constriction. Furthermore, the optical path from the objective lenses to the sample is free, while using a telescope from the top, the AFM probe is between the lenses and the sample precluding the sample view in the correspondence of the AFM tip. For the first time, the author employed a confocal laser scanning microscope (CLSM) ¹⁴⁵. The advantage of CLSM is related to its capability to study the inner part of the cell in details. CLSM can define the optical plane of acquisition, having the potential to

characterize the internal part of the cell with a better signal to noise ratio, being able to distinguish the structures that are present at the basal plane at the mid-plane, or the apical plane of the cell. This possibility is precluded in the AFM. The quality of the correlated images increased with respect to the previous works.

A similar design was also proposed by Henderson et al. just a few months later, integrating a commercial inverted optical microscope to a head-scanning AFM, at least in part commercially available ¹⁴⁶ (Fig. 3.10 b). The basis of correlative AFM-optical microscopy was built, paving the avenue for future developments.

3.7.2 The advent of super-resolution: AFM-STED correlative microscopy

In 2012, for the first time, Harke and co-workers reported the combination of different AFM modes with the super resolution STED microscopy ¹³². A nominal multi photon fluorescence microscope equipped with a custom-made STED microscope was boosted by an AFM (Fig 3.11). Initially, the main aim was to provide chemical specificity to the AFM, by coupling it with a fluorescence technique with a comparable lateral resolution. The choice of STED microscopy, among all the available super-resolution techniques was suggested by the unique capability of STED such as fast image acquisition (in particular if compared with the slow acquisition rate of the AFM), the possible integration with other fluorescent based techniques as fluorescence correlation spectroscopy (FCS)¹⁴⁷, fluorescence lifetime imaging ¹⁴⁸, fluorescence recovery after photo-bleaching ¹⁴⁹, etc.

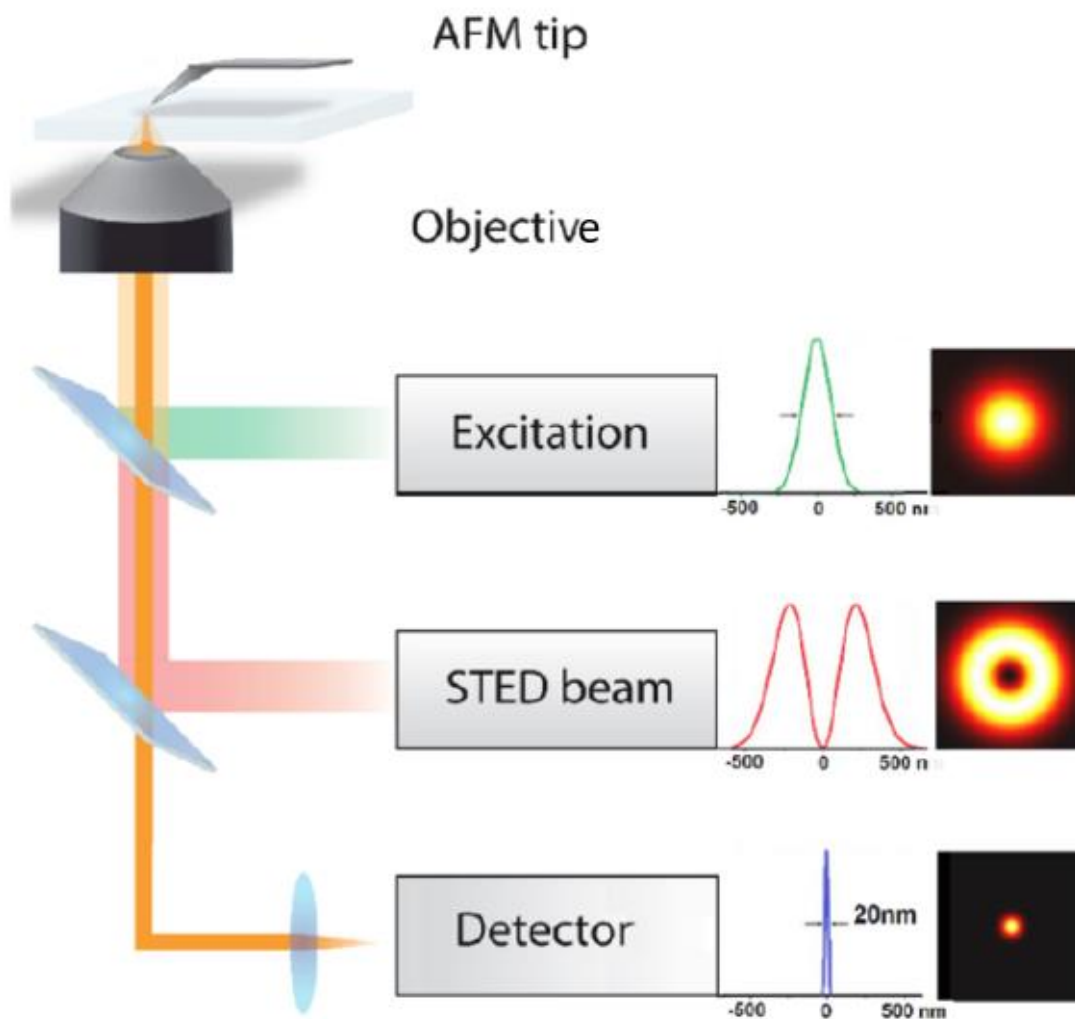


Figure 3.11 | Schematic representation of a combined AFM and STED microscope components. The sketch includes the excitation source, the STED beam, the detection unit, and the AFM. (Image from JPK instruments, application notes)

As a first test sample to check the platform performance, fluorescent spherical beads with specified diameter of 40 nm were measured under dry conditions. The results shown in Fig. 3.12 b, d represented single fluorescent beads in STED image and in topographical AFM of the same area of interest, respectively. It was possible to associate each single feature displayed by AFM

with the fluorescent trace in the STED image. On the contrary, the confocal image (Fig. 3.12 a) showed only blurred fluorescent spots in which the trace of the single beads was lost. From the results, they pointed out that the targeting capability of STED technique is compatible with that of the AFM, but is enriched with chemical specificity, and this confirmed STED as an ideal candidate to improve AFM analysis.

As a second test sample, the labeled microtubules of cos7 cells were measured under aqueous conditions. Also, in this case the confocal data could not confirm the AFM data, whereas the number of the microtubuli was clearly distinguished from the rest of the cell area in the images acquired with STED (Fig. 3.12 c). The AFM force maps of an area of interest was acquired to extract the Young's modulus gaining information about the cell stiffness. The Young's modulus map matched with the data extracted from the STED images suggesting interesting application in nano-mechanics (data not shown). The higher precision given by STED microscopy also reduced the total number of force spectroscopy cycles for the adhesion determination which thereby reduced the risk of possible tip contamination.

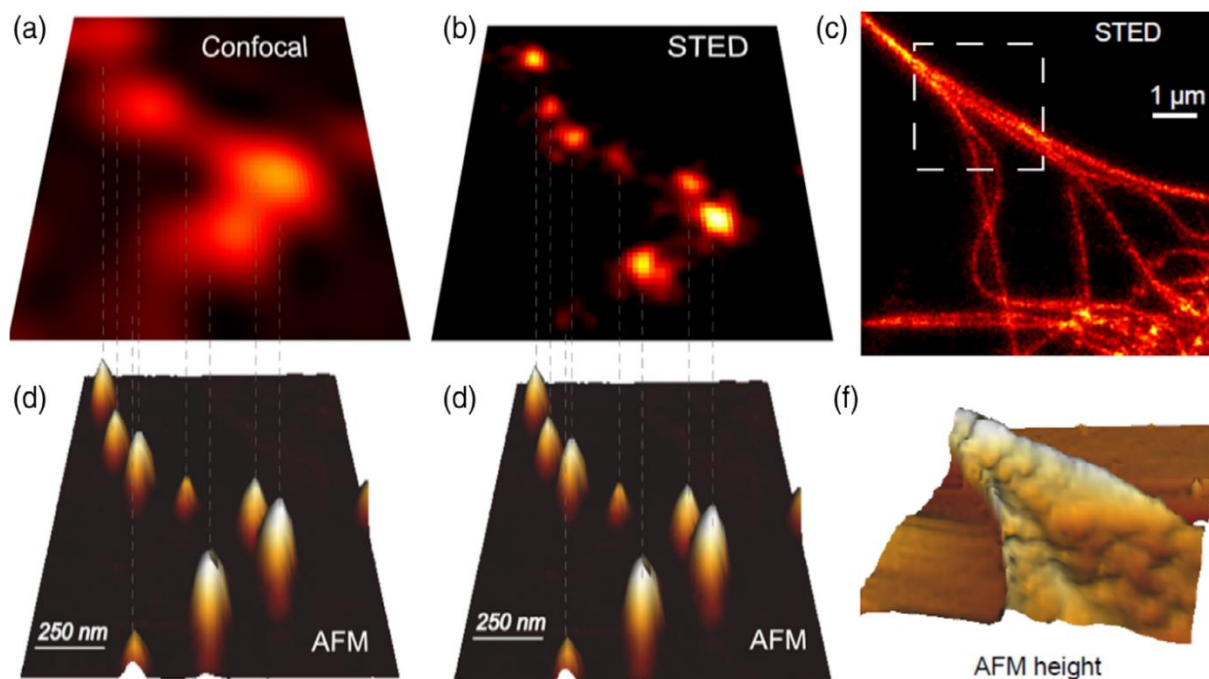


Figure 3.12 | (a, b) Confocal and STED images of fluorescent spheres of 40 nm size, respectively. (c) STED image of fluorescently labelled microtubules in cos7 cells (d, e, f) 3D rendered view of height analysis extracted from AFM images of A, B, and C, respectively. Part of the figure was modified from ¹³².

More recently, Cosentino et al. performed correlative AFM-STED measurements to study amyloid aggregates formation starting from different monomeric peptides ¹⁵⁰. This work shows that the AFM, i.e., a high-resolution label free microscopy technique, can be a fundamental tool able to detect artifact induced by the presence of the fluorescent molecules. My work started from this point to refine the work of Cosentino et al., extending it to the case of larger peptides and to the use of more specific and well-defined labeling strategies that I describe in detail in chapter 5.

Chapter 4

Interplay between cell membrane and amyloidogenic α -syn

Membrane-mediated protein physiological alterations are linked to amyloidogenic diseases making the study of membrane-protein interactions of particular interest ⁴⁹. One of these proteins is the intrinsically disordered and amyloidogenic protein, α -synuclein (α -syn), involved in Parkinson's disease (PD). As monomeric α -syn is natively unfolded, its conformation is determined by the interplay with its cellular environment, in particular with lipids and fatty acids ^{74,151}. Due to the close interplay between α -syn and lipid membranes, not only α -syn aggregation is affected by the presence of lipids, but also monomeric α -syn can alter membrane structure and properties in a variety of ways depending on different parameters, including lipid/protein ratio. At low lipid/protein ratios, the negatively charged lipids enhance the aggregation rate of α -syn by enabling penetration of hydrophobic residues into the hydrophobic region of the bilayer, providing nucleation sites ⁷⁴. Increasing the lipid/protein ratio usually prevents α -syn aggregation due to a reduced amount of free monomeric α -syn available for aggregation, as the protein is mainly bound to the membrane in an alpha-helical state ⁷⁵. However, some cases in which aggregation is enhanced at high lipid/protein ratios have also been reported ⁵². In fact, α -syn binding to membranes can cause membrane remodelling, including membrane thinning ^{49,152} and expansion ⁵³, formation a variety of structures, including tubules, small vesicles, and lipoprotein nanoparticles ^{153–156}.

α -syn is present in the cytoplasm and in the extracellular region as well. It is clear that the asymmetry of the neuronal membrane plays a role in the interaction with the peptide and its aggregates. The proximity of the two leaflets and the difficulty to reach the cytoplasmic region of

the cell is precluding a systematic and reliable characterization of the interaction of α -syn with the two membrane sides working at the cell level. To shed light on how α -syn interacts with biomembranes, we employed supported lipid bilayers with different compositions, mimicking the inner and outer leaflets of the neuronal membrane. This approach allows the clear decoupling of the interaction with the two membrane leaflets.

In this chapter, I will describe the procedures used in the preparation of supported lipid bilayers (SLBs) with the two compositions. Then, I will give a detailed analysis of the influence of α -syn on the SLBs. Different techniques have been employed. The AFM was used to detect morphological changes in SLBs induced by α -syn. Quartz crystal microbalance with Dissipation monitoring (QCM-D) investigation, performed in collaboration with Dr. Silvia Dante, at the Materials Characterization Facility of the Istituto Italiano di Tecnologia in Genoa, provided a characterization of protein uptake. Vesicle leakage experiments, performed in collaboration with Dr. Ester Canepa at the Physics Department of the University of Genoa, provided complementary information on the membrane destabilization level.

4.1 Materials and methods

The experimental works described in this chapter are based on in situ AFM applications. The main aim is related to the characterization of the influence of monomeric α -syn on SLBs. SLBs were prepared by vesicle fusion technique on mica substrate. Vesicle fusion consists in the deposition of a large unilamellar vesicles (LUVs) suspension onto a hydrophilic substrate: vesicle adsorption and spreading on the surface may lead, under specific conditions, to bilayer formation (Fig 4.1).

α -syn is a cytosolic protein mainly expressed in presynaptic terminals, but it is also found in the extracellular space^{157,158}. Therefore, both the inner and outer leaflets of neuronal membranes can face the protein and can be involved in the first step of its interaction with the membrane. To this purpose, we selected a five-component (DOPC/SM/DOPE/DOPS/chol) and a four-component (DOPC/SM/GM1/chol) lipid mixture, mimicking the inner and the outer leaflet of the cell membrane, respectively. We used AFM to test the effect of α -syn on the morphology of supported lipid bilayers. We considered different α -syn concentrations, always keeping the lipid-protein ratio high enough to avoid protein aggregation in the solution. As lipid mobility is strongly affected by the presence of the substrate, our study was performed considering both pre-formed supported lipid bilayers which were exposed to the protein and supported lipid bilayers formed from liposomes pre-incubated with the protein; in the latter case, protein-membrane interaction could take place without any constraint from the substrate.

4.1.1 Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, 18:1 (Δ 9-Cis) PC), sphingomyelin (SM, porcine brain), ganglioside GM1 (ovine brain, sodium salt), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, 18:1 (Δ 9-Cis) PE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS, 16:0-18:1 PS, sodium salt) were purchased as lyophilized powders from Avanti Polar Lipids (> 99 % purity for all species). Cholesterol (chol, $\geq$ 99 %, lyophilized powder), 2-[tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid (TES, $\geq$ 99 %), L-histidine ($\geq$ 99 %), ethylenediaminetetraacetic acid disodium salt (EDTA, 99 %), and sodium chloride ($\geq$ 99.5 %) and calcium chloride ($\geq$ 99.5 %) were purchased from Sigma Aldrich. All chemicals were used without

further purification. Before use, water was always purified with a Milli-Q ultrapure water system (18.2 M Ω -cm resistivity at 25 °C; Millipore).

4.1.2 Lipid vesicle preparation and characterization

Two lipid mixtures were used to model the composition of the extracellular and cytoplasmic leaflets of mammalian plasma membranes, i.e. DOPC:SM:chol:GM1 63:31:1:5 (molar ratio) and DOPC:SM:DOPE:POPS:chol 45:20:20:10:5 (molar ratio), respectively. The lipid powders were dissolved in chloroform/methanol (2:1), and then mixed in the amount needed to obtain the desired lipid bilayer compositions. Then the solvent in the mixture was evaporated with a gentle nitrogen flux, and incubated under vacuum overnight, to remove any possible solvent trace. The dried lipid film was then re-hydrated, either with deionized water or PBS, and let to swell for at least one hour, in order to induce a spontaneous formation of multilamellar liposomes. Unilamellar lipid vesicles were prepared via thin-film hydration and subsequent extrusion at 60 °C using the mini-extruder kit (Avanti Polar Lipids) equipped with 100 nm pore size filters (Nuclepore, Whatman). Lipid films were hydrated with water at 1 mg/ml. The final lipid concentration (0.2 mg/mL) was estimated by considering the dilution ratio of the vesicle suspension before and after filtration. All vesicle suspensions were stored at 4 °C and used within a few days.

4.1.3 Peptide Preparation

Lyophilized α -syn was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved at the final concentration of 1 mg/ml in ultrapure water, aliquoted, and stored at -20 °C. For the

experiments, frozen aliquots were freshly diluted at selected concentrations in ultrapure water and used for lipid interaction studies.

4.1.4 AFM experiments

AFM samples

Alpha-synuclein interaction with mEXT and mINT bilayers was studied using two different incubation methods, in which (a) the protein was administered to preformed supported lipid bilayers (SLB) (method I, incubation method), or (b) it was pre-incubated with vesicles before their deposition on mica and planar bilayer formation (method II, pre-incubation method). In details:

Method I: SLB formation

For each bilayer, a diluted vesicle suspension (60 μL , 1 mg ml^{-1}) and a fresh CaCl_2 solution (10 μL , 10 mM) were deposited onto an approximately 2.0 x 2.0 cm^2 freshly cleaved mica foil glued on a glass slide microscope. Samples were stored for 10 min at room temperature and then incubated for 15 min (mEXT) and 25 min (mINT) at 60 $^\circ\text{C}$ in a close chamber at 100% relative humidity. The samples were let to cool down at room temperature for 2 h and finally gently rinsed with ultrapure water. This step is necessary to remove non-deposited vesicles from the liquid before AFM investigation. AFM imaging usually started at least two hours after rinsing. SLBs were characterized before the interaction with the protein. α -syn was administered to the samples at different concentration (1 nM, 40 nM, or 200 nM), and it was let to interact for 30 minutes before starting the AFM imaging. At least four replicates were prepared for each condition in each experiment.

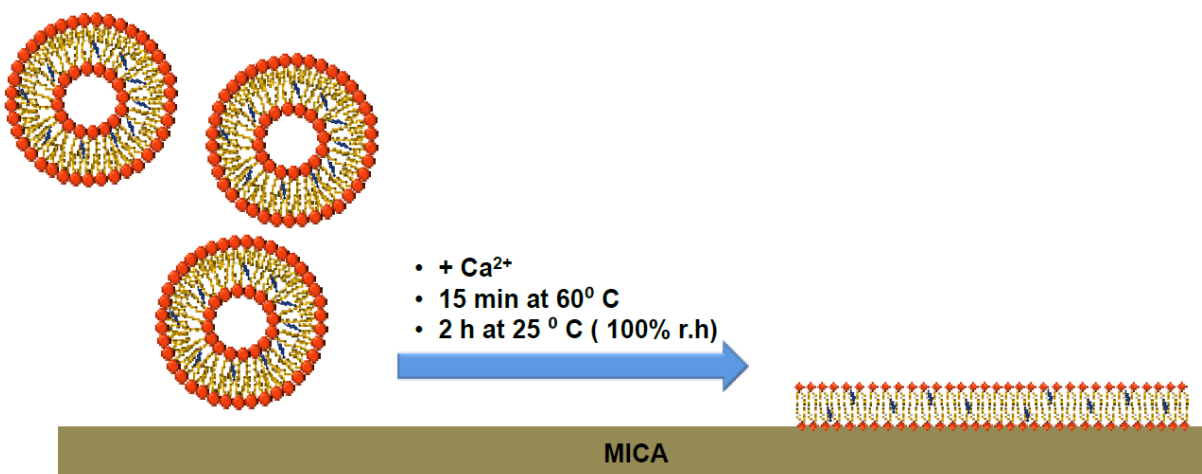


Figure 4.1 | Schematic representation of the vesicle fusion protocol employed in this work. (Image from Reinier Oropesa Nunez' thesis)

Method II: vesicle pre-incubation

With the pre-incubation method, vesicles (30 μ L, 1 mg ml⁻¹) and α -syn were pre-incubated for 30 minutes before their deposition on mica. Also in this case, the pre-incubation occurred at final concentrations of α syn was 1 nM, 40 nM, or 200 nM. The formation of the bilayer on mica followed the same steps described in the previous section. At least four replicates were prepared for each condition in each experiment. Each SLB sample prepared with this protocol was rinsed at least 2 h before AFM imaging.

In both methods, α syn was let to interact with the multidomain membrane before AFM imaging. In the first method, α syn interacts directly with a supported planar membrane, whereas in the second case the interaction takes place in the presence of the curved and freestanding membrane of the vesicle, without any constraints from the substrate.

AFM measurements

AFM images were acquired by using a Nanowizard III (JPK Instruments, Germany) mounted on an Axio Observer D1 (Carl Zeiss, Germany) inverted optical microscope (Fig 4.2). V-shaped DNP silicon nitride cantilevers (Bruker, MA, USA), with a nominal spring constant 0.24 N/m, resonance frequency in air ranging from 40 kHz to 75 kHz, and tip typical curvature radius of 20-60 nm were used. The actual spring constant was determined in situ for each cantilever using the thermal noise method, finding values in the range 0.3-0.4 N/m. Images of SLBs were acquired working in water, by using both Quantitative imaging (QI) mode, and intermittent contact mode (Fig. 4.3). QI images were produced through the collection of 256 x 256 force-distance curves, with maximum force load of 1 nN, the length of FD curves was 100 nm, and the tip velocity 25 $\mu\text{m/s}$. Intermittent contact mode AFM images were performed in the constant-amplitude mode, using the same type of probes, working in water with an oscillating frequency of 10-20 kHz. The scan velocity was between 0.7 to 1 Hz. In all the cases, the amplitude setpoint was kept above 70% of free oscillation amplitude to prevent possible damages to the sample. Before α -syn administration, the morphological features of SLBs were evaluated through AFM imaging. the protein was administered to the bilayer at final concentrations of 1nM, 40 nM, or 200 nM and let incubate for 30 minutes before further measurements. The determination of the difference in thickness between the ordered and disordered lipid phases was determined by using the JPK Data Processing (JPK Instruments, Berlin, Germany). The statistical analysis of image height distributions was obtained with the help of OriginPro 2020 software.

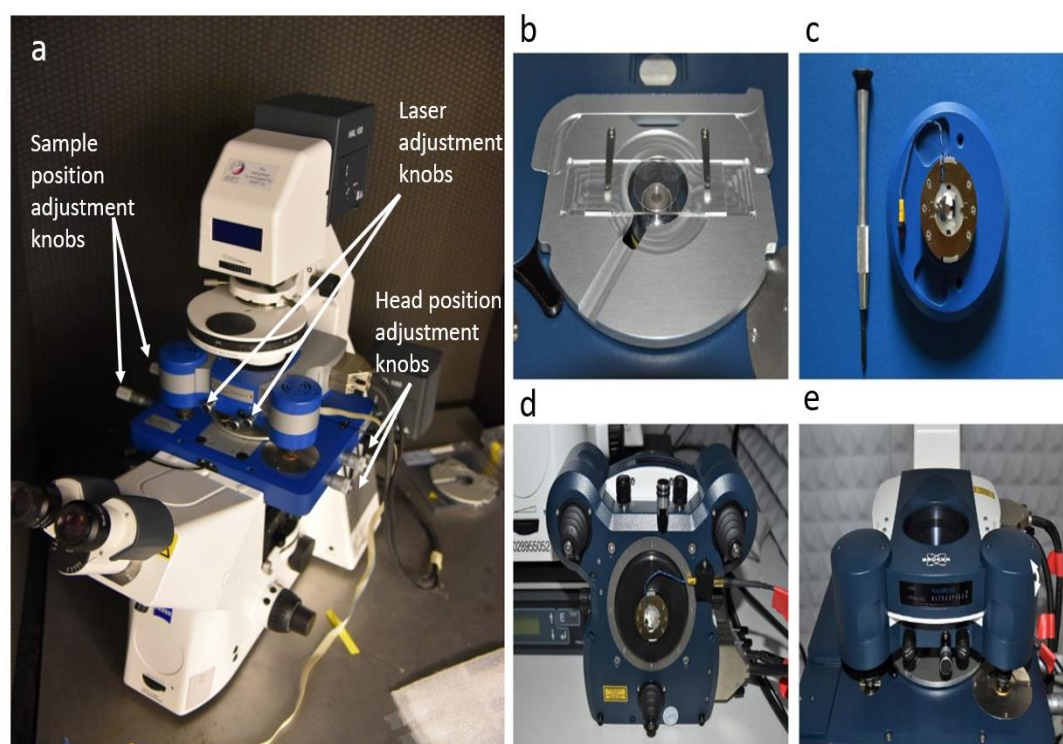


Figure 4.2 | JPK Nanowizard III with Zeiss inverted optical microscope. (a) Description of JPK Nanowizard III (b) AFM stage with sample holder. (c) Cantilever holder inside the cantilever changing tool. (d) AFM head. (e) AFM head positioned on the stage adapted for the Zeiss inverted microscope. Images modified from: <http://www.jpk.com>.

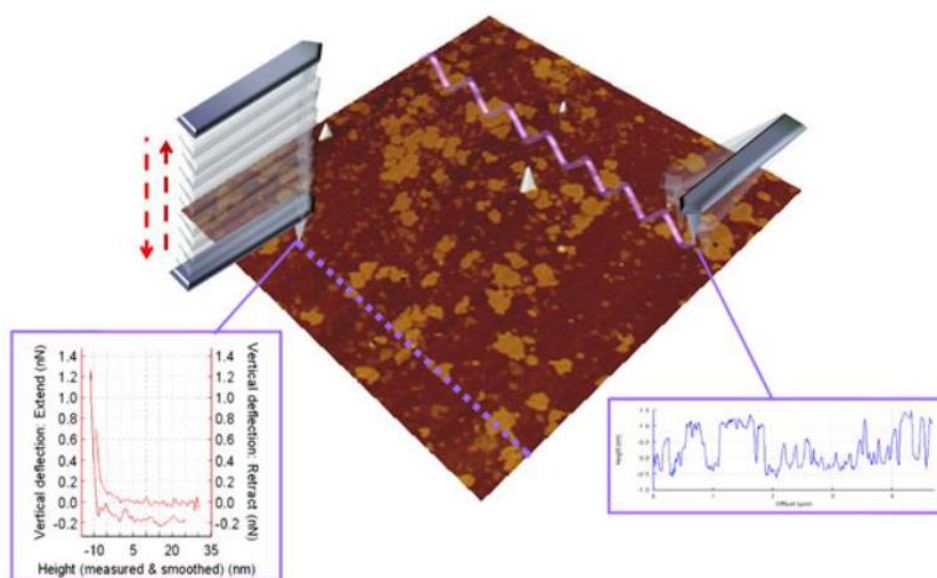


Figure 4.3 | Schematic of imaging modes on SLBs: QI mode and intermittent contact mode. (Image from Silvia Seghezze's thesis)

Force-distance curves on SLBs

Force spectroscopy is an approach to characterize the mechanical stability of supported lipid bilayers (Fig 4.4)^{154–158}. In this technique, AFM probe is brought towards the supported lipid bilayer and a load increasingly applied until the bilayer ruptures and the probe senses the underlying hard substrate. Afterwards the probe is withdrawn, and the cycle is repeated many times. The rupture events are manifested by a well-defined discontinuity associated to tip penetration inside the bilayer in the force-distance approach curves, which can subsequently be analyzed to determine the magnitude of the rupture force or break-through-force (F_b), corresponding to the force at which local bilayer failure occurs. Such breakthrough event, which typically occurs at several nanonewtons of force, marks the penetration of the cantilever through the lipid bilayer and has been shown to be a fingerprint for the intrinsic properties of the bilayer. Therefore, the breakthrough force unequivocally identifies the maximum force that the membrane can withstand before being indented by the cantilever tip. In the case of a double lipid bilayer, i.e., two overlapped membranes, the breakthrough events are two per each curve. In my work, I used this technique to demonstrate the presence of membrane patches formed on top of the SLB. QI mode was employed during preliminary analysis to evaluate tip penetration inside the sample after breakthrough event: images constituted by 256 x 256 force-distance curves with setpoint above (18 nN) F_b distribution have been acquired, with tip vertical velocity 25 $\mu\text{m/s}$, and curve length 150 nm.

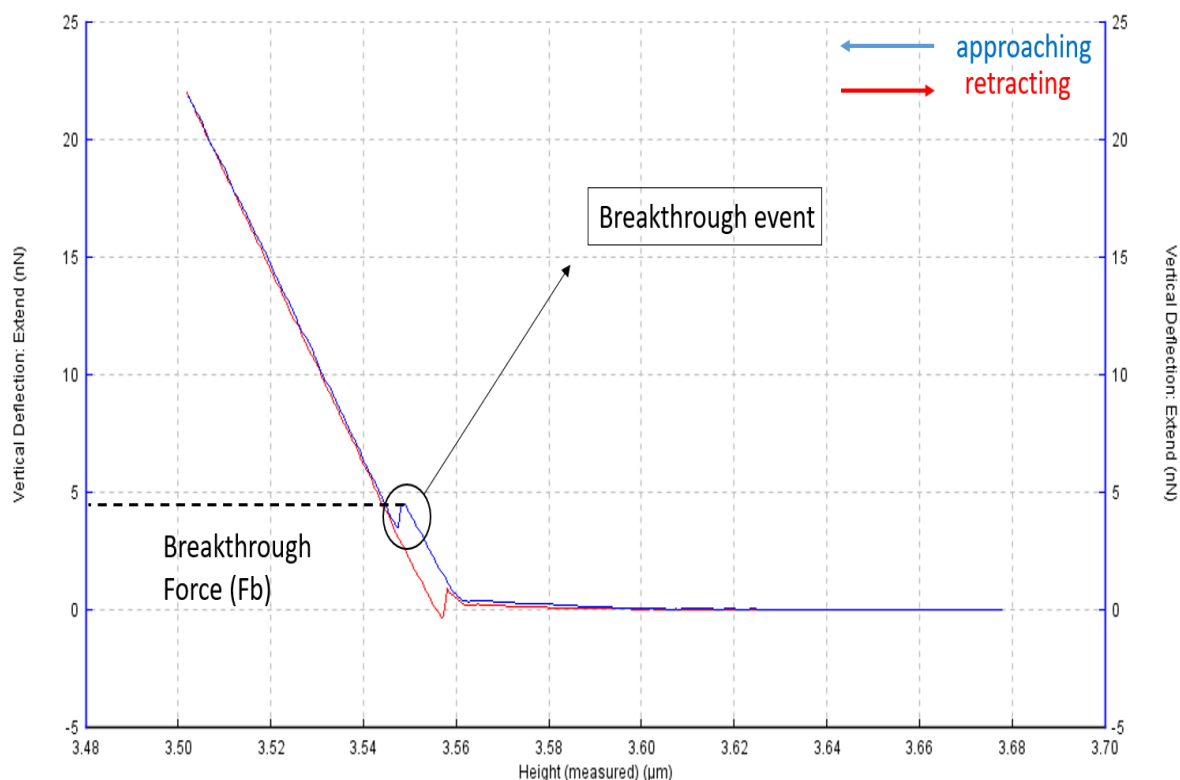


Figure 4.4 | Characteristic force-distance curve on a SLB.

4.1.5 QCM-D analysis

QCM-D measurements were performed with a QCM-Z500 (KSV, Finland) microbalance equipped with a thermostated flow chamber. Depending on the experiment, the interaction of α -syn with preformed SLB or the interaction of α -syn with intact vesicles adsorbed to the sensor, i.e., a supported vesicle layer (SVL) SiO₂-coated, and gold-coated quartz sensors (resonance frequency 5 MHz) were utilized, respectively. To enhance the hydrophilicity of the surface, the sensors were exposed to UV/Ozone for 10 min before usage. The higher harmonics of the fundamental frequency (3rd–11th overtones) were recorded every 1 s during all experiments. Frequency and dissipation changes (ΔF and ΔD , respectively) were recorded simultaneously.

Before starting the measurement, the QCM chamber and the access tubing to the chamber (pre-chamber) were filled with PBS and let to equilibrate at 40 °C until the frequencies of all overtones were stable.

For SLB formation, a concentrated mEXT or mINT vesicle suspension (1 mg mL⁻¹) was diluted in PBS to a final concentration of 0.25 mg mL⁻¹. The vesicle suspension (2 mL) was injected into the thermostated prechamber and let to equilibrate at 40 °C for 30 min. When all overtones were stable, the recording was started, and the frequencies of all overtones set as baselines (i.e., frequency, f , and dissipation, D , zero values); after 600 s, the vesicle suspension was injected in the chamber, carefully avoiding microbubble formation. After the SLB formation, the buffer was exchanged to PBS (thermostated at $T = 40$ °C) to remove the vesicle excess and subsequently exchanged to ultrapure water (thermostated at $T = 40$ °C). Finally, the temperature was set to $T = 22$ °C. Two mL of α syn (1 nM, 40 nM, or 200 nM) were injected in the pre-chamber to thermalize, then injected in the chamber and let to interact with the SLB for several hours, recording ΔF and ΔD for all overtones. After careful rinsing with ultrapure water, the recording was stopped.

In case of SVL, first the vesicle suspension (either mEXT, or mINT) was injected in the chamber, following the same protocol described above. When ΔF and ΔD were stable respectively at their minimum and maximum values, α syn was injected in the chamber, and the recording was continued for at least 10 h. At the end of the measurement, a detergent (Triton) was injected in the chamber.

4.1.6 Calcein release assays

Calcein release can be utilized to probe the ability of α -syn to perturb the membrane integrity of lipid vesicles^{159,160}. A Fluorolog-3 spectrofluorometer (Horiba Jobin-Ivon) equipped with temperature control of the cell holder was utilized to perform the experiments. Each experiment was performed at 25 °C in a quartz cuvette (Hellma, optical path length 10 mm $\times$ 4 mm) filled with 1170 μ L sample volume. Freshly extruded vesicles were diluted to 13.2 mM using a 100 mM NaCl, 2 mM histidine, 2 mM TES, 0.1 mM EDTA buffer (pH adjusted to 7.4). Calcein fluorescence was monitored as a function of time ($\lambda_{\text{ex}} = 490$ nm, $\lambda_{\text{em}} = 520$ nm; sampling rate of 1 s during the measurement. Vesicles were stirred with a magnetic bar for 5000 s to check the system stability, before addition of α -syn (1, 40, or 200 nM). The stirring was then kept constant till the end of the recording. Control experiments in the absence of α -syn were performed to check the vesicle integrity during this time frame. In general, vesicle leakage in the absence of protein was negligible. The time-dependent leakage rate (%) was then normalized as follows:

$$\text{leakage \%}(t) = \frac{F(t) - F_0}{F_{\text{max}} - F_0} \% \quad (4.1)$$

where F_0 is the mean fluorescence level immediately before α -syn addition and is the mean fluorescence level immediately after detergent-induced vesicle rupture (both values were averaged over at least 600 s of recording). F_{max} corresponds to complete calcein release and thus maximum fluorescence emission. Sodium cholate (0.5 % w/v) was added to the vesicle/ α -syn suspension after 54400 s of incubation. Results shown in Table 4.1 correspond to the mean leakage (%), resulting from the last 10 min of vesicle/ α -syn incubation before detergent addition; error bars were calculated by applying the Student's statistics (95 % confidence level, n=3).

4.2 Results

4.2.1 Atomic force microscopy on supported lipid bilayers

SLBs prepared on mica from either lipid mixture displayed segregation between ordered and disordered phases. For mEXT, the ordered phase is composed of SM, chol, and GM1, while for mINT, enrichment of the ordered phase with SM, and chol is expected. Characteristic raft domains for each lipid composition are shown in Fig 4.5, together with representative cross-sections, showing the height profiles of the ordered domains for the two lipid mixtures. The morphology shown in Fig 4.5 is related to the microdomains thickness in which the ordered lipid domains are thicker than the disordered phase, resulting in brighter areas in the AFM images. The difference in thickness between the ordered domains and the disordered phase background (ΔZ) was evaluated. For mEXT SLBs, ΔZ is 2.1 ± 0.2 nm, while for mINT SLBs, a lower value of 1.0 ± 0.1 nm was found. The higher ΔZ found for mEXT agrees with previous measurements on ordered domains containing GM1^{161,162}.

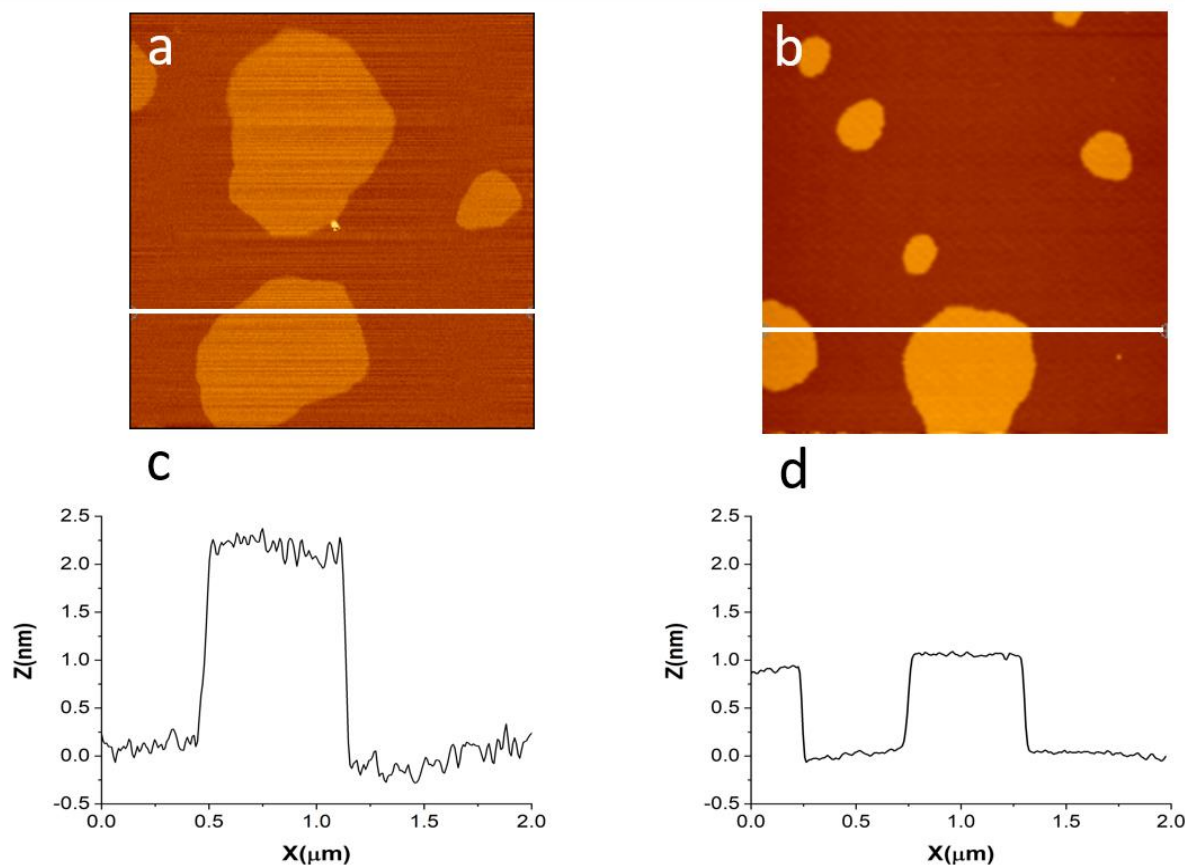


Figure 4.5 | AFM images showing ordered (brighter areas) and disordered domains (darker areas) in mEXT (a), and mINT (b) SLBs. The image size is 2.0 μm , the Z range is (a) 20.5 nm; (b) 4.6 nm. Typical cross-sections obtained on mEXT (c), and mINT (d) SLBs.

In Fig 4.6 and Fig 4.7, the morphology of the SLBs before and after the interaction with α -syn is compared. The typical appearance of SLBs before interaction with the protein is shown in the top panel images for both the lipid mixtures under investigation. After the complete formation and stabilization of the ordered and disordered phase domains, SLBs were incubated with peptide solutions at 1 nM, 40 nM, and 200 nM concentrations, following the procedure described in the materials and methods section. We observed that the morphological changes induced by α -syn are concentration dependent. In case of outer leaflet, the first visible effects were observed already at

1 nM α -syn. At this concentration, the formation of defects/holes in the bilayer is evident (Fig 4.6 d). Furthermore, increasing the protein concentration to 40 nM and 200 nM, an increasingly pronounced destabilization was observed. At all peptide concentrations, the damage caused by the peptide was mainly found in the fluid phase, indicating that the mechanical properties of the lipid membrane play a central role in defining the mechanism of interaction between the membrane and α -syn. In case of inner leaflet, the first visible effects on the lipid bilayer morphology were found at 40 nM. However, at this concentration, and also at 200 nM very small defects (insets in Fig 4.7 e,f) were observed. The damages were localized in the fluid phase domain as well, although it was very limited compared to the outer leaflet mixture. This means that mEXT is more sensitive to the presence of α -syn while the inner leaflet mixture is only slightly impaired by the presence of α -syn, at least at the concentrations under study.

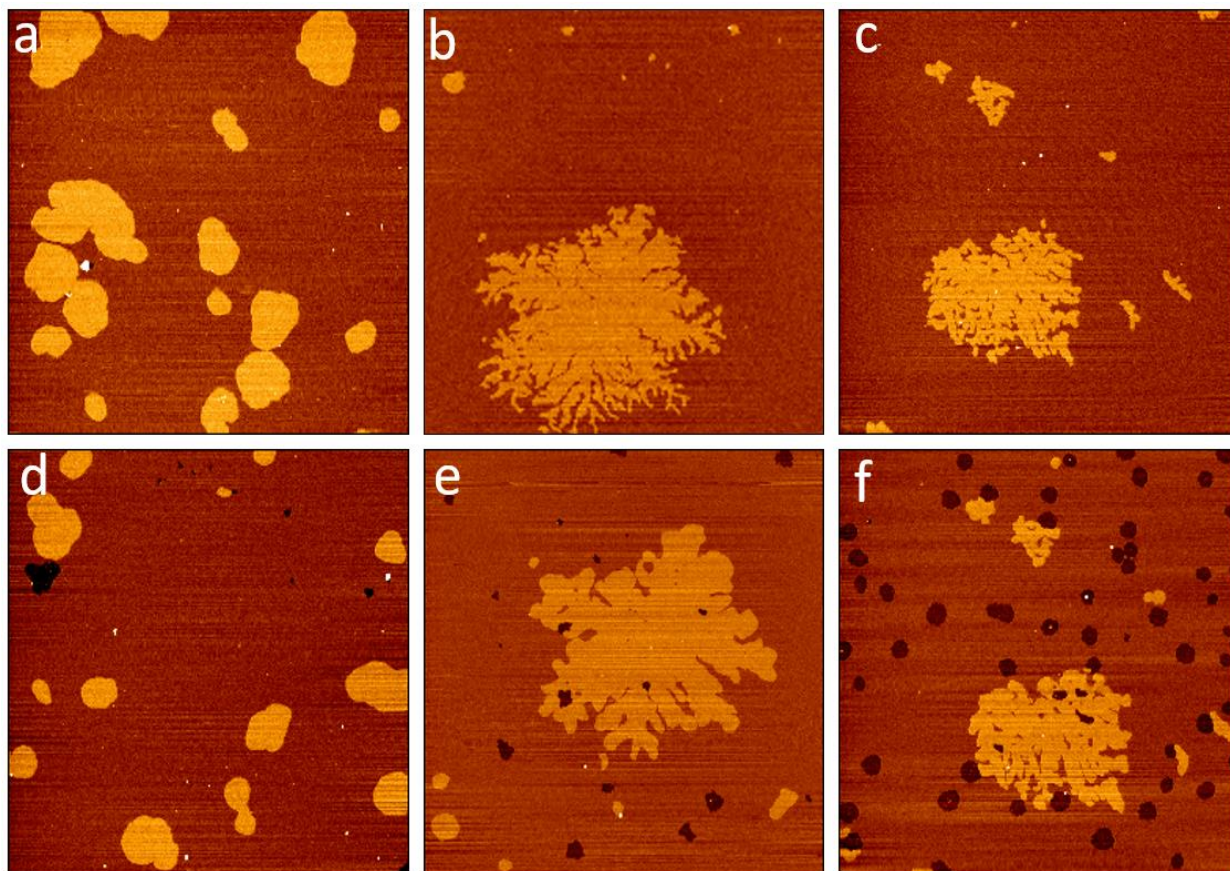


Figure 4.6 | Representative AFM images of mEXT SLBs sampled before (a-c) and after incubation with α -syn at concentrations of 1 nM (d), 40 nM (e), and 200 nM (f). α -syn induces destabilization of the defect-free bilayer, mainly in the liquid disordered phase. The image size is 7.5 μ m, the Z range is 10 nm.

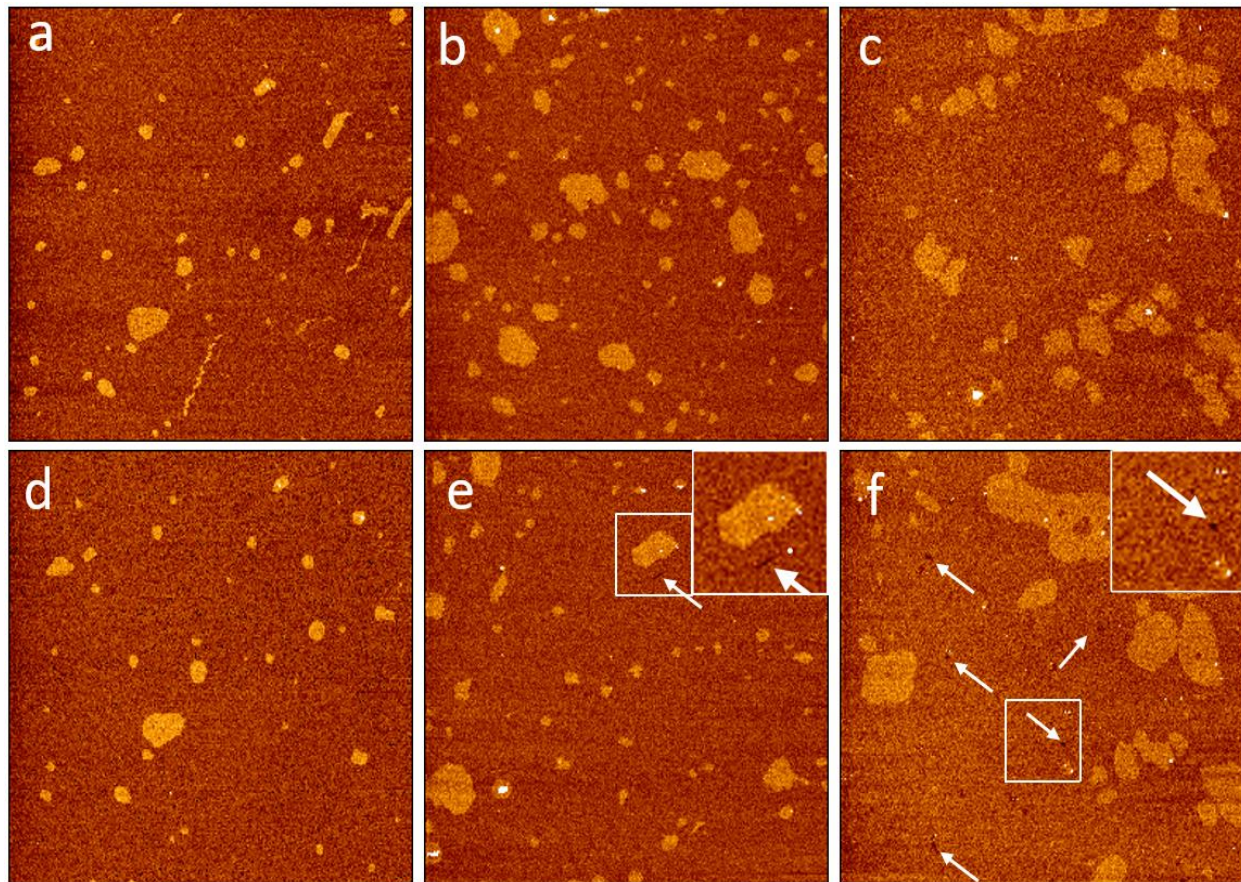


Figure 4.7 | Representative AFM images of mINT SLBs sampled before (a-c) and after incubation with α -syn at concentrations of 1 nM (d), 40 nM (e), and 200 nM (f). At 40 and 200 nM, α -syn causes the formation of small defects (indicated by arrows in Fig 4.5 e,f) in the liquid disordered phase. The image size is 7.5 μ m, the Z range is 5.4 nm. In panels e,f, the inset in the upper right corner shows a magnification of the framed image portion.

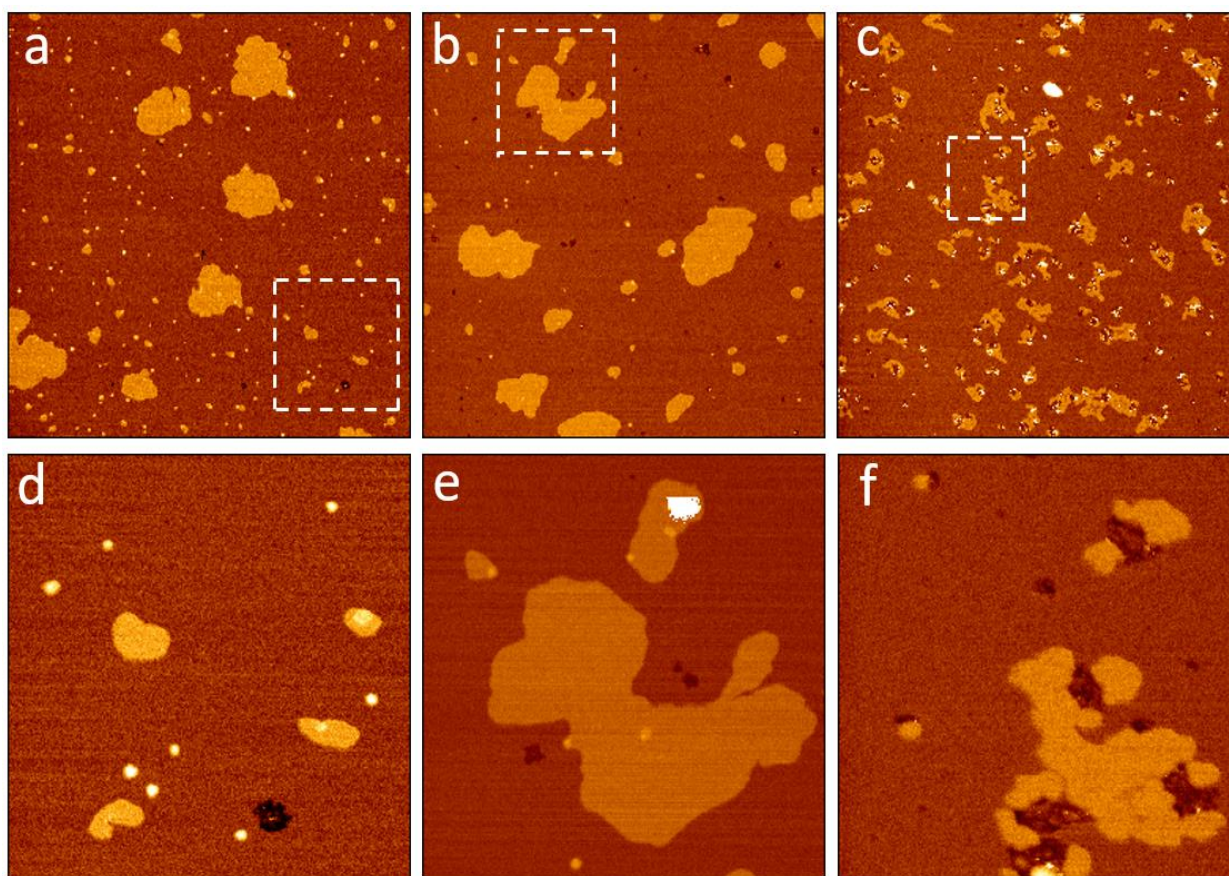


Figure 4.8 | Representative AFM images of mEXT SLBs obtained from liposomes pre-incubated with α -syn before SLB formation, at α -syn concentrations of 1 nM (a,d), 40 nM (b,e), and 200 nM (c, f). Images (d-f) correspond to the framed portions of images (a-c). The image sizes are 7.5 μ m (a-c) and 2.0 μ m (d-f), the Z range is 12 nm. Interaction with the peptide caused the removal of bilayer parts with consequent holes formation.

It is known that the diffusion of lipids is affected by the presence of the supporting rigid substrate. In particular, the interaction between the SLB and the substrate induces a mechanical stabilization of the membrane, restricting its capability of remodeling in the presence of external destabilizing agents, such as α -syn¹⁶². To unbundle the effect of the substrate, an alternative approach, proposed by Canepa et al.¹⁶², was employed. In this approach, first, we let the peptides interact with liposomes in solution for 30 min and formed a planar bilayer after this interaction. In this way, α -syn interacts with free diffusing vesicles, thus better resembling the conditions found

in the cell membrane. Fig 4.8 and Fig 4.9 show the morphology of SLBs formed from mEXT and mINT lipid mixtures, respectively, after preincubation of liposomes with α -syn. In the case of mEXT (Fig 4.8), the protein induced hole formation (i.e., lipid removal) especially at the highest concentrations (40 nM and 200 nM), although small defects were already present at 1 nM α -syn. Again, the fluid phase of the bilayer was mostly involved in the interaction with the protein. In the case of mINT (Fig 4.9), no significant damage to the bilayer was observed. Only at the highest concentrations of 40 and 200 nM, some small defects were present in the fluid phase, but very limited in size and number compared to what was observed in the case of mEXT. The most pronounced effect in these experiments was the appearance of white patches at the domains of the ordered phase, characterized by a constant height of 12.2 ± 0.5 nm. We then performed tapping mode AFM scans on the same areas, and observed that the islands of increased height detected by QI imaging on the ordered phases disappeared, as shown Fig 4.9 d-f.

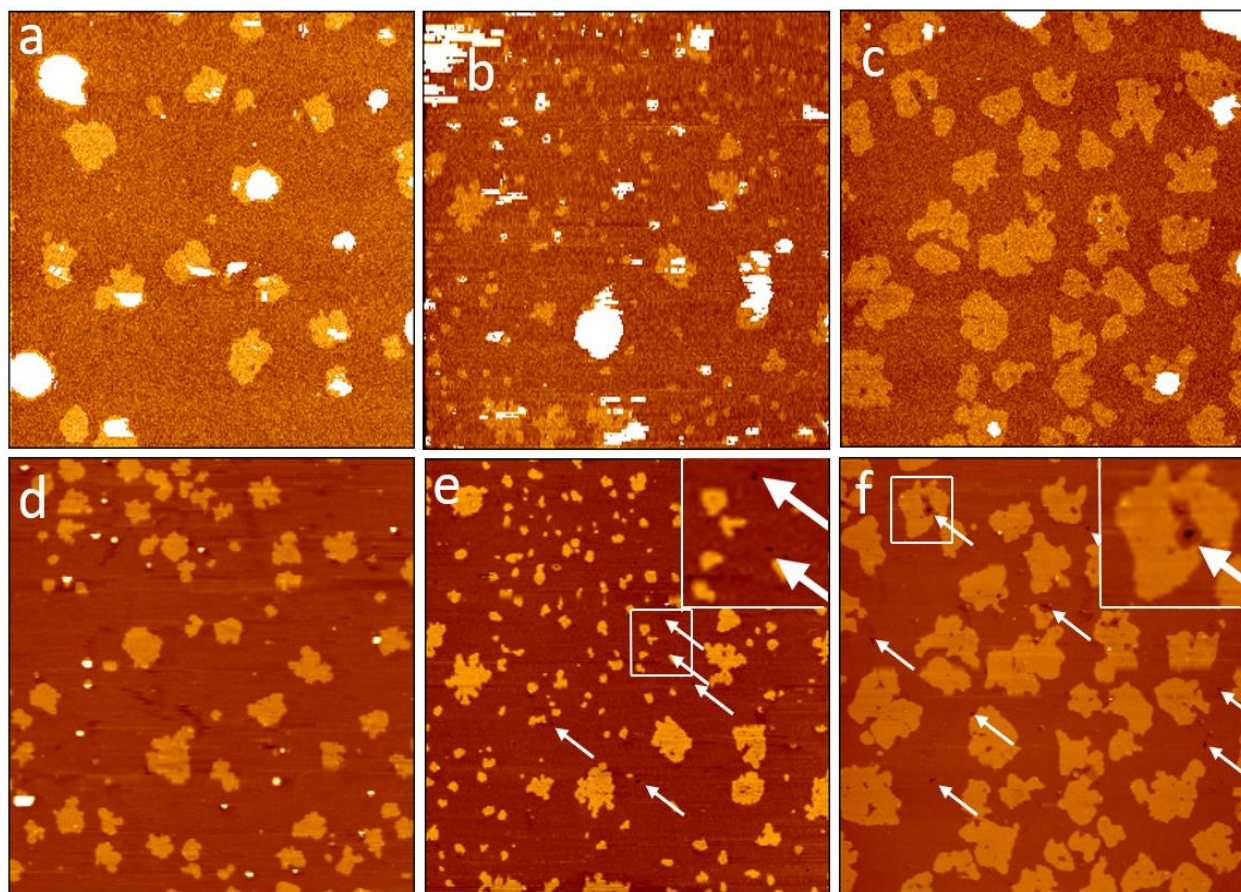


Figure 4.9 | Representative AFM images of mINT SLBs obtained from liposomes pre-incubated with α -syn before SLB formation. The α -syn concentrations employed are 1 nM (a,d), 40 nM (b,e), and 200 nM (c,f). (a,b,c) QI images; (d, e, f) tapping mode images. The presence of α -syn induces small defects in the samples only at high α -syn concentration. The image size is 7.5 μ m, the Z range is 12 nm. In panels e,f, the inset in the upper right corner shows a magnification of the framed image portion.

Force-distance curves in different points of the SLBs were acquired to figure out the nature of such constant-height white patches weakly bound to the ordered phase domains. Force-distance curves on SLBs, shown in Fig 4.10, represent a characteristic discontinuity associated with the tip penetration within the bilayer (breakthrough event) present on the disordered phase (Fig 4.10, points 1 and 2). The ordered phase was not punctured by the AFM tip under the setpoint conditions applied in this experiment¹⁶³. In contrast, when the tip was pushed onto the higher height islands,

two breakthrough events, likely associated with the presence of two bilayers above the ordered phase domains, were detected (Fig 4.10, points 3 and 4). The corresponding tip advancement was 4.0 ± 0.2 nm on the fluid phase domain, compatible with the penetration of a lipid bilayer, and 9.7 ± 0.3 nm on the taller island, compatible with the subsequent penetration of two bilayers by the AFM tip, but slightly lower than the expected thickness of the membrane/membrane patches due to the elastic deformation induced by the AFM tip on the sample before the breakthrough event.

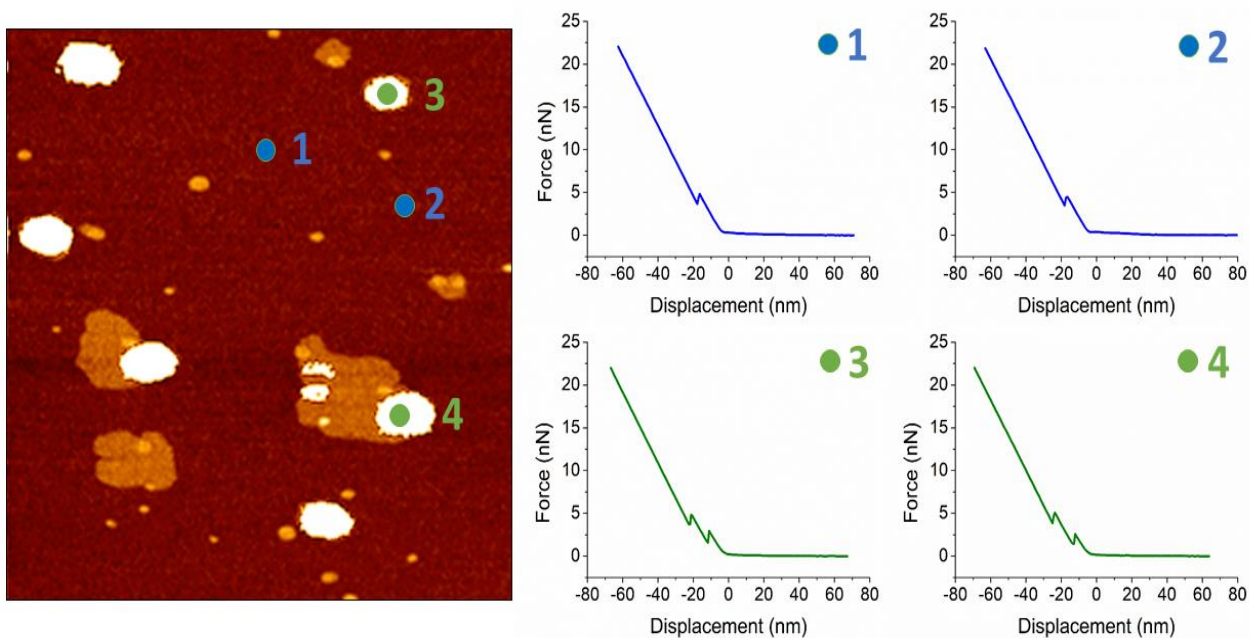


Figure 4.10 | Force distance curves collected in correspondence of different SLB regions. Exemplifying curves extracted from high force setpoint QI data set, acquired on mINT SLBs. These curves have been selected either from the disordered phase background (1, 2), or from ordered domains (3, 4).

4.2.2 QCM analysis

Two lipid systems, including SLBs obtained via vesicle fusion on SiO₂ interface and intact vesicle layers (i.e., supported vesicle layers, SVL) adsorbed on gold were studied by QCM-D. The behavior of each lipid system, composed of mEXT or mINT lipid mixture, after interaction with α -syn was monitored. Following the experimental method applied for the AFM measurement, α -syn was administered at three different concentrations, i.e., 1 nM, 40 nM, and 200 nM. The experiments were conducted in two steps: in step I, lipid systems (i.e., SLB or SVL) were created with the help of two different interfaces, SiO₂ vs Au. In step II, the formed lipid layer was allowed to interact with α -syn.

In case of SLBs, it is indeed well established^{159,164} that multi-domain lipid bilayers based on DOPC and containing negatively charged lipids in PBS spontaneously adsorb and fuse onto a SiO₂ substrate, originating a planar SLB. In contrast, lipid vesicles tend to adsorb and accumulate on the gold sensor, forming a viscoelastic vesicular layer, or SVL (supported vesicular layer).

After the stability of the formed lipid bilayers, α -syn was injected into the chamber, changes in frequency and dissipation were recorded in overnight measurements (step II) as summarized in Fig 4.11 (ΔF vs. t). In the case of mEXT, α -syn first adsorbed to the SLB (Fig. 4.11 a), as indicated by the decrease in frequency recorded in the first 5h; after reaching a minimum value, the frequency increased again, indicating a loss of material from the sensor. This behavior was observed for all α -syn concentrations, but with different kinetics. The mass removal from the surface was faster and particularly pronounced at the highest α -syn concentration (200 nM). The concentration-dependent behavior of the interaction was also observed in the SVL experiments with mEXT (Fig. 4.11 c). Again, the highest concentration of α -syn resulted in a loss of material from the surface after an adsorption phase. A slow and continuous increase in frequency was

recorded at lower concentrations. It is evident that the frequency changes recorded in the experiments for mEXT lipid systems (Fig. 4.11 a, c) were larger than mINT (Fig. 4.11 b, d). This suggests that the inner leaflet lipid mixture has less propensity to interact with the protein, remaining mostly stable in either SLB or SVL form. For the mEXT, α -syn adsorption was accompanied by a disordering effect in SLBs, as it is typically observed when peptides are embedded in the lipid head group region of the bilayer¹⁶⁵. In mINT SLBs, less viscoelastic effect was observed, and protein adsorption, followed by a little mass removal, was detected at the highest α -syn concentration. Interestingly, in the experiments with SVLs and at the low protein concentrations, the initial adsorption of the α -syn to the mINT was followed by a change in dissipation at constant mass, indicating an internal remodeling of the membrane.

At the end of the experiments, in case of SVLs, the integrity of the vesicles was evaluated using a detergent (Triton), injected into the chamber. In all cases, a sudden frequency shift of several hundred Hz occurred, bringing the frequency back to baseline and demonstrating SVL integrity before detergent addition.

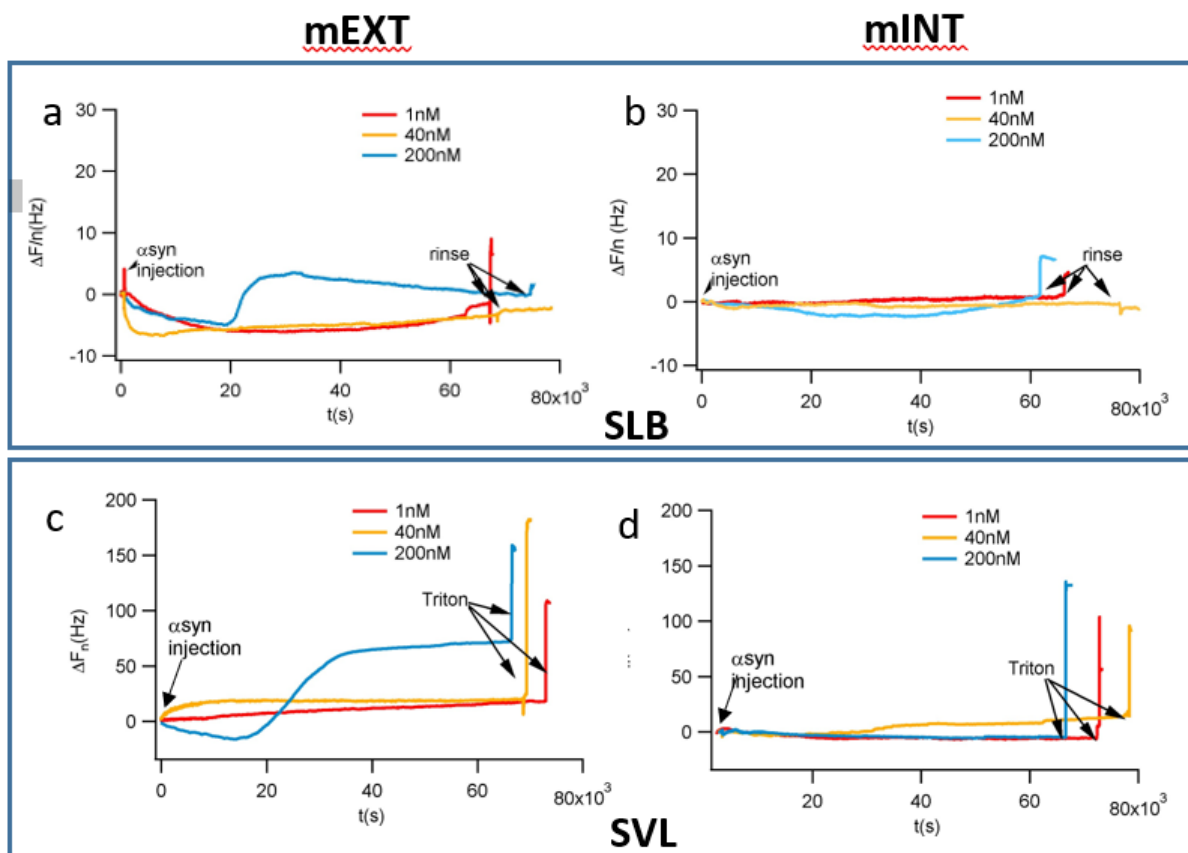


Figure 4.11 | Frequency changes (3rd overtones) recorded by QCM-D after the interaction of SLB (a,b) and SVL (c,d) lipid systems with α -syn at different concentrations. The left panels refer to mEXT and the right panels to mINT.

4.2.3 Vesicle leakage experiments

The ability of α -syn to permeabilize mEXT and mINT membranes was evaluated by conducting calcein leakage experiments. Calcein is easily encapsulated at self-quenched concentration within the vesicle aqueous compartment and readily de-quenches upon external dilution after membrane leakage. Because of this characteristic, calcein is widely utilized for vesicle release assays as a water-soluble fluorescent probe. Being membrane-impermeant, calcein leaks from vesicles only when the formation of membrane permeation pathways, such as pores, occurs. Fig. 4.12 b, c shows typical fluorescence traces obtained from an overnight recording on

mEXT (b) and mINT (c) vesicles, after addition of α -syn at the same concentrations, employed in AFM and QCM experiments.

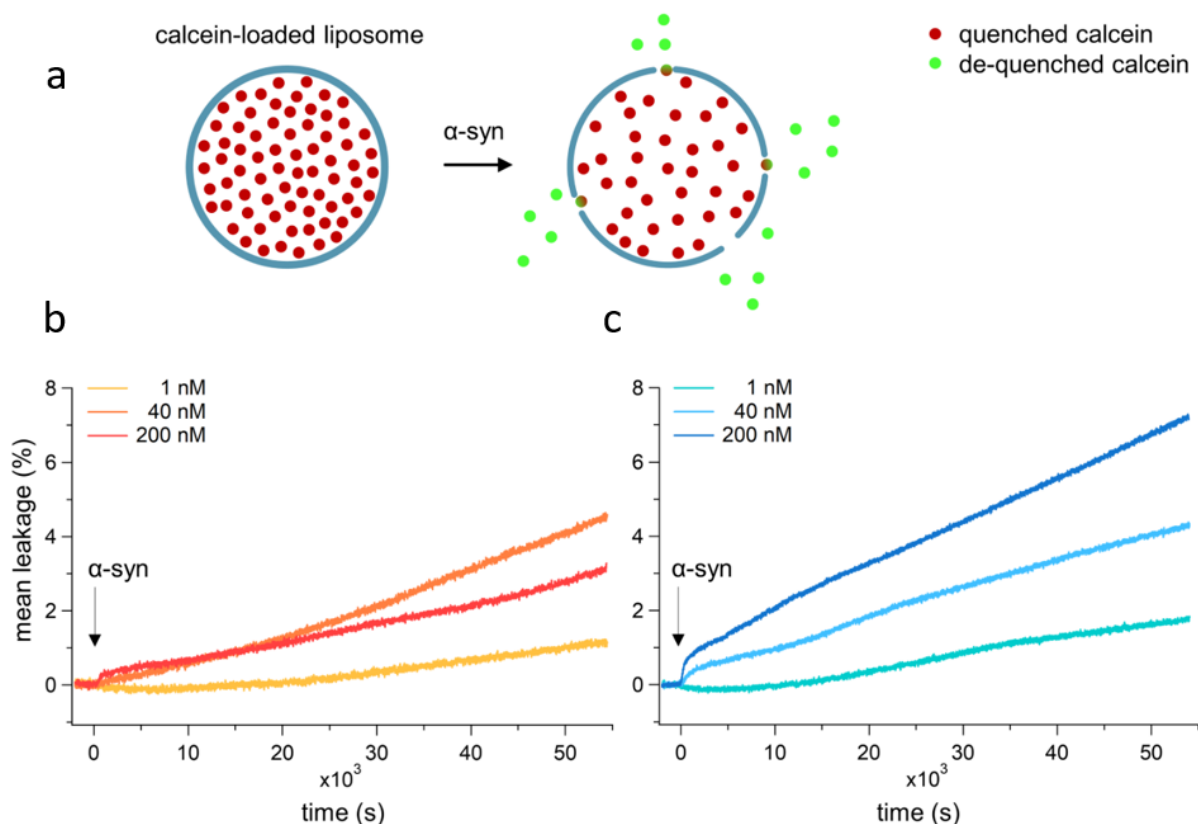


Figure 4.12 | Content release assays on calcein-loaded liposomes. (a) Scheme (not to scale) illustrating a typical calcein-leakage assay. Fluorescence results – expressed as mean leakage (%) (equation 4.1, see Methods) – are reported in (b) for the extracellular membrane and (c) for the cytoplasmic membrane. In all experiments, α -synuclein was added to lipid vesicles after $\sim 1\text{h}30$ min of stirring, and the recording was kept overnight (~ 15 h). In panels (b,c), each point corresponds to an average over a 20 s time interval.

The mean extent of leakage observed in the different conditions is reported in Table 4.1

For both lipid mixtures, increasing α -syn concentration from 1 to 40 nM corresponded to a significant increase in membrane destabilization. Considering the experimental uncertainties, the leakage measured at 200 nM α -syn was not significantly different from that observed at 40 nM α -syn. The observed calcein leakage is indeed due to interaction of α -syn with the liposome

membrane. In fact, no relevant leakage was recorded overnight in control experiments in which the protein addition was replaced with an addition of the same volume of buffer.

<i>Membrane</i>	<i>Cα-syn</i> (nM)	<i>Mean leakage</i> (%)
Outer membrane	1	1.1 $\pm$ 0.4
	40	4.5 $\pm$ 1.6
	200	3.2 $\pm$ 1.2
Inner membrane	1	1.8 $\pm$ 0.7
	40	4.3 $\pm$ 1.6
	200	6.3 $\pm$ 2.3

Table 4.1 Mean calcein leakage induced by different α -synuclein concentrations and recorded immediately before detergent addition. See Methods for full details of statistical analysis.

4.3 Discussion and conclusion

Depending on α -syn location, cytosolic or extracellular space, its interaction with cell membrane could be different due to the typical asymmetry of the plasma membrane. To investigate this behavior, a range of peptide concentration was used starting from a concentration of 1 nM, a concentration that resembles the physiopathological one, to 40 nM, and 200 nM. Moreover, two different lipid mixtures to mimic the composition of the outer (mEXT) and inner (mINT) leaflets of neuronal cell membranes were employed to gain insight into the mechanism of action of α -syn in different situations, e.g., when it acts from the extracellular space or from the cytosol. Both mixtures include phosphatidylcholine (PC), sphingomyelin (SM), and cholesterol (chol), and both mixtures exhibit coexistence of the ordered and disordered phase. The main difference between the two mixtures lays in the localization of the negative charge of the lipid headgroup, which was confined into the ordered domains (GM1) for mEXT, and into the fluid phase (PS) for mINT. Further, the inner leaflet mixture included PE, a zwitterionic lipid characterized by a small head

group, prone to facilitate negative curvature at the bilayer, which confers high fluidity to the lipid membrane and a self-healing towards the membrane pores formation.

In this work, we performed experiments with three different techniques, AFM, QCM-D, and vesicle leakage experiments, to investigate the effect of α -syn on lipid systems of different composition and organization (SLBs, SVLs, vesicles).

The first experimental evidence we acquired was the higher stability against the action of monomeric α -syn of the mINT mixture compared to the mEXT mixture. This property turned out to be common to SLBs with both incubation method, and SVLs. An exception is the vesicle leakage experiment, where the calcein release was comparable for mINT and mEXT mixtures. This experiment, however, is sensitive to the short-term interaction between free diffusing vesicles and α -syn, a different situation from the others used in this work.

AFM images clearly showed a higher stability of the internal leaflet mixture to the action of α -syn. Damage to membranes made of the external leaflet mixture was detected in a concentration-dependent manner by the peptide, with the damage mainly affecting the fluid phase. The destabilizing effect of monomeric α -syn on mEXT SLBs was detected when α -syn was administered at a concentration as low as 1 nM. At the highest concentration, the mEXT SLBs heavily damaged by the action of α -syn after a short-term interaction. The lower tendency of mINT SLBs to interact with α -syn is likely due to the presence of PE, inducing local negative curvature to the membrane, which confers a self-healing capability towards pores/defects formation. In this case, the appearance of the small holes in the fluid phase in the AFM images was evidenced. The membrane damage did not correlate to the localization of anionic lipids (GM1 or PS) confined into different regions (ordered and fluid, respectively) in the employed lipid mixtures. In addition, in case of outer leaflet, AFM images confirmed that the presence of negatively charged GM1

facilitates protein recruitment in the very vicinity of the fluid phase as proposed by Galimzyanov et al. that GM1 is not evenly distributed within ordered domains, its concentration being enriched at the domain boundary ¹⁶⁶. The lower propensity of SLBs formed from the mINT mixture to interact with α -syn was confirmed by the QCM-D investigation as well, where deposition and subsequent loss of material was detected in the mEXT system, but an almost constant frequency response was obtained in the case of mINT SLBs, at all α -syn concentrations under investigation. In this system, little protein adsorption followed by mass removal was observed at α -syn 200 nM, a finding consistent with the small holes appearing in the fluid phase in the AFM images. The destabilizing effect of monomeric α -syn on mEXT SLBs was detected when α -syn was administered at a concentration as low as 1 nM. At the highest concentration, in agreement with AFM results showing the mEXT SLB heavily damaged by the action of α -syn after a short-term interaction, spontaneous removal of large amounts of material after several hours was detected in the QCM-D investigation. Interestingly, the membrane damage was related to the fluidity of the lipid domains and not to the presence of negatively charged lipids.

The greater tendency of SLBs, formed after pre-incubating vesicles with α -syn, to undergo destabilization upon interaction with the protein demonstrated that the presence of the supporting substrate induces a mechanical stabilization of the membrane, hindering the free in-plane diffusion of lipids ¹⁶⁷, and the possibility of their rearrangement upon protein action. The obtained results were similar to the ones observed with pre-formed SLBs: removal of material from the mEXT membrane, and presence of small defects in mINT, in a manner proportional to the protein concentration but to a lesser extent. In QCM-D experiments with SVL, we considered the effect of the interaction of α -syn with intact vesicles, although supported on a substrate and not freely

diffusing. This experiment also confirmed the lower propensity of mINT to be damaged by α -syn, whereas with mEXT at high concentrations of the protein, the damage was so high as to partially destroy the SVL. For SVLs made of mINT, α -syn appeared to change only slightly the viscoelastic behavior of the vesicles, leaving them intact at all concentrations employed. Interestingly, in these experiments, the initial adsorption of the α -syn at low concentration to the mINT was followed by a change in dissipation at constant mass, indicating an internal remodeling of the membrane.

The detection of weakly bound lipid patches on top of the gel domains after the incubation of mINT vesicles with α -syn was a further finding derived by AFM experiments and in agreement with previous observations, confirming that α -syn can bind to vesicles and induce a morphological change or anisotropic curvature strain as well as vesicle aggregation, resulting into tubulation, vesicle budding, and formation of lipoprotein nanoparticles^{52,154}. The nature of such lipid patches, with their extremely uniform thickness and a regular 12 nm height, observed above the ordered domains, was revealed by force spectroscopy on SLBs. They were characterized by a double lipid bilayer and represented by a double breakthrough event in force-distance curves when punched by the AFM tips. This behavior is peculiar of the mINT mixture in which bridging between bilayers is likely to be mediated by α -syn inserted in the lipid vesicles. Besides, PS and α -syn were shown to be able to form lipid-protein particles similar to high density lipoproteins¹⁶⁸. Also, for mEXT, in some cases, the formation of a higher phase on top of the ordered domains was observed. Such higher phase, however, has not a uniform thickness, but it is rough, and the recruiting of material by the negatively charged ordered phase domains is still taking place during AFM scanning, as shown by the comparison of the framed areas in Fig 4.8 b, with the next scans in Fig 4.8 e.

Chapter 5

Correlative AFM-STED nanoscopy applied to the in- vitro study of protein misfolding aggregates

Correlative techniques emerged in the last decades to provide information that one, or the other microscope alone cannot. The integration of different microscopy techniques enables a precise local multifunctional characterization of biological features that no single technique is yet able to achieve ^{169–171}. In particular, the coupling between electron and optical microscopy was successfully applied in material science investigation, was also applied to biological study, but with the elimination imposed by the working condition of the electron microscope, not perfectly fitting with biological system ^{172–175}. However, in the last decade, the integration between AFM and super-resolution (SR) fluorescence microscopy has been proposed to provide fundamental understanding of numerous comprehensive studies in medicine, biology, and material science ^{132,171,176} (Fig 5.1). Integration of AFM with STED enables high-resolution fluorescence, topographical, and nano-mechanical imaging of living systems, such as cells, or dynamic molecular processes, in a physiological-like environment, and with resolution at the molecular scale. In the first attempts ^{132,176}, the main idea was to provide chemical specificity to the AFM, by coupling it with a fluorescence technique with a comparable lateral resolution. More recently, new results demonstrated that the AFM could provide a deeper control to validate the results deriving from fluorescence microscopy, and it can drive the experiments toward more accurate and less invasive methods of labeling.

Although the final influence of the fluorescent dye on the biological processes under investigation is hardly predictable, it is well known that the covalent linking of fluorophores to the molecular system under investigation can interfere with biological processes^{177,178}. I focused on amyloid aggregates formation. Several studies observed that the kinetics of aggregation was slightly delayed by the presence of a fraction of labeled monomers, at an extent that is proportional to the ratio of labeled molecules^{32,179}. By coupling the AFM to a stimulated emission depletion (STED) fluorescence microscope, it has been demonstrated that unexpected phenomena could take place during the aggregation of a partially labelled peptide solution, caused by the structural changes induced in the molecules¹⁵⁰.

Following the standard methods to induce the aggregation and to label the molecules at different dye to protein ratios, it was shown that starting from a partially labeled solution of insulin or beta-amyloid (A β) monomers, only a fraction of the final fibrillary aggregates was fluorescent¹⁵⁰. The results suggested the coexistence of different aggregation pathways. Labeled molecules can follow just some of these pathways.

These results are important since they highlight a selectivity in peptide aggregation induced by the presence of the small dye molecule. On the other hand, this effect was proved for a limited number of peptides, in particular for small peptides such as insulin and β -amyloid (MW~ 6kDa, and 4kDa, respectively); besides, in the case of insulin, we covalently bind the fluorophore to free amines, i.e., more than one fluorophore can bind to a single peptide, and the addition of the dye molecule strongly changes the mass of the peptides. For these reasons, the phenomenon described cannot be considered as a general issue.

In my research work, I tried to extend the analysis proposed by Cosentino et al., considering the in vitro aggregation of α -syn, the amyloidogenic protein responsible for Parkinson's disease

(PD), which is significantly larger (MW~ 14kDa) with respect to insulin, previously investigated. Furthermore, the peptide labeling was performed by using a site-specific labeling method. In this well-controlled method, the structure of the α -syn monomer is modified, introducing a cysteine residue at the C-terminus of the peptide. The fluorophore covalently binds to the free cysteine at the C-terminal group of the peptide. C-terminal domain of α -syn is considered not to be or scarcely involved in fibrillation¹⁸⁰. Our aim was to verify whether using this approach all the aggregates were fluorescent, i.e., the aggregation process is a stochastic process where the labeled and unlabeled molecules are acting as a same population of monomers. As control experiments, we also studied the aggregation of α -syn labeled with an NHS-dye, following the same procedure employed by Cosentino et al., to reveal whether the increased mass of the peptide could be sufficient to preclude any influence induced by the presence of the small dye molecules, or, whether, on the contrary, the fluorophore position in a particular part of the polypeptide chain is the key point towards the definition of a reliable experimental method.

5.1 Materials and methods

5.1.1 Site-specific labelling of α -syn and fibrillation

To produce ATTO 488-labelled fibrils, an α -syn monomer, with Gly-Cys residues at C-terminal (α -synGC) was prepared as described previously¹⁸¹ and labeled with Thiol-Reactive ATTO 488-Label (Maleimides, ATTO-TEC GmbH) following a “solid state-based labeling” technique¹⁸². Briefly, expressed, and purified α -synGC cysteine dimers were reduced with 5.23 mM TCEP (tris(2-carboxyethyl) phosphine (Sigma-Aldrich) in NaP buffer 100 mM at pH 7 for 6 hours at RT. Resulting monomer containing solution was diluted 1:1 with MQ water and EDTA (1mM) pH 8, followed by the addition of 70% ammonium sulfate salt (w/v). Labelling reaction

was achieved by adding 7 molar equivalents of Thiol-Reactive ATTO 488-Label dye (in DMF) and incubating at RT ON, no agitation. Labelled asynGC was precipitated by centrifugation (20.000 $\times$ g, 10 min, 4°C) and the pellet was washed five times with NaP buffer 50 mM pH 7.0 added with 70% ammonium sulfate. Finally, the pellet was resuspended in PBS and the exceeding dye was removed through PD-10 column (GE Healthcare). ATTO 488 labelled α -synGC was confirmed by RP-HPLC (protein labelling more than 90%). ATTO 488-labelled α -syn fibrils were obtained from LPS free recombinant human α -syn monomers and ATTO 488 labelled α -syn, incubated in 1:20 ratio at a final concentration of 5 mg/ml in sterile PBS (Biowest). Fibrils were recovered by centrifugation after shaking the sample (ThermoMixer F1.5 Eppendorf, 1000 rpm) at 37°C for seven days. Labeling and fibrillation of α -syn were performed in collaboration with the group of Prof. Bubacco in the Department of Biology of the University of Padova.

5.1.2 Labelling α -syn with ATTO NHS-esters and fibrillation

To produce ATTO 488-labelled fibrils, an α -syn WT monomer was prepared as described previously¹⁸¹ and labeled with NHS ATTO 488-Label (ATTO-TEC GmbH) following manufacture instructions. Briefly, expressed, and purified α -syn WT protein were labeled with 1.5 molar equivalents of NHS-Reactive ATTO 488-Label dye (in DMSO) and incubating at RT 2 hours, no agitation. To remove the exceeding dye reaction was loaded in a PD-10 column (GE Healthcare) and only the conjugated protein was collected.

ATTO 488-labelled α -syn (NHS) fibrils were obtained from LPS free recombinant human α -syn monomers and ATTO 488 labelled α -syn (NHS), incubated in a 1:20 ratio at a final concentration of 5 mg/ml in sterile PBS (Biowest). Fibrils were recovered by centrifugation after shaking the sample (ThermoMixer F1.5 Eppendorf, 1000 rpm) at 37°C for seven days.

Labeling and fibrillation of α -syn were performed in collaboration with the group of Prof. Bubacco in the Department of Biology of the University of Padova.

5.1.2 AFM measurements

AFM control measurements were performed by using a Nanowizard III (JPK Instruments, Berlin, Germany). Images have been acquired in air in intermittent contact mode, using rectangular silicon cantilever (TESPA, Bruker, MA, USA) with nominal spring constant 42 N/m, resonance frequency 320 kHz, and a tip with curvature radius 8 nm. All images were acquired as 512 x 512 pixels images with a scan rate between 0.6 and 1.0 Hz. The fibrils thickness was determined by using the JPK Data Processing (JPK Instruments, Berlin, Germany). The statistical analysis was obtained with the help of OriginPro 2020 software.

5.1.3 Correlative AFM-STED

A NanoWizard II AFM head (JPK Instruments, Berlin, Germany) was mounted on a commercial Leica TCS SP5 gated STED-CW microscope (Fig 5.1a). The optical microscope stage was exchanged with an AFM compatible stage (JPK Instruments, Berlin, Germany), which features minimum mechanical coupling of noise to the AFM cantilever.

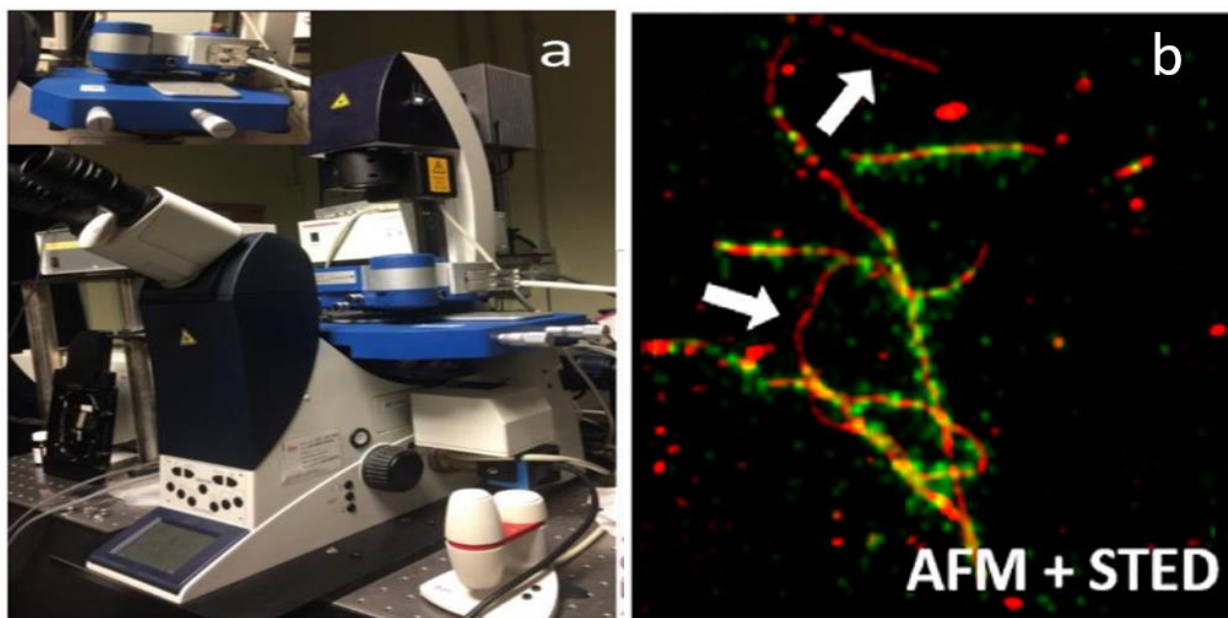


Figure 5.1 | (a) Representative correlative AFM/STED setup with NanoWizard II AFM stage and head in the inset. (b) The STED image of Insulin amyloid fibrils labeled with ATTO488-NHS, overlaid with AFM. The white arrows indicate two aggregates that are present only in the AFM image.

This procedure is made simple by the availability of suitable mountings for microscope commercial models. The Direct Overlay software, which is available in the AFM software, uses the automatically recognized cantilever position to map the optical image and calibrate it before importing the image into the SPM software. It requires an image calibration file created for an objective and field-of-view from an image sequence of AFM movements.

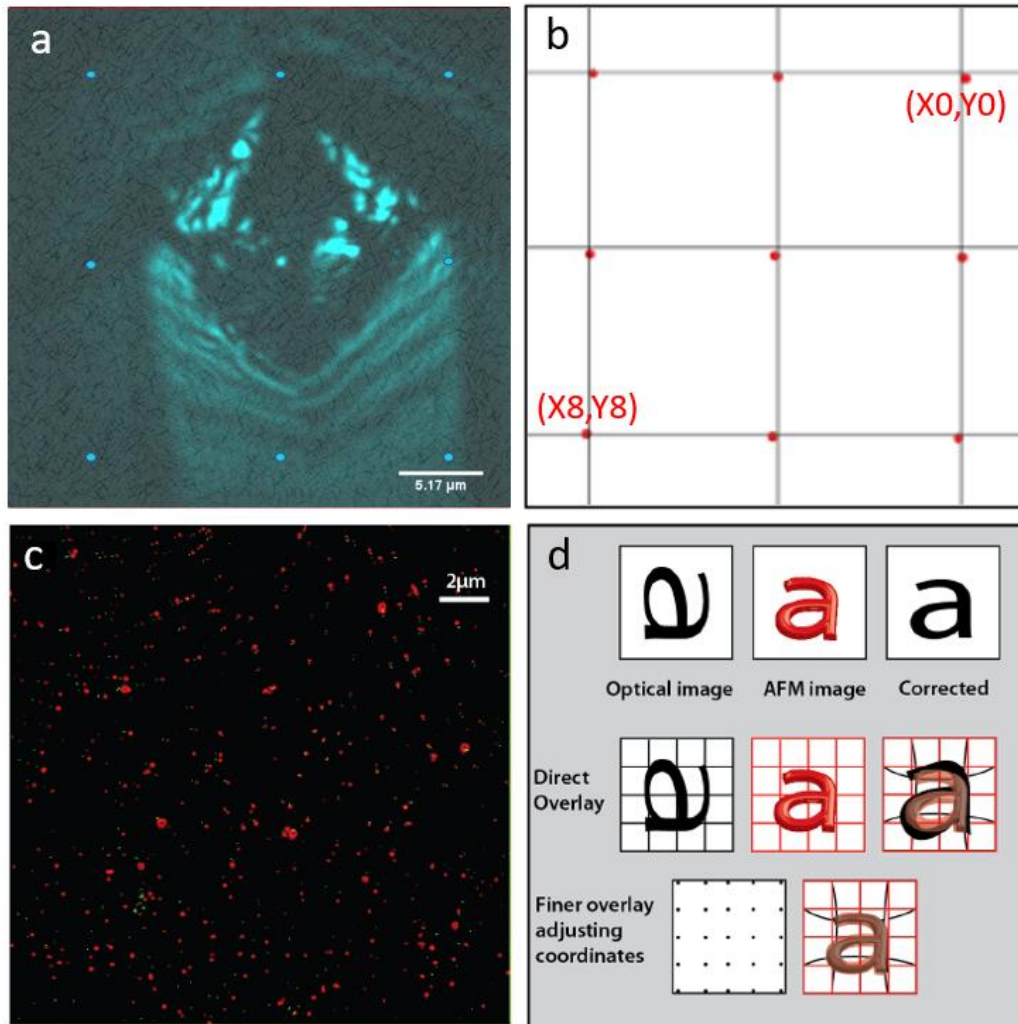


Figure 5.2 | Representative direct overlay procedure. (a) Panel a show the reflection images of the tip apex, made on a 30x30 μm sq area of optical field of view by moving the tip in 25x25 μm sq in 9 different positions. (b) Panel b shows how the grid is formed in the AFM software by a calibration. (c) Panel c is an overlay of AFM on STED image using these calibration points given by AFM software. (d) Panel d shows the total overlay procedure. The first row of the panel shows a cartoon of the collected optical image with inherent aberrations, AFM, and the corrected image. The second row depicts the direct overlay software, which calibrates the pictures and makes a grid automatically. The third row shows a finer overlay, obtained by adjusting these grid points manually. (Image modified from doi:10.1371/journal.pone.0066608.g001).

In detail, this overlay image is a grid of at least 9 points which nonlinearly shifts, rotates, and stretches the optical image to correct for all possible optical aberrations. The AFM software makes this grid from sequential reflection images of the tip's actual movement in specific piezo positions with a finite pixilation and scan size. The grid has been done for a 30x30 μm square area

(shown in Fig 5.2b) and the AFM software visualizes the respective overlay of AFM on 25x25 μm square area inside this calibrated area.

The STED Images have been collected by a Hybrid detector in the spectral range 500-570 nm. We have used an HCX PL APO CS 100x 1.4NA oil objective (Leica Microsystems, Mannheim, Germany) and a scan speed of 1400 Hz for 1024 pixel per line with a 128-line average and a time-gated detection of 1.4 ns. The selected dye was ATTO 488 for α -syn, which is suitable for 488 nm excitation and 592 nm depletion wavelengths.

AFM Images have been acquired in air in intermittent contact mode, using rectangular silicon cantilever (TESPA, Bruker, MA, USA) with nominal spring constant 42 N/m, resonance frequency 320 kHz, and a tip with curvature radius 8 nm. All images were acquired as 512 x 512 pixels images with a scan rate between 0.6 and 1.0 Hz.

The level of co-localization between AFM and optical images is calculated by using the colocalization function of ImageJ (Bethesda, USA) and deriving the ratio of colocalization. The ratio of colocalization is the ratio between the number of co-localized pixels and the total number of pixels that composed the fibrils in the AFM images. In particular, the Colocalization Threshold plugin was used. The threshold was automatically determined by the software using the Costes auto threshold method¹⁸³. The scatter plots of the pixel-by-pixel correlation were also calculated using the same ImageJ function.

5.2 Results

Correlative AFM-STED technique has been applied in the study of α -syn fibrils, a protein significantly larger (MW~ 14kDa) with respect to insulin, previously investigated. An example of correlative AFM-STED experiment, performed in vitro, is shown in Fig 5.3. STED microscopy

visualizes α -syn fibrils, covalently labeled based on a site-specific labeling method, introduced by Pinotsi et al. and described in materials and methods.

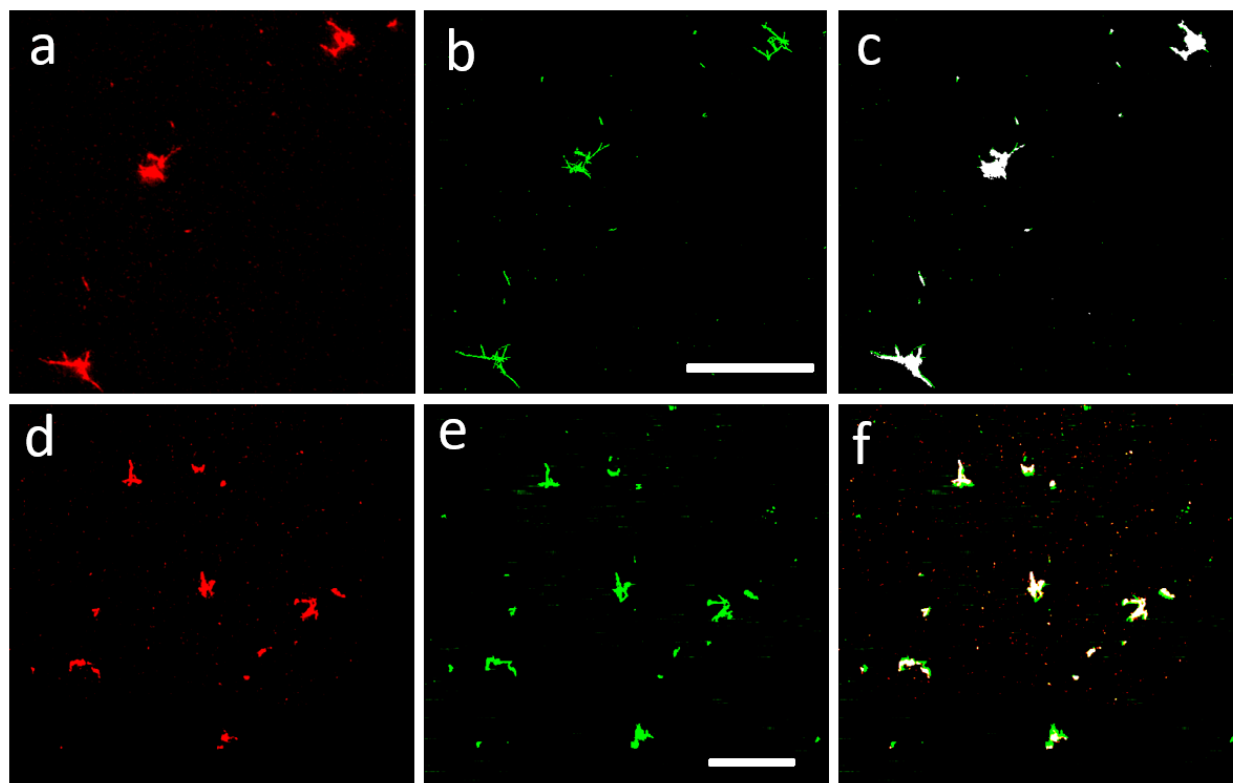


Figure 5.3 | Representative α -syn fibrils site-specific labeled with ATTO488 with a functional maleimide at a dye-to-protein ratio of 1:20 visualized by STED microscopy (a, d), AFM (b, e), and the correlative AFM-STED microscopy (c, f). All the population of fluorescent fibrils visualized by AFM are also visible in STED image, confirming that the presence fluorescent dye does not interfere with the aggregation process. The ratio of co-localization is 0.94 (c) 0.73 (f). Scale bar: 5 μ m. Z range in AFM image: 1.96 nm (b) 5 nm (e).

We observed that all the fibrillar aggregates displayed by AFM were also visible in the STED image. The entire population of α -syn fibrils within the sample was fluorescent (Fig 5.3), verifying that the fluorescent dye labels do not interfere with the aggregation process in a site-specific labelling method.

The co-localization ratio between AFM and STED of all the acquired images has been quantified to 0.71 ± 0.32 , calculated from 29 images derived from 15 different samples. However,

I noted a failure of superimposition in some images, likely due to the non-perfect correction with Direct Overlay (Fig 5.3 f). This slight mismatching could be negligible on micro-sized sample features, but it is particularly important in α -syn fibrils samples. Due to the very small size of fibrils, a misalignment in the order of tens of nanometers results in a significant decrease in the co-localization ratio that affects the average values considering the whole dataset. Repeating the analysis, excluding the images that have a clear mismatch due to a non-ideal correction of the optical image, we obtained a colocalization-ratio of 0.92 ± 0.05 , close to 1 (calculated from 9 images).

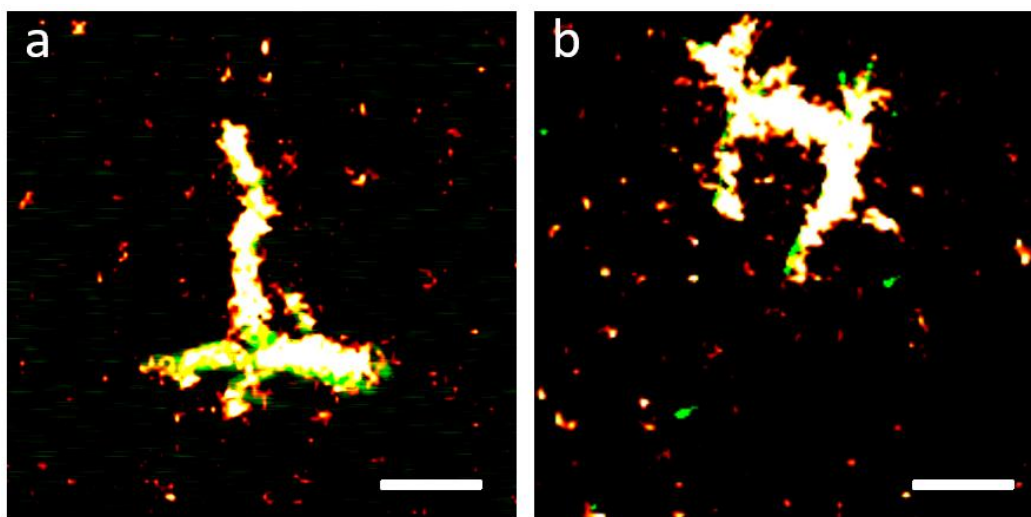


Figure 5.4 | Representative correlative AFM-STED images of α -syn fibrils at a dye-to-protein ratio of 1:20. The scale bars are 500 nm. The ratio of co-localization is 0.923 (a) 0.942 (b). Generally, the colocalization ratio for the images with small field of views is higher than for larger field of views. The effect of optical aberrations, partially remaining after the calibration procedure, is more evident on large size field of view.

Fibrils have a variable length and the thickness, slightly not affected by the presence of the fluorescent dye. In particular, the calculated mean thickness for α -syn fibrils with dye-to-protein ratio 1:20 was 9.7 ± 2.3 nm and for unlabeled ones 9.6 ± 3.5 nm. In both cases, fibrils are relatively short, showing a length above the micron rarely.

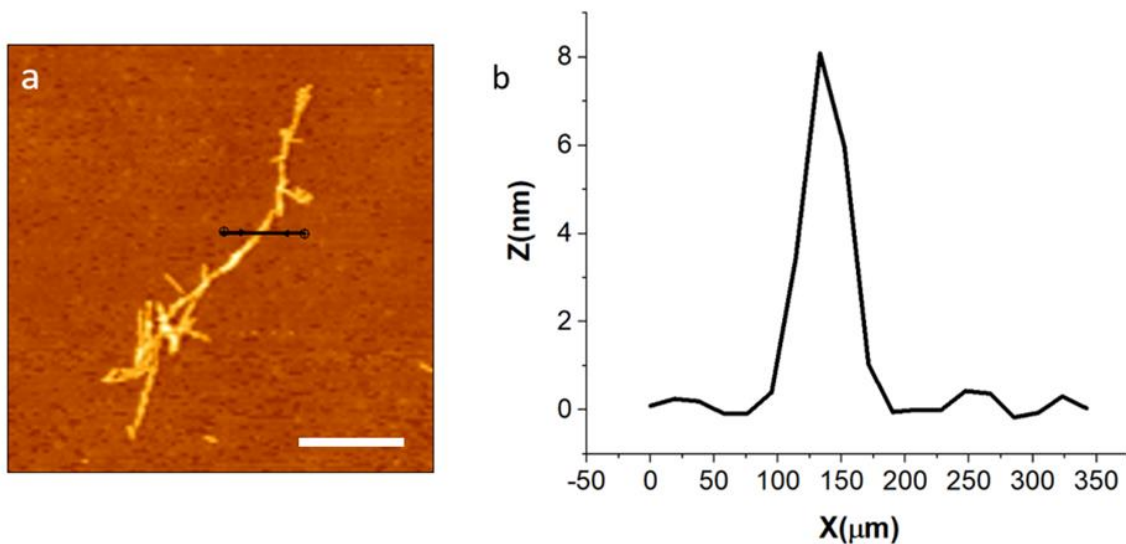


Figure 5.5 | The typical cross-section of the α -syn fibrils, measured by AFM. (a) AFM image in dry condition of α -syn fibrils structure (Scale bar: 500 nm. Z-range: 5 nm). (b) The profile cross-section indicates a height of 8 nm.

Figure 5.6 shows fibrils obtained from alpha-syn labeled with ATTO488 NHS. A large population of unlabeled fibrils is present and clearly displayed in the image, confirming the coexistence of different aggregation pathways in which labeled molecules can follow just some of these pathways which agreed with what was previously observed in the experiments performed on insulin fibrils¹⁵⁰. The ratio of co-localization calculated on 8 images from 6 different samples were 0.32 ± 0.2 .

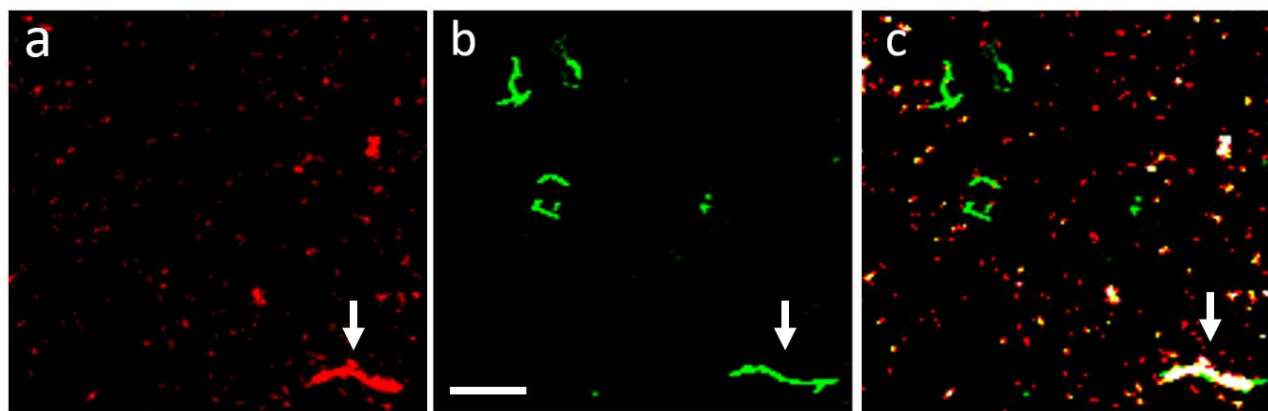


Figure 5.6 | Representative α -syn fibrils labeled with ATTO488 NHS at a dye-to-protein ratio of 1:20. STED image (a), AFM image (b), and the correlative AFM-STED image (c) of the

same area of interest. A large population of fibrils are not fluorescent in STED image compared to AFM image. Only one fibril is displayed with both imaging modalities (see arrows). The scale bar is 1 μm (a–c). The ratio of co-localization is 0.648. Z range in AFM image: 1.69 nm.

5.3 Discussion and conclusion

The characterization of aggregation process of amyloidogenic peptides by advanced correlative techniques is a new way of shedding light on understanding high heterogeneity and multi-pathways behavior of the aggregation process ¹⁸⁰. In particular, the mutual and complementary abilities of correlative AFM-STED nanoscopy is enabling the observation of unexpected phenomena, happening behind the formation of fibrillar aggregates. A previous study, performed by Cosentino et al. ¹⁵⁰ on insulin and β -amyloid peptides suggested that the presence of fluorophores not only decreased the kinetics of the aggregation, a very well-known effect, but also promoted the coexistence of different aggregation pathways, and that labeled molecules can follow selectively just some of these pathways. On the other hand, both insulin and β -amyloid ($A\beta$) are small peptides (MW~ 6kDa, and 4 kDa, respectively), furthermore, in the case of insulin, the fluorophore is covalently bound to the free amines. Considering that all the lysine residue presents an amine group, more than one fluorophore can bind to a single peptide, with a maximum of 2 fluorophores per each insulin molecule; one lysine is present in the insulin polypeptide chain. For these reasons, the phenomenon described cannot be considered as a general issue. In my work, we investigated the in vitro aggregation of α -syn, which is significantly larger (MW~ 14kDa) with respect to insulin and $A\beta$, previously investigated. A site-specific labeling method has been used to covalently label the C-terminal group of the peptide, an area of the polypeptide chain that is considered not or scarcely involved in fibrillation, as suggested in previous works ^{180,181}. In this way, a single fluorophore is present per each monomer and in a controlled position along the

protein polypeptide chain. To follow the procedure proposed by Pinotsi et al., in this labeling strategy, the structure of the α -syn monomer must be modified, introducing a cysteine residue at the C-terminus of the peptide. We employed ATTO488 with a functional maleimide group that covalently bind to the free cysteine at the C-terminal domain. The peptide was labeled at the dye-to-protein ratio 1:20.

Looking at the AFM and STED images acquired on the same areas, it is clear that all the aggregates display in the AFM images are also visible in the fluorescence images. Despite this, the co-localization ratio, the ratio between the number of co-localized pixels and the total number of pixels that composed the fibrils in the AFM images, calculated from all the AFM and STED images acquired, has been quantified to 0.71 ± 0.32 . Looking at some of the correlated images in details, I noted a failure of superimposition in some parts of the fibrils between AFM and STED images, which is likely due to the non-perfect correction with Direct Overlay, and that results in a significant decrease in the co-localization ratio, with a particular extent on large field of view (Fig 5.3 f). I decided to consider only the images that displayed a good correction of the optical images after calibration. Considering this sub-group, the co-localization ratio is 0.92 ± 0.05 .

From the analysis, I deduced that the fibrillary aggregates are all fluorescent (Fig 5.3), indicating that the labeled and unlabeled molecules are acting as a same population of monomers, indistinctly contributing to the fibrillation process. My work suggested the adoption of site-specific labelling method designed for each peptide.

As a control, I also tried to label α -syn with ATTO 488-NHS. In this case, I observed that a large population of fibrillary aggregates were not fluorescent, bolding coexistence of labeled and unlabeled pathways and a selectivity in peptide aggregation induced by the presence of the small

dye molecule. The ratio of co-localization was calculated to 0.32 ± 0.2 on 7 images from 6 different samples.

This work provides evidence of a fundamental drawback in fluorescence microscopy, generating a warning for all the fluorescence users: a deeper control for possible artifacts induced by the fluorophore is needed. Overall, these results indicate that labeling artifacts can be avoided, applying labeling strategies designed to the molecular system under investigation. The use of a correlative label-free microscopy technique would play a key role in the control of the setting of these conditions. In fact, the integration between AFM and super-resolution fluorescence microscopy represents not only a complement but a fundamental tool to validate the results deriving from fluorescence microscopy, and that it can drive the experiments towards more accurate and less invasive methods of labeling.

Chapter 6

A future perspective: correlative light and electron microscopy (CLEM)

The application of STED-AFM correlative nanoscopy indicates that the in-vitro fibrillation of small peptides is in some cases affected by the presence of a sub-population of fluorescent monomers. In my work, I went deeper in the analysis of these kind of processes, demonstrating that such affections can be avoided by adopting a correct approach for the direct labeling of the molecules. In particular, I found that binding the fluorophore to amines group via NHS-ester functional fluorophore interferes with the aggregation process of the peptide, characterized by the visualization of a large population of unlabeled fibrils. My work confirmed what suggested by Cosentino et al.¹⁵⁰, extending the results to larger peptides. A question is still open: what is the difference, if any, between the fibrils that contain labeled monomers and the others? The AFM was not able to answer this question, also due to the fact that when it is mounted on an optical stage for correlative microscopy, the AFM is inherently noisy, precluding the acquisition of high-resolution images. Trying to solve this issue, in the last part of my activity, I conceived a new approach, based on the correlation between optical images and transmission electron microscopy (TEM) images. These two techniques cannot be exploited simultaneously, at least in our laboratory, and in general exploiting the maximum capability in terms of resolution of TEM. However, TEM is able to acquire images faster than the AFM, hence, to investigate a large portion of the samples in some minutes. My idea was to deposit fibrils on a TEM finder grids that help the identification

of the same areas under investigation. I studied these samples with optical microscopy and after with TEM, trying to identify the same structure with both techniques. In principle, also with TEM, it would be possible to display both fluorescent and non-fluorescent fibrils. TEM could be able to show some morphological/structural differences between the two fibrils population.

This is a short Chapter since this activity, started in the last year of my Ph.D., is still at an initial stage. Despite this, I think that it is important to show this new approach that provided promising, although preliminary results.

6.1 Electron microscopy

In 1920, a French physicist, Louis de Broglie, discovered that accelerated electrons in vacuum can act as light while the wavelength of these electrons is about 100,000 times smaller than the wavelength associated to photons. This was experimentally proven, in the year 1927, by Clinton Davisson and Lester Germer using electron beam as a source of particles, behaving as waves, forming a diffraction pattern which was significantly smaller than the one caused by photons. It is also discovered that electric and magnetic fields influence electrons similarly to how lenses and mirrors influence the light. Ernst Ruska, who assembled the first electron microscope in 1931, was awarded Nobel prize in physics more than 50 years after his invention.



Figure 6.1 | The electron microscope built by Ruska (in the lab coat) and Knoll, in Berlin in the early 1930s. (Image from ¹⁸⁴)

In electron microscopy, an electron beam is produced by an electron gun in which a tungsten filament can be placed as the electron source. The beam is produced by heating the filament up to 2,700 °C and connected to high voltage. The electrons are accelerated from the cathode to the anode by high voltages (20-300 kV). The higher the voltage, the higher the energy of electrons. However, to reach a high resolution, the accelerating voltage and a series of magnetic lenses must be highly stable. Since electrons move freely only in a vacuum, there must be a vacuum in the entire column. The difference between common air pressure and residual pressure in the microscope is about 10 orders of magnitude. Since the electrons are charged particles, they can be easily focused on the examined sample using a positively charged anode and series of electromagnetic lenses in an optimal way. A positively charged anode in front of the electron gun

drives the electrons moving towards the sample. During its entire journey, the electron beam goes through several apertures with various diameters. These apertures stop electrons undesirable for creating the image. According to the way the electron beam hits the sample, two types of electron microscopy are distinguished into scanning electron microscopy (SEM) and transmission electron microscopy (TEM) which are the highest spatial resolution imaging techniques.

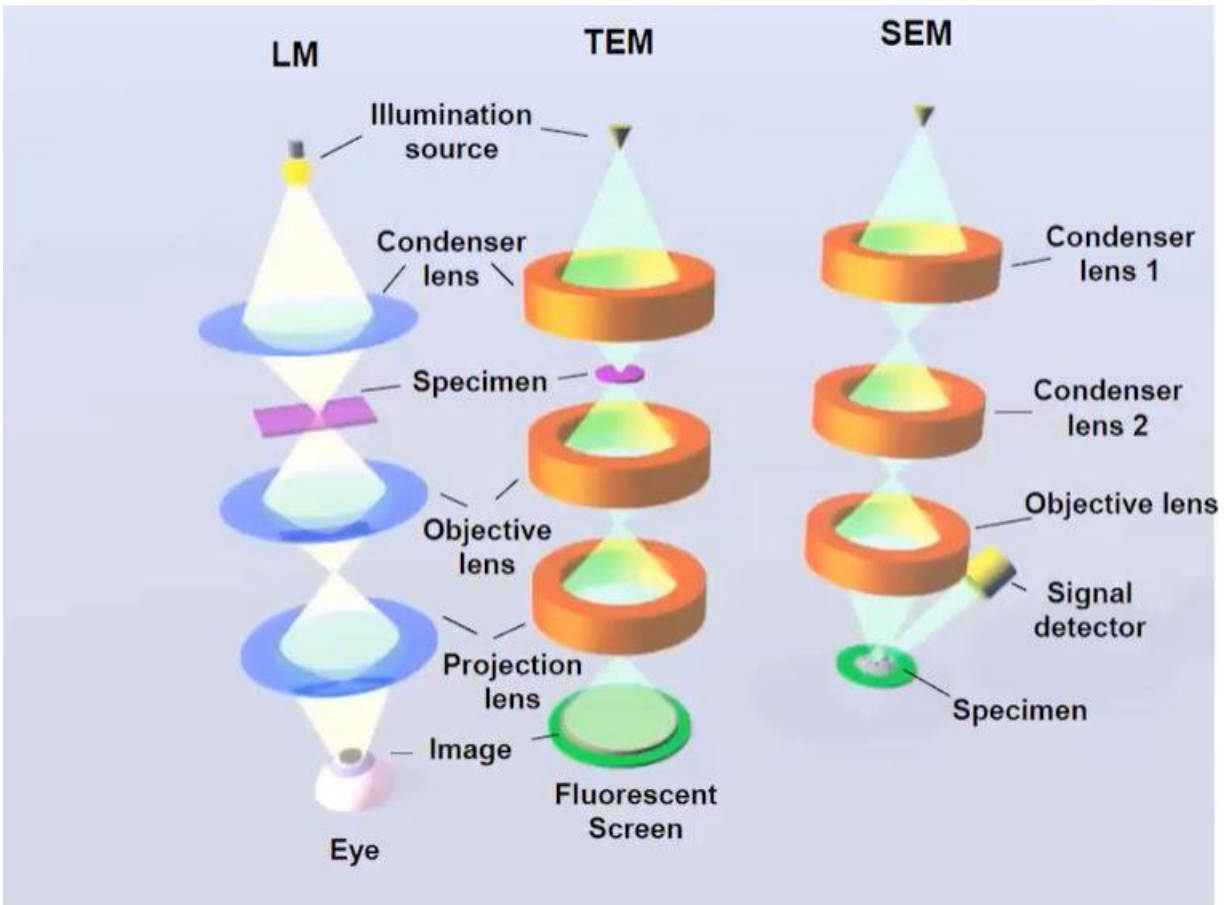


Figure 6.2 | Comparison between the lens configurations for imaging mode in light microscopy, TEM, and SEM.

6.1.1 Transmission electron microscopy (TEM)

In TEM imaging mode, electrons have very high energies in the order of hundreds of keV, meaning that they move at close to the relativistic speed to penetrate through the sample and come

out the other side in order to form a bright field image. In fact, the incident electron beams with uniform intensity go through a thin sample, bounce off the nucleus of the atom, and be bent. This is a process of elastic scattering in which electrons do not change energy, speed, but change the direction to generate the contrast in the image. The electrons, which go through the atom and interact with the electrons in the sample, lose their energy and dissipate it to the sample. These inelastically scattered electrons only contribute as noise in the image, while at the same time damaging the sample. TEM can visualize the sample using elastically scattered electrons to generate contrast in the image by means of an aperture. The aperture is placed after the objective lens, allowing the elastically scattered electrons to go through, but it blocks the un-scattered ones, and that generates the contrast in the image (Fig 6.4). Basically, regions with high density of scattering appear dark and regions with less density of scattering appear bright in a TEM image.

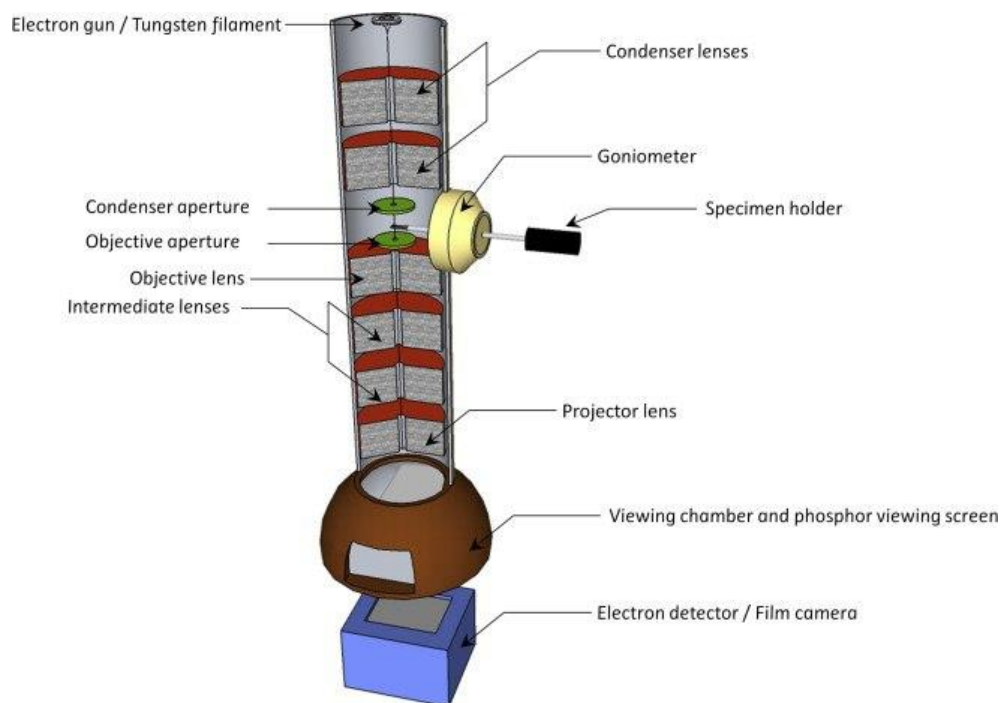


Figure 6.3 | Schematic diagram of vacuum column in TEM with all the lens configuration inside. (Image from ¹⁸⁵)

Fig 6.3 shows the schematic lens configuration for imaging mode in TEM. As shown, moving electrons from electron gun as a source of electrons, are focused on the desired size of the specimen using the condenser. The magnification is controlled by condenser lens and how large an area is illuminated by the beam. The refracted electrons by the specimen are collected by the objective lens. The electrons from the objective lens move to the projector lens which projects the image of the specimen on the fluorescence screen, film, or CCD camera. Notably, in TEM imaging mode, since the beam must go through the sample, the samples must be thin in the order of hundred nanometers thickness. The sample is mounted on the copper grids of about 3mm across and loaded in special sample holders. The ultimate resolution of the TEM is dictated by the wavelength of the electrons.

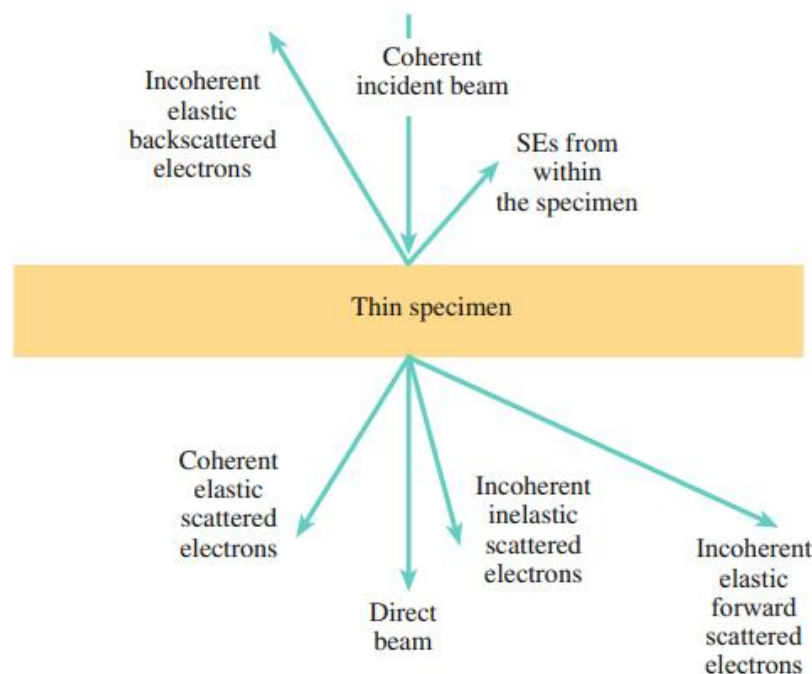


Figure 6.4 | Different kinds of electron scattering from a thin specimen. A thin specimen permits electrons to be scattered in both the forward and back directions. (Image modified from ¹⁸⁴⁾

6.1.2 Negative staining in TEM microscopy

High vacuum in the column of the electron microscope is not suitable for biological investigations. Besides, the atoms that biological materials are made of, nitrogen, carbon, and oxygen have the same scattering power as the water is surrounding them. In another word, they have very low intrinsic contrast. Ultimately and most importantly, the biological samples are very radiation sensitive, meaning that when inelastic scattering occurs, the sample gets ionized, generating radicals that move around the sample and break all the chemical bonds within the sample. Therefore, in TEM, the samples are usually stained with uranyl acetate. In this case, the sample is embedded in a low concentration of a salt solution of a very heavy atom, typically uranium. The solution is then dried to a thin layer and introduced into the electron microscope. In this case, the uranium generates very high contrast in the image.

6.2 Insulin: a model protein for the in vitro study of amyloid fibrils

Insulin was used as a model system to study the formation of amyloid fibrils. Insulin from bovine pancreas is not considered an amyloidogenic peptide since it is not involved in any protein misfolding disease in-vivo. Insulin aggregates in-vitro after inducing a destabilization of its native structure, producing amyloid aggregates that have the same morphological and structural (they have a cross- β structure) properties of the typical fibrils found in brains affected by Alzheimer's, or Parkinson's diseases. These fibrils also show the same tinctorial properties, while early aggregates display a toxicity pathway that resembled that of A β oligomers. Insulin is a small peptide (51 amino acids) with a molecular weight of $\sim$ 6kDa. Insulin is a good model to study amyloid fibrils formation,

since it forms long fibrils that bind strongly to negative charged surfaces such as mica or plasma treated glass, being an ideal system for AFM and fluorescence microscopy investigation.

6.3 Materials and methods

6.3.1 Insulin labeling and fibrillation

Bovine insulin was purchased from Sigma-Aldrich (St Louis, MO, USA) and used without further purification. The protein was labelled with fluorescent dyes, ATTO 488 NHS ester (Sigma-Aldrich, St Louis, MO, USA). NHS-esters readily react with amino groups of proteins, i.e., the ϵ -amino groups of lysine or the amino-terminus, forming a chemically stable amide bond. Labelling was done in a solution of sodium bicarbonate 100 mM at pH 8.3 and mixing monomeric insulin at a final concentration of 2 mg/ml (0.35 mM), with 1 mM ATTO 488, previously diluted in dimethylsulfoxide (DMSO) (Thermofisher, Waltham, MA, USA). After 1 hour under stirring, the proteins were separated from the excess of free dye via filtration with Amicon Ultra, centrifugal filters (MWCO 3K, Merck Millipore, Milan, Italy). Immediately after filtration, the concentration of the labelled proteins was determined spectrophotometrically (Cary spectrophotometer, Agilent, Technologies, Santa Clara, CA, USA). The unlabeled insulin monomers were added in pH 2 Hydrochloric acid (HCl) 37% (Sigma-Aldrich, St Louis, MO, USA) diluted in deionized water at the final concentration of 2 mg/ml. Finally, labeled and unlabeled insulin were mixed together to have a final dye-to-proteins ratio (i.e., labelled: unlabelled insulin) of 1:20 with final concentration of 2mg/ml. Fibrillation took place at 60°C, without agitation. The aggregation process was followed for at least 2 weeks.

6.3.2 Sample preparation for correlative confocal-TEM imaging

Firstly, labeled insulin fibrils 2 mg/ml were diluted 500 times with MQ water. 5 μ l of solution were retrieved on a 200-mesh carbon coated copper grid (Film Gilder Finder F1 Cu Grids, CF200F1-CU) already plasma cleaned (medium bench-top plasma system, Tucano, Gambetti Kenologia s.r.l., Italy) for 1 min under 20W power. These grids are composed of an asymmetric mark in the center and the numbers that are imprinted in the letters to identify a particular grid square of interest. After 5 min incubation, the grids were rinsed with distilled water. The grids were blotted with filter paper in between each washing step.

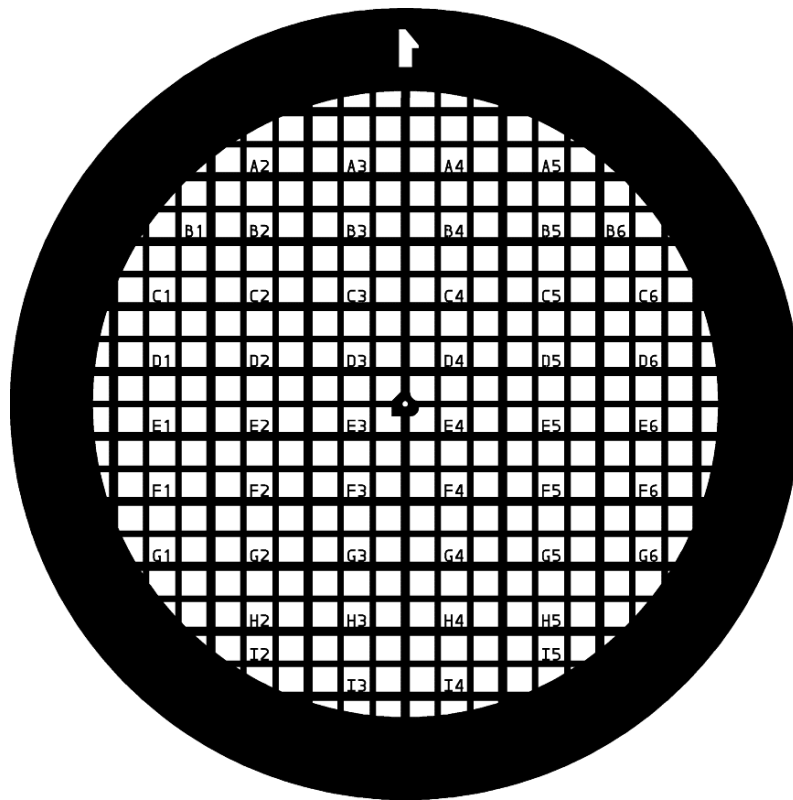


Figure 6.5 | Representative a typical TEM finder grid. (Image from www.emresolutions.com)

6.3.3 Confocal microscopy measurements

At first, I tried to image the TEM grid with STED microscopy. I found that the depletion beam was heavily damaging the TEM grid, precluding the application of STED microscopy. For this reason, I used confocal microscopy, with some changes in sample preparation. In order to distinguish the single fibrils by confocal microscopy the sample must be sparse, since the broadened effect of confocal microscopy precluded the identification of single structure in crowded samples. For imaging with the confocal microscope, the grid is sandwiched between two glass coverslips, inserted into a custom-made liquid chamber. First, in the bright-field (BF) mode, the center of the grid, which contains the asymmetric mark for the orientation, is retrieved. Switching to fluorescent mode, an image of the region of interest is acquired with a very low excitation intensity and exposure to avoid photobleaching and damaging the carbon layer of the grid. The confocal images are acquired at different magnifications to later be used for the correlation. A lower magnification can be used to locate enough fibrils for the correlation, and a higher magnification for higher resolution images of the areas positive for the fluorescence. For each field of view, the position of interest is determined. For electron microscopy measurement, the grid must be removed from the coverslip. In this step, the grid carbon film tends to stick the surface of the coverslip. In order to remove the grid from the coverslip sandwich, a drop of water is placed onto the grid and carefully pipette up and down until the grid is released from the surface. A filter paper is used to blot off the excess liquid, and then the grid is left dry in air and placed back in the storage box. the asymmetric mark in the center of the grid is used to navigate the region of interest during TEM analysis. Commercial Leica SP8 FALCON has been used for confocal microscopy measurements. The confocal images have been collected by a Hybrid detector in the spectral range 500-570 nm. We have used an HCX PL APO CS 100x 1.4NA oil objective (Leica

Microsystems, Mannheim, Germany) and a scan speed of 1000 Hz for 1024 pixel per line with a 96-line average.

6.3.4 Transmission electron microscopy measurements

After confocal analysis the same finder grids, containing insulin fibers were stained using 1% uranyl acetate in distilled water. The same areas, already imaged with the confocal, were then acquired on a JEM-1011 (JEOL) transmission electron microscope (TEM) equipped with a thermionic source (W filament) operating at 100 kV and a Gatan Orius SC1000 series CCD (charge coupled device) camera. TEM experiments were performed in collaboration with Dr. Roberto Marotta, at the EM Facility of the Istituto Italiano di Tecnologia in Genoa, provided a characterization and correlation of insulin fibrils.

6.4 Results and discussion

In order to study the structural diversity between labeled and unlabeled fibrils, correlative confocal-TEM was performed to visualize the deposited labeled insulin fibrils on a TEM finder grid that helps the identification of the same areas under investigation. In Fig 6.6, it is shown that individual fibrils can be imaged and correlated precisely with confocal and TEM. TEM imaging showed that insulin fibrils are of irregular shape and size. The coexistence of labeled and unlabeled fibrils was revealed by our CLEM method, consistently with the observations made during previous works.

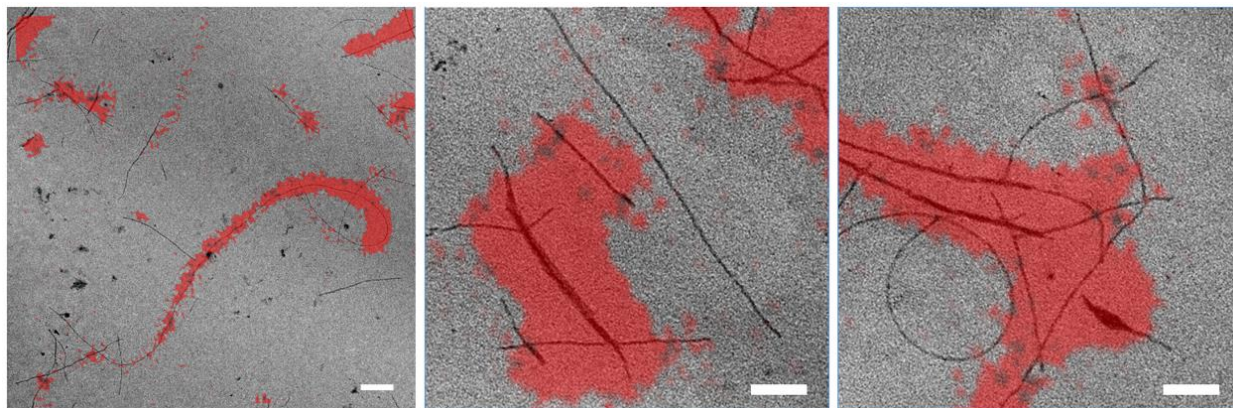


Figure 6.6 | Representative correlative confocal-TEM image of insulin fibrils labeled with ATTO488 NHS-ester at dye-to-protein ratio of 1:20. Only a fraction of the fibrillary aggregates is fluorescent, and some fibrils are completely missing, indicating that the labeled monomers were not taking part in the formation of these aggregates. Scale bar: 0.2 μm .

The work is promising, but in the first set of experiments, we were not able to reach a very high resolution by TEM imaging. In the first images, we can only note that some of the aggregates are larger and formed by the twisting of more fibrils. These supercoiled structures are all fluorescent, as was expected, being formed from the assembly of several fibers, having each one a significant probability to be labeled. Despite this, the resolution obtained in TEM images is still far to allow the detection of structural difference, likely at the sub nanometric scale.

6.5 Discussion and conclusion

In this work, we performed the correlating fluorescence microscopy and TEM, not simultaneously but using finder TEM grids. In particular, the sensitivity of carbon coated copper grid to high intensity power of the laser in STED microscopy limited the application of STED microscopy in this correlation. However, the success of correlation between confocal microscopy and TEM could reveal some hidden aspects behind the formation of labeled and unlabeled fibrils. Specifically, we were able to observe the coexistence of labeled and unlabeled fibrils in agreement

with what we observed in a previous work with correlative AFM-STED recordings¹⁵⁰. The new capability in terms of resolution offered by negative stain TEM paves the way towards a better comprehension of the molecular mechanism that regulates the aggregation of peptides in the presence of fluorescent molecules.

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