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XXVI CICLO DEL DOTTORATO DI RICERCA IN
NANOTECNOLOGIE

NANOSCALE PLATFORM TO STUDY
UNSTRUCTURED PROTEINS INTERACTIONS.

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Riassunto

Le proteine intrinsecamente disordinate (IDP), nello stato nativo non ripiegate, sono inclini all’aggregazione e direttamente correlate con lo sviluppo di malattie amiloidi. Tra queste, ci siamo focalizzati sullo studio dell’alfa-Sinucleina (AS), una proteina coinvolta nella malattia di Parkinson, un disturbo neurodegenerativo caratterizzato dalla degenerazione dei neuroni dopaminergici e l’accumulo di AS in placche amiloidi. Sebbene lo studio delle interazioni AS-dopamina sia di importanza cruciale nella comprensione dei meccanismi responsabili dello sviluppo della malattia, tuttavia, non sono disponibili dati in letteratura riguardo all’affinità di questo legame nei diversi stati ripiegati/funzionali dell’AS. L’elevata tendenza all’aggregazione delle IDP rende difficile il loro studio in soluzione e anche una stima approssimativa dei parametri relativi all’affinità di legame è estremamente difficile. Nuove tecniche di superficie potrebbero pertanto permettere lo studio delle interazioni di legame di molecole e di farmaci capaci di favorire/inibire l’aggregazione. In particolare, le tecniche di superficie potrebbero permettere un migliore controllo dell’immobilizzazione di queste proteine, particolarmente instabili in soluzione, e di studiare con alta precisione fenomeni di legame.

Con questa visione, la microscopia di forza atomica (AFM) rappresenta un’opportunità. Il nanografting, una tecnica litografica per mezzo AFM, permette l’immobilizzazione di proteine orientate con il preciso controllo dei parametri di immobilizzazione. Con AFM e AFM-nanografting, abbiamo prodotto una nuova piattaforma per lo studio di diversi aspetti dell’aggregazione/interazione dell’AS. Abbiamo quindi studiato l’affinità di legame tra AS e dopamina misurando variazioni nell’altezza e nella rugosità della topografia AFM su AS immobilizzata in aree confinate, in funzione della concentrazione di dopamina. Il valore micromolare della costante di dissociazione (Kd) è stato inoltre confermato dallo studio dello spiazzamento di un anticorpo legato all’AS in seguito all’aggiunta di dopamina. Sebbene la Kd calcolata con il nostro approccio sia una stima della reale Kd calcolata in soluzione, in quanto influenzata dall’affollamento delle molecole o ai limiti di diffusione, i nostri risultati sono comunque degni di nota.

Infine, abbiamo testato il nostro saggio per lo studio delle fasi preliminari dell’aggregazione dell’AS. In particolare, abbiamo osservato come aggregati confinati di AS siano disciolti dall’azione della dopamina, confermando il suo ruolo nell’inibizione della fibrillazione. In questo modo, abbiamo creato un
Abstract

Intrinsically disordered proteins (IDPs) are natively unfolded, prone to aggregation and directly correlated with amyloid diseases. Among them, we focused on alpha-Synuclein (AS), a protein related to Parkinson’s disease, a neurogenerative disorder characterized by the degeneration of dopaminergic neurons and the accumulation of AS into amyloid plaques. In particular, the study of AS-dopamine (DA) adducts is crucial for the comprehension of the mechanisms responsible of Parkinson’s disease development. However, according to our knowledge, there is no evidence in literature about AS-DA binding parameters at different folding/functional state of AS. IDPs have in fact a strong propensity to aggregate, making it extremely difficult to get even rough estimation of binding affinities in solution. Therefore, new methods able to study surface immobilized IDPs could be useful for both fundamental studies of binding interactions and drug screening of new compounds able to interact favouring/inhibiting the aggregation process. In particular, the use of a surface-based technique could help to better control the immobilization of such proteins, particularly unstable in solution, and to study with high precision binding events.

With this perspective, atomic force microscopy (AFM) offers a challenge. Nanografting, an AFM mediated nanolithographic technique, allows for the nanoscale immobilization of proteins in a well-oriented manner with the precise control of the immobilization parameters. By means of AFM and AFM-nanografting, we produced a new platform able to study different aspects of AS aggregation/interactions. First, we studied AS-dopamine binding affinity by measuring the variation of AFM topographic height on AS nanopatches due to binding of DA on the patch as a function of its concentration in solution, and the correspondent surface roughness variation. The value of the dissociation constant, in the micromolar range, was confirmed by studying the displacement of AS-bound mAbs, after the addition of dopamine. We think that this result is noteworthy, even though we are aware that the $K_d$ values obtained with our assay are probably a very rough estimate of the $K_d$ of the AS/DA interaction occurring in solution, due for instance to AS surface crowding and restrictions to diffusion.
Finally, the assay was used to study the early stages of the aggregation process. In particular, dopamine dissolved confined AS aggregates confirming its role in the inhibition of fibrillation. In this way, we created a potential screening assay able to study the interaction of AS with new molecules virtually usable as new drugs.

Moreover, the interaction of AS with lipid rafts, specialized microdomains of the cell membrane, is object of a strong debate. For this reason, we created a model supported lipid bilayer with composition able to mimic lipid rafts. In particular, we characterized by both AFM and Grazing Incidence Small Angle X-ray Scattering (GISAXS) a three components membrane presenting a lipid phase separation, focusing on the effect of humidity and cholesterol percentage on the structure of each phase. Then, we studied the effect of AS binding on this membrane model system. In particular, trough AFM imaging in liquid we discriminated the topology of filamentous structures resulting from AS binding. The phenomenon is dependent on the membrane’s lipid phase and order and is favoured by mild packing of lipids. From GISAXS measurements, the occurrence of a strong rearrangement of the membrane upon AS binding was suggested.
1. Intrinsically disordered proteins: α-synuclein

1.1 Protein folding, misfolding and aggregation

One of the most fascinating characteristic of the living cell is the ability of auto-assembly in a precise and functional manner. The folding of the protein in the three dimensional conformation is one of the most fundamental example of biological self-assembly and contribute to the correct functions of the cell. After water, proteins are the most abundant component of the cell. The proper three-dimensional configuration of the protein gives stability to the molecules and is fundamental for correct protein activity as in cell trafficking, regulation of cell growth and differentiation. It can happen that protein folding does not occur in a proper way and it is probably the starting point for a cascade that leads to pathological conditions 1,2.

1.1.1 The fundamental mechanism of protein folding

The specific three-dimensional conformation of a polypeptide chain is still unclear in many aspects. The total number of the possible conformations of a polypeptide is huge but usually the native folded state of a protein is the most thermodynamically stable in physiological conditions 3. The folding mechanism does not require mandatory intermediate folding steps but incompletely folded polypeptides, during inherent fluctuations, enable residues in various chain position to come in contact. In this way, the native, more stable protein form is selected. From here, the concept of an energy landscape was developed, in which the free energy of the polypeptide as a function of the protein configuration is described. During the folding process, a decrease of the free energy is observed (Fig. 1.1). The early stages of the folding process were studied on small proteins (less than 100 residues) and analysing the intermediate folding stages, a nucleation-condensation process has been suggested, in which the formation of a folding nucleus is the starting point for the following condensation (Fig. 1.1).
Moreover, integration of computer simulation of limited region of the protein with experimental observation suggested that intermediate folding state, despite the high rate of disorder, is characterized by topology very similar to that of the native state. In other words, the early interaction between a small number of key residues (especially between hydrophobic and polar residues) force the protein to adopt a certain structure, similar to the native three-dimensional conformation. In the final stages of the folding, the native structure is almost invariable. Probably, the transition state works as a “quality control” for the folding, in fact if these interactions do not occur, the protein does not assume the correct topology and does not arrange in a stable globular structure.

1.1.2 Protein folding and misfolding in cells

The genetic information for the synthesis of a protein is carried out by the DNA sequence responsible for the codification of the proteins. The synthesis occurs in the ribosome and brings to the formation of the amino acidic chain. In vivo, the folding of the protein is usually co-translational, i.e. it occurs usually during
the synthesis of the polypeptide, while the chain is still attached to the ribosome. In some other cases, the folding happens in the cytosolic space, after the release from the ribosome, whereas sometimes it happens in other compartments such as the endoplasmatic reticulum (ER). Incompletely folded proteins, exposing regions normally hidden in the native state (e.g. hydrophobic core, highly prone to the aggregation), can interact aspecifically with other molecules in the crowded environment of the cell. For this reason, the cell uses a strategy to prevent this behaviour using molecular chaperones that bind and guide the folding of the polypeptide. These chaperones recognize and bind hydrophobic regions and unstructured protein chains promoting the correct folding by cyclic binding and release of the substrate. Some chaperones are able not only to protect nascent proteins, but also, interacting with misfolded or solubilizing aggregated proteins, can give them the possibility to refold correctly. The expression of some chaperones increases in high stress condition for the cell (e.g. heat shock proteins, Hsp family, highly expressed after temperature increase), where some native proteins are unfolded.

In the eukaryotic cell, many proteins are secreted in the extracellular environment.

Figure 1.2. Regulation of protein folding in ER. Some proteins, synthetized on the ribosome, are translocated in the ER where the folding occurs. Correctly folded proteins are addressed to the Golgi apparatus for the secretion in the extracellular space, while misfolded ones are identified by the quality control, ubiquitinated and addressed to the proteasome where they are degraded. Adapted from Dobson, 2003.
In this case, the folding occurs in the endoplasmic reticulum (ER) and then they are addressed to the Golgi apparatus for the secretion. The ER is highly enriched in chaperones for enhancing the correct protein folding. Moreover, here proteins have to overcome a quality control that involves a series of glycosylation and deglycosylation reactions that permit to distinguish between a folded and a misfolded protein. According to that signal, the major part of the folded proteins are addressed to the endoplasmic reticulum (ER) and Golgi apparatus for the secretion, while misfolded ones are recognized and addressed to the ubiquitine-proteasome system where they are degraded (Fig. 1.2).

Whether the cell machinery devolved in misfolded protein elimination is lacking, proteins fail to fold correctly cannot work properly and give rise to pathological conditions, some of them summarized in table 1. Some of these diseases are due to alterations in the function of the protein (e.g. cystic fibrosis), in some others the misfolded protein starts to aggregate in the cytosolic or extracellular space of patients with amyloid disease (e.g. Alzheimer and Parkinson’s diseases).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Protein</th>
<th>Site of folding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypercholesterolemia</td>
<td>Low-density lipoprotein receptor</td>
<td>ER</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>Cystic fibrosis transmembrane regulator</td>
<td>ER</td>
</tr>
<tr>
<td>Phenylketonuria</td>
<td>Phenylalanine hydroxylase</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>Huntingtin</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Marfan syndrome</td>
<td>Filamin</td>
<td>ER</td>
</tr>
<tr>
<td>Osteogenesis imperfects</td>
<td>Pre-collagen</td>
<td>ER</td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>Hemoglobin</td>
<td>Cytosol</td>
</tr>
<tr>
<td>α-Antitrypsin deficiency</td>
<td>α-Antitrypsin</td>
<td>ER</td>
</tr>
<tr>
<td>Tay-Sachs disease</td>
<td>β-Hexosaminidase</td>
<td>ER</td>
</tr>
<tr>
<td>Scertry</td>
<td>Collagen</td>
<td>ER</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>Amyloid β-pleit peptide/amyloid</td>
<td>ER</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>α-Synuclein</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Scrapie/Creutzfeldt-Jakob disease</td>
<td>Prion protein</td>
<td>ER</td>
</tr>
<tr>
<td>Familial amyloidosis</td>
<td>Trefoil protein/lysozyme</td>
<td>ER</td>
</tr>
<tr>
<td>Retinitis pigmentosa</td>
<td>Rhodopsin</td>
<td>ER</td>
</tr>
<tr>
<td>Cataracts</td>
<td>Crystallin</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Canut</td>
<td>p53</td>
<td>Cytosol</td>
</tr>
</tbody>
</table>

*Table 1.1.* The table reports protein folding alteration, the site of folding and related diseases.

### 1.1.3 Protein aggregation and amyloidosis

During the last decade, the interest in the study of amyloid diseases increased tremendously and the number of discovered protein with little or no ordered structure was growing exponentially. This class of proteins was defined for the first time as “natively unfolded” in 1994. Regarding amyloid proteins, their soluble forms are usually very different but a certain relation between the amino
acidic chain sequence and the propensity of the protein to be “natively unfolded” has been observed. In particular, the presence of a net charge at physiological pH (often negative) and a low content of hydrophobic amino acids has been noted for several “natively unfolded” proteins.

The deposits found in patients with amyloid pathologies have in common many aspects. It was observed in fact, that in all of these cases there is the presence of amyloid fibrils composed by copies of the same protein. These fibrils are long (>1 µm), thin (10-20 nm), straight and unbranching. The protein, in these three-dimensional aggregates, is organized in β-sheet conformation, perpendicular to the main axis of the fibril. Amyloid fibrils (either ex vivo or in vitro) are hystorically defined by three main criteria: green birefringence upon staining with Congo Red, fibrillar morphology, and β-sheet secondary structure. Moreover, amyloid fibrils were widely studied by the use of fluorescent intercalating agents as Thioflavin T to study fibrils both in tissue samples and other in vitro studies.

1.2 Parkinson’s disease

Parkinson’s disease (PD) is one of the most common neurodegerative disease and affects 1-2% of the population over 65 years. James Parkinson in the classic monograph “An essay on the shaking palsy” described for the first time in 1817 six patients suffering of the movement disorders which came to bear his name. Clinically, it is characterized by three cardinal symptoms: muscle rigidity, bradykinesia and resting tremor. In addition, postural instability, a mask-like facial expression and festinating gait are often observed. Cognitive impairment is present in a significant proportion of patients at advanced stages of the disease. Although the pathological features were well described, the molecular mechanisms were obscure and it was considered initially a neuropsychiatric disorder. Neurologically, the pathology is characterized by the substantial loss of dopaminergic neurons in the substantia nigra pars compacta, the area of the brain involved in the activation of the movement (Fig. 1.3). The early symptoms appear after the death of the 70% of the dopaminergic neurons which are less than 1% of the total number of brain neurons but, depending on the brain regions that they innervate, are fundamental for motor behaviour, motivation and working memory.
Figure 1.3. Schematic representation of Substantia nigra pars compacta. Parkinson’s disease is characterized by alterations in this region of the brain. The loss of dopaminergic neurons is evident in post-mortem brains of PD patients as a depigmentation of this area.

In 1992 Lewy described for the first time the inclusions characteristic of PD, called Lewy bodies (Fig. 1.4). These eosinophilic, round cytoplasmic plaques are composed by fibrillaceus inclusions of α-Synuclein (AS) and are confined largely to nerve cells, with glial cells being only rarely affected.

Figure 1.4. Substantia nigra from patients with PD immunostained for AS. a. Two pigmented nerve cells, each containing an AS-positive Lewy body (thin arrows). Lewy neurites (thick arrows) are also immunopositive. Scale bar, 20 µm. b. A pigmented nerve cell with two AS-positive Lewy bodies. Scale bar, 8 µm. c. AS positive, extracellular Lewy body. Scale bar, 4 µm. From Goedert, 2001.
Transgenic model animals, expressing high levels of human wild-type or mutant AS, show the formation of lesions very similar to those found in PD patients, providing evidence of the central role of AS deposits in the pathogenesis of PD 19–22. In the late 1950s it was identified the presence of dopamine (DA) in the mammalian brain 23,24. The high concentration of DA in the striatum was compatible with its role as neurotransmitter 25,26. Carlsson demonstrated a strategic role of DA in the control of the motor function and suggested that a deficiency in the DA level could be fundamental for the development of PD. He showed that L-dihydroxyphenylalanine (L-DOPA), a precursor of DA (Fig. 1.5), passing through the blood brain barrier, could be used to restore the DA brain normal level and the motor functions in animals 24,27. Despite the great importance of L-DOPA in the therapy of PD, its efficacy is limited. In particular, the major benefices are greatest during the early stages of the disease, while they decrease as the disease progresses. This therapy in fact is not able to repair the brain damages due to the nerve cell degeneration in the substantia nigra, but it can only reduce the symptoms associated with the disease. The ability to identify a therapy able to interfere with the molecular mechanism at the basis of the neurodegeneration requires a more fundamental knowledge of the causes of the disease, still obscure in many aspects.

Figure 1.5. Metabolic pathway of catecholamines synthesis. L-DOPA, precursor of DA is converted into DA inside the brain.
1.2.1 Genetics of PD

The 90% of the cases of PD are sporadic. The causes are unclear but a role of the exposure to chemicals and a toxic environment was suggested. The remaining 10% is familiar and a genetic correlation with the development of the disease was identified. Genetic defects, both autosomic recessive and dominant, were characterized. In particular, 16 PD-related loci (PARK loci) and 11 genes associated were described (table 1.2). Among these genes, α-synuclein (also known as SNCA) and leucine rich repeat kinase 2 (LRRK2) are autosomal dominant, while parkin, PTEN-induced putative kinase 1 (PINK1), DJ-1, are autosomal recessive genes. Defects in the ubiquitin carboxyl-terminal esterase L1 (UCHL1) gene were observed only in one case of familiar PD and its correlation with the disease is still unclear. The causative genes of the four remaining loci are not yet identified.

<table>
<thead>
<tr>
<th>Locus &amp; PARK</th>
<th>Gene</th>
<th>Chromosome</th>
<th>Inheritance</th>
<th>Probable function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK1 &amp; PARK4</td>
<td>α-synuclein</td>
<td>4q21</td>
<td>Dominant</td>
<td>Presynaptic protein, Lewy body, lipid and vesicle dynamics</td>
</tr>
<tr>
<td>PARK2</td>
<td>parkin</td>
<td>6p25.2-p27</td>
<td>Recessive</td>
<td>Ubiquitin E3 ligase, mitophagy</td>
</tr>
<tr>
<td>PARK3</td>
<td>UCHL1</td>
<td>2p13</td>
<td>Dominant</td>
<td>Ubiquitin-Ceramidase hydroxylase</td>
</tr>
<tr>
<td>PARK5</td>
<td>PINK1</td>
<td>1p35-36</td>
<td>Recessive</td>
<td>Mitochondrial kinase</td>
</tr>
<tr>
<td>PARK7</td>
<td>DJ-1</td>
<td>1p36</td>
<td>Recessive</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>PARK8</td>
<td>LRRK2</td>
<td>12p11.2</td>
<td>Dominant</td>
<td>Kinase signaling, cytoskeletal dynamics, protein translation</td>
</tr>
<tr>
<td>PARK9</td>
<td>A TP1A2</td>
<td>1p36</td>
<td>Recessive</td>
<td>Unknown</td>
</tr>
<tr>
<td>PARK10</td>
<td>Unknown</td>
<td>1p32</td>
<td>Dominant</td>
<td>UCHL1</td>
</tr>
<tr>
<td>PARK11</td>
<td>GIGYF2</td>
<td>2p37</td>
<td>Dominant</td>
<td>IGF-1 signaling</td>
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<tr>
<td>PARK12</td>
<td>Unknown</td>
<td>Xq21-q25</td>
<td>X-linked</td>
<td>Unknown</td>
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<tr>
<td>PARK13</td>
<td>Gm13222A2</td>
<td>2p13</td>
<td>Unknown</td>
<td>Mitocondrial serine protease</td>
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<td>PARK14</td>
<td>PLA2G6</td>
<td>22q13</td>
<td>Recessive</td>
<td>Phospholipase enzyme</td>
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<tr>
<td>PARK15</td>
<td>FBXO7</td>
<td>22q11</td>
<td>Recessive</td>
<td>Ubiquitin E3 ligase</td>
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<tr>
<td>PARK16</td>
<td>Unknown</td>
<td>1q21</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Table 1.2. 16 PD-related loci are associated to 11 genes. In the table the chromosome position, their inheritance and the probable function is reported. From Thomas et al., 2011

Sporadic and familiar forms of PD have many clinical and pathological aspects in common. For this reason, the identification of the physiological function of the proteins encoded by these genes is necessary in order to find a novel target in the treatment of PD. However, mitochondrial dysfunction, protein phosphorylation, oxidative stress, protein misfolding and impairment of the ubiquitin proteasome system (UPS) have been identified in both forms of PD. Studying familiar autosomal-dominant forms of PD, mutations affecting AS locus have been characterized. In particular, point mutations (A53T, A30P and E46K) and multiplications (duplication and triplications) were identified in
the AS gene as related to PD. The fact that patients with triplications are usually younger than ones with duplications suggests that higher level of AS is probably toxic for the cell. 

1.3 Alpha-synuclein

The gene SNCA encodes for alpha synuclein (AS), a small protein (14,460 Da, 140 residues) that lacks of a specific structure in the native state as reported by previous spectroscopic and NMR studies. It is considered an Intrinsically Disordered Protein (IDP) and a naturally unfolded protein at physiological conditions. This characteristic is presumably due to the aminoacidic composition of the protein that has an high negative charge at neutral pH and low hydrophobicity.

![Figure 1.6. Primary structure of AS.](image)

**Figure 1.6. Primary structure of AS.** The scheme on top shows the three main domains of AS. The N-terminal (red) is an amphipathic domain, the central region (green) is a highly hydrophobic domain known as non amyloidogenic component of the extracellular senile plaque (NAC), which promotes the protein aggregation. The C-terminal domain (blue) has an acidic character. The scheme at the bottom reports the aminoacidic AS sequence. Basic amino acid are coloured in blue, acidic ones are red.
The primary sequence of the protein can be divided into three main regions (Fig. 1.6):

- residues 1-60 that contains imperfect repetitions of 11 residues with a highly conservative hexamer motif (KTKEGV). It is the N-terminal region and is predicted to form amphipathic α-helices, typical of the lipid-binding domain of apolipoproteins\textsuperscript{37,38};
- residues 61-95, known as non-amyloid β-component (NAC region) with high propensity to the aggregation. It contains two additional KTKEGV repeats;
- residues 96-140, the C-terminal region, enriched in acidic residues and prolines.

Despite the correlation with PD, the functions of AS are not completely understood. AS is highly expressed in the central nervous system and represents the 0.5–1% of total cytosolic proteins of brain homogenates. AS is concentrated at the presynaptic terminal, pointing out a possible role in the regulation of the synaptic functions\textsuperscript{39}. In order to study \textit{in vivo} the normal functions of AS, knockout mice have been produced. In these transgenic model animals, the lack of AS decreased the number of synaptic vesicle available and the expression of synapsis-associated proteins at the presynaptic level. Although AS is not fundamental for synaptic formation and cell survival, a role of AS in the regulation of the vesicles release at the presynaptic membrane, in the neuronal plasticity\textsuperscript{40–42} and in neurotransmission\textsuperscript{40,43–46} was speculated. Some evidences confirm these hypothesis. It was observed indeed in yeast that one third of the genes involved in the toxic accumulation of AS are involved in lipid metabolism and vesicle trafficking. In \textit{Drosophila melanogaster} as well, expression of genes involved in lipid transport are associated with that of AS\textsuperscript{47}. Moreover, \textit{in vitro} experiments suggest a possible interaction of AS with phospholipase D2 and its inhibition\textsuperscript{48,49}. Phospholipase D2 is a membrane-bound enzyme, involved in the lipid-mediated cascades and vesicle trafficking. AS knockout mice have a decrease in the lipid uptake with a decrease in the assimilation of fatty acid inside the brain, suggesting that AS has a role in lipid metabolism\textsuperscript{50,51}. This is evident also at mitochondrial level, with the disruption of the mitochondrial membrane. Since AS does not bind the mitochondrial membrane, a role in lipid uptake and trafficking was speculated as well.
Finally, AS could be involved in the regulation of the concentration of DA at presynaptic level, acting on the anabolic pathway of the synthesis of DA or on the transporters of DA modulating the permeability of the plasma membrane to the molecule \(^{52–54}\). It is also interesting to highlight a role of AS in the regulation of the transporters of serotonin and norepinephrine, suggesting a key role of AS in the homeostasis of these neurotransmitters and its presumable involvement in many brain functions \(^{55}\) (Fig. 1.7).

**Figure 1.7. AS is highly enriched in presynaptic terminals.** A fraction of AS is associated with synaptic membranes, the other is cytosolic. When bound to phospholipid membranes, AS changes its secondary structure to \(\alpha\)-helix. An important neuronal function of AS might be to regulate the formation of synaptic vesicles from early endosomes through interactions with phospholipase D2 (PLD2). Mutations in AS might result in a reduced number of vesicles being available for dopamine storage, leading to an accumulation of dopamine in the cytoplasm and increased levels of oxidative stress and cytotoxic dopamine by-product. *From Lotharius et al, 2002* \(^{56}\).

### 1.3.1 AS interaction with membranes

It is reported in literature that AS binds lipid membranes. *In vitro* studies, describe the binding of AS to synthetic lipid vesicles composed by mixtures of phospholipids such as phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidic acid (PA), or neutral phospholipids like phosphatidylcholine (PC) and phosphatidylethanolamine (PE) \(^{57–59}\). In order to understand the
conformation of the protein upon binding, a series of studies was performed. Early studies using optical spectroscopies such as circular dichroism (CD) demonstrated an high transition to α-helical structure of AS bound to vesicles of different type 57,58. NMR measurements performed on membrane-mimetic sodium dodecyl sulfate (SDS) micelles suggested that the first 100 residues of AS are directly involved in the interaction and form two curved antiparallel helices with a linker represented by residues 38 to 44, maintaining the C-terminal tail unfolded 60–62 (Fig.1.8). In this structure, lysine side chains of the repeated region point sideward from the helices which allow favourable interactions with the acidic sulfate groups on the SDS micelle.

Figure 1.8. Frontal and side view of micelle-bound AS. The membrane binding domain (gray), non-amyloid β component region, and acidic region are colored in gray, light blue, and red, respectively. Lysine residues found within the amphipathic repeats are shown. From PDB ID: 1XQ8.

According to the structure determined, it was suggested a partial insertion of the NAC region into the membrane 63,64 but no transmembrane arrangement was found 65–67. The AS binding to lipid membrane was supposed to occur into three main steps: first, electrostatic interaction between the amphipathic helices and the membrane interface occurs; second, a local rearrangement takes place and hydrophobic side chain insert partially into the membrane; finally, the protein folds into α-helices 68–70.

In vitro, a series of factors influence the binding of AS to lipid membrane. First, it was observed a direct correlation with chemical properties of lipids. The first
study appeared in 1998 when it was suggested a preferential binding to anionic lipid (such as phosphatidylserine (PS), or phosphatidylinositol (PI)) probably due to electrostatic interactions with the lysine residues of the N-terminal (Fig.1.9).

Figure 1.9. Model of AS vesicle bounded. (A) and (B) are representations of the interaction of AS with a curved lipid surface. (C) A more detailed image of the protein–lipid interaction. The N terminus of the α-helix is in the foreground. Lysine residues 58 and 60 are shown in space-filling format. (D) Cartoon representations of the structures of AS on micelles and SUVs. The small and highly curved micelles cannot accommodate the extended helical structure present on the membrane. (E) The repeat region residues are plotted onto a helical wheel in which 11 aa make up three turns. Lipid-exposed sites (red) fall onto one side, while solvent-exposed sites (green) lie on the opposite side. Adapted from Jao et al, 2008

For this reason, the interaction is also strongly dependent on the ionic strength of the solution, with a decrease of α-helix as the concentration of salt in solution increases, although the binding is not completely absent even at higher concentration (>500 mM). Thus, although electrostatic forces play a key role in the binding process, they are not the only forces affecting the binding. Furthermore, the use of lipids with small head group facilitate AS binding,
probably because of the presence of more space for AS insertion. The chemical characteristics of the lipid chain can also influence AS binding. In particular, AS seems to prefer the binding of polyunsaturated lipids because they are responsible of a less packing of lipids leaving more defects available for AS insertion.\(^{72,73}\)

Moreover, the curvature of the lipid membrane seems to be crucial in AS-membrane interaction. Experiments of incubation of same amounts of AS in presence of lipid vesicles composed by PC/PA of different size, suggest higher affinity for small unilamellar vesicles (around 25 nm diameter) rather than to large unilamellar vesicles (around 125 nm diameter), indicating the higher membrane curvature as a factor favouring the binding.\(^{57}\) Subsequent fluorescence correlation spectroscopy, site-specific fluorescence, isothermal titration calorimetry and gel-filtration studies confirmed the dependence of AS binding on vesicle size and therefore on membrane curvature\(^{74–77}\), although the protein binds anionic large unilamellar vesicles\(^{78,79}\) and even planar supported bilayers.\(^^{80}\) The preferential binding of AS to vesicles with higher curvature could be due to the presence of a major number of packing defects in the membrane which facilitate the protein insertion.\(^^{81,82}\) For this reason, complementary measurements with surface techniques on planar supported lipid bilayer could elucidate the role of AS-lipid membrane interaction removing the contribution of membrane curvature.

Finally, AS interaction/colocalization with gangliosides (GMs) was described using CD spectroscopy and molecular simulations.\(^{83,84}\) GMs are glycosphingolipids composed of a ceramide backbone and one or more sugars as the headgroup and are supposed to bind AS through the formation of hydrogen bonds between sugar alcohols and AS side chains.\(^^{84}\)

AS-lipid interaction was widely studied in vitro but few is known about the interaction in living cells. GM1 ganglioside, together with cholesterol, is proposed to enrich lipid rafts, liquid ordered domains of the cell membrane involved in many cellular processes. In HeLa cells, it was observed the co-localization of AS with GM1 and the specific association with lipid rafts was speculated.\(^{83}\) The same study was performed on the detergent resistant fraction extracted from HeLa cells membrane and supposed to be the raft domain of the cell, demonstrating AS association. The situation is anyway controversial and the AS binding to lipid rafts is still under debate. A confocal laser scanning microscopy study seems to highlight for example the preferential binding of AS to the liquid disordered phase of giant unilamellar vesicles (GUVs) instead to
lipid rafts. It is therefore still obscure whether AS prefer liquid ordered environment of lipid rafts or if the binding is mediated by its components (e.g. GM1 or GPI-anchored proteins).

The cellular dysfunctions caused by the interaction of amyloid proteins with the cell membrane are usually due to a membrane destabilization upon binding. In particular, AS binding can cause the permeabilization of anionic membrane, alteration of the calcium flux, dispersion of intra-vesicular dopamine and depolarization of mitochondrial membrane, all phenomena potentially involved in PD. Moreover, atomic force microscopy studies and vesicle dye leakage assays reports on the capability of AS oligomers and fibrils of enhancing membrane permeability. In presence of anionic lipids and lipid disordered domains, the ability of AS of disrupting the bilayer is affected, although the specific mechanism of this disruption is still unclear. Other works suggest that AS can also transform membrane surface topology. In particular, AS binding to SUVs can induce the formation of tubular structure, multilamellar vesicles, and can increase the membrane curvature. These results highlight some points still unclear. Here, the necessity to validate these hypothesis with complementary techniques able to elucidate the effects of AS binding on membranes, the structural rearrangement in different conditions, the role of the lipid charge and lipid phase, fundamental for the comprehension of AS functions and its correlation with PD progress.

1.3.2 The aggregation process

The ability of AS to aggregate and form fibrils is abundantly described in literature but many aspects are still unknown. It seems clear that AS accumulation in form of aggregates and fibrils inside dopaminergic neurons is one of the early stages of the PD pathogenesis that lead to AS accumulation in Lewy bodies, but is still unclear how the formation of these aggregates triggers the cell death. AS forms indeed amyloid fibrils in vitro, similar to those observed in Lewy bodies in a concentration-dependent manner.

It is important to underline that aggregates resulting from the AS self-association in vitro are not actually always the same but it is possible to observe different types, depending on the experimental conditions. In particular, it is possible to create fibrils, oligomers and insoluble amorphous aggregates following three different pathways (Fig. 1.10).
The abnormal aggregation and accumulation of AS starts from a partially folded intermediate that exposes contiguous hydrophobic residues able to enhance hydrophobic interactions between the molecules involved in the aggregation. The probability of the interaction between two of these species, even if it is low, can manifest after the formation of hydrogen bonds. The dimer can interact with other proteins starting the growth of soluble oligomers. Early formed metastable protofibrils typically comprise around 10 to 50 monomers, and appear as spherical in shape. These spheres may anneal to form chain-like protofibrils, which can further form annular pore-like species or proceed to amyloid fibrils.

All the amyloid fibrils have in common a β-sheet structure in which beta strands are perpendicular to the main axis of the fibril (Fig. 1.11). This means that the protein has to rearrange its secondary structure in order to allow the conformational changes required to obtain this structure.
It was demonstrated that oligomers are the most toxic species $^{99,100}$ and affect the cell viability. It is possible moreover to observe the formation of annular intermediates $^{101}$ able to disrupts membranes $^{86}$.

For this reason, the comprehension of the conversion rate from the monomer to oligomers and, from here to fibrils, could be a crucial step in the understanding of PD evolution and will increase our knowledge of the process at the basis of this phenomenon.

When AS is incubated at pH 7.4 at 37 °C, it starts to aggregate exponentially, with a kinetic typical of a nucleated polymerization (Fig. 1.12, blue line) where transient soluble oligomers are observed in the lag phase $^{96,99,102}$. These oligomers were studied with many different biophysical techniques in order to understand their role in fibrillogenesis. Using Dynamic Light Scattering (DLS), rapid formation of these oligomers was observed soon after AS incubation (Fig. 1.12, red line). Their concentration grow up to 15-20% at the end of lag period, and rapidly decrease as soon as fibril grow increase (Fig. 1.12). Similar results were obtained by FTIR and FRET measurements $^{102}$. 

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**Figure 1.11. Schematic representation of an amyloid fibril.** Beta-sheets cross perpendicularly to the main axis of the fiber.
Small angle X-ray Scattering (SAXS) measurements on AS solution soon after the incubation displayed the presence of the monomer only, while oligomers were found after 2 hours. These species are not stable in time, dissociate very rapidly and they are difficult to detect by other techniques such as size exclusion chromatography (SEC) high-performance liquid chromatography (HPLC). Atomic force microscopy (AFM) imaging displayed two populations of oligomers, one in the early lag phase having height of 2-3 nm, the other in the late one, having height of 5-7 nm, size more similar to mature fibrils. The decrease of these oligomers with the same rate of fibril growth suggest that fibrils probably could be formed by oligomers association, rather than by binding of monomeric species to the growing end of fibrils.

It was reported that, as expected, an increase in the concentration of AS in solution, increasing the intermediate concentration, enhances the rate of fibrillation $^{103}$. Moreover, the presence of ions (anions and cations) in solution is critical in the aggregation process because can influence the formation of the folded intermediate and accelerate the fibrillation $^{104}$.

The partially folded intermediate is stabilized by many factors such as an increase in temperature $^{105}$, low pH $^{105}$, metal ions $^{103,106}$ and organic solvents $^{104}$.

In particular, it was indeed observed in literature that solvents are able to influence the secondary structure of AS suggesting that the stability of the

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**Figure 1.12. Formation of oligomers during the AS aggregation measured by Dynamic Light Scattering.** The red line represent the formation of transient oligomers measured as scattering intensity increase whereas the rate of formation of fibrils, monitored by thioflavin T fluorescence, is shown in blue. *Adapted from Fink, 2006* $^{94}$. 

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protein is highly complex. In particular, Munishkina et al. 104 studied the effect of simple and fluorinate alcohols on AS secondary structure:

- hexafluoroisopropanol (HFIP) starts an alpha-helix transition at 2.5% v/v which is complete at 30% HFIP in water;
- ethanol (EtOH) is able to induce β-sheet conformation with the complete transition at 60% v/v;
- methanol (MeOH) is able to induce β-sheet conformation with the complete transition at 40% v/v;
- trifluoroethanol (TFE) is able to induce a more complex transition pathway, depending on the concentration. AS assume β-sheet conformation at 16% v/v TFE while it evolves to α-helix at 40% TFE. These forms are initially monomeric but start to aggregate over the time.

Incubation of AS at 37 °C in presence of alcohols starts an aggregation process dependent on the solvent concentration. At low concentration, the fibrillation enhances probably because of the stabilization of the partially folded intermediate, previously described as the key form in the aggregation process. Increasing the concentration (e.g. >40% EtOH or MeOH), AS starts to self-associate forming β-enriched oligomers that do not fibrillate, suggesting that oligomers formed in these conditions are quite stable. The incubation in these stabilizing conditions for several days gave indeed amorphous aggregates as the major component, even if some short fibrils were observed.

AS aggregation could be inhibited in some other cases. It was observed in fact that nitration of tyrosine residues led to the formation of stable oligomers that occurs in vivo through the oxidation of tyrosine residues. In vivo, this nitration occur through the interaction with oxidative products of NO, which could lead to additional oxidative products of AS such as di tyrosine 107. Since dopaminergic neurons are the most sensitive in PD, and dopamine shows high propensity to the oxidation, this suggested that, oxidized products of DA, stabilizing oligomeric forms of AS in vitro, could be involved in PD.

1.3.3 AS-DA interaction

The preferential vulnerability of dopaminergic neurons in PD is still unclear but it has been hypothesized a role for DA in the biology of AS 108. In particular, DA, forming AS-DA adducts, stabilizes the oligomeric, presumably toxic,
protofibrillar form of the protein and inhibits the formation of AS fibrils \textit{in vitro} \cite{109} (Fig. 1.13).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure13.png}
\caption{AS aggregation pathway. AS amyloid fibrils formation occurs through the formation of AS small oligomers that elongate in fibrils (route 1) as shown by electron microscope micrograph (scale bar 200 nm). DA disaggregates these unsoluble fibrils into soluble oligomers (route 2). AS monomer, in presence of DA produce off-pathway intermediates (route 3a) inhibiting the conversion into amyloid (route 3b). \textit{From Leong et al, 2009} \cite{110}.}
\end{figure}

The hypothesis that protofibrils are actually the pathogenic species and that fibrils might represent a protective cellular response is consistent with pathological studies that suggest the neuroprotective role of Lewy bodies \cite{111,112}. For this reason, DA binding, stabilizing AS protofibrils, could promote PD pathogenesis \cite{92}. Another important function of the DA-modified AS, recently demonstrated, is to block chaperon mediated autophagy \cite{113} but it is still unclear the ensemble of function and effects in living cells. The binding of DA to AS is suspected to be involved in PD pathology, as dopaminergic neurons are the most affected. Among the possible culprits, from oxidative stress to AS fibrillation, the interaction of DA to AS, whether in its native or oxidized forms, has in fact been reported as possible pathogenic events in the cascade leading to AS oligomerization and fibrillation.
In order to understand the nature of the AS -DA interactions, *in vitro* studies were performed. Truncations in the C-terminal of the proteins demonstrated a key role of the residues 125-129 (YEMPS) in the formation of the AS -DA adduct 114. Recently, theoretical modelling suggest a role of the long-range interactions with residues in the NAC region in the stabilization of the ligand 115,116. Covalent 114,115,117,118 and/or non covalent 109,119,120 crosslinking of DA to AS has been reported.

The morphology of dopamine-induces oligomers was studied with a range of biophysical techniques indicating a variety of different shapes and sizes 109,121,122, without providing information about the binding affinity of DA to AS, important for the comprehension of the mechanism at the basis of this interaction.
2. Proteins at solid interface

The immobilization of proteins on solid supports was introduced for analytical research some decades ago\(^1\) and has found applications ranging from fundamental studies to biosensors. Although the study of biorecognition phenomena in solution is in fact often the easiest choice, this is not always the best one, especially in the case of IDPs, highly prone to the aggregation and difficult to handle with the classical in-bulk techniques. While the study of binding parameters in solution benefits of the isotropic distribution of the ligand, it is difficult to discriminate between bound and unbound molecules and therefore to quantify the portion of the bounded ones. For this reason, a careful labelling of the molecules, together with a sensitive way to discriminate between bound and free molecules is required, for the accurate monitoring of the reaction kinetics.

The immobilization of the receptor on a solid support can overcome these problems allowing for the easy spatial separation of the bound from the unbound molecules. The main advantage of surface immobilization, compared to solution, is the possibility to tune the parameters of the immobilization process, and to control the localization and the stability of the molecules. As a drawback, the presence of a surface close to the protein could alter its function both because of unfavourable orientations with respect to the analyte in solution and because of electrostatic interactions.

Among the analytical techniques for biorecognition studies on solid surfaces, surface plasmon resonance (SPR) is one of the most used. It measures the variation of the angle of minimum reflection of polarized light through the interaction with a functionalized metal. Such minimum (resonance angle) is due to the interaction of light with activated surface plasmons, and is proportional to the mass on the sensor surface. Monitoring the position of the resonance angle versus time during a bioaffinity reaction, allows getting information on the binding parameters, in real-time. Although this technique is label-free, many examples for heterogeneity of surface binding sites caused by immobilization have been reported\(^{124–126}\).

Another well-known example of solid-phase assay is the Enzyme-Linked ImmunoSorbent Assay (ELISA). This test is usually performed in a sandwich configuration: protein binder molecule, generally antibodies, are immobilized on a surface. The bound protein are detected through a secondo, enzyme-linked antibody. After the addition of the enzyme’s substrate, a reaction gives
colorimetric signal used to quantify the analyte. The first biomolecular layer is usually immobilized either via physisorption or grafted on the surface through a grafted site which is often randomly picked along the sequence and could even be buried in the middle of the protein. Clearly, a better control of surface heterogeneity is of critical importance for the development of biosensors and the characterization of macromolecular interactions.

In this context, we propose here to carefully control the density and orientation of protein layers on gold surfaces for optimal biorecognition, by using nanografting, an innovative lithographic technique based on atomic force microscopy (AFM). A self-assembled, nano-sized monolayer of protein in controlled orientation is promoted by the tip of the AFM, and is embedded inside a bio-fouling carpet of self-assembled organic molecules. This allows at the same time for the miniaturization of the assay, making in principle possible to conduct the analysis in small sample volumes. Controlling the availability of active sites to the molecules in solution increases the sensitivity of the assay, particularly crucial when dealing with small volumes and a reduced number of analyte in solution. In our layout, AFM nanografting is used to localize the binder molecules. Sensitive AFM topography read-out is then used to measure the characteristics of the binding monolayer, and the successive binding of analytes.

2.1 Atomic force microscopy

The Atomic force microscope (AFM) is a surface imaging technique. It was developed in 1986 by Gerd Binning, Calvin F. Quate and Christopher Herber as “a combination of the scanning tunnelling microscope and the stylus profilometer”\(^ {127}\).

It is considered part of the Scanning Probe Microscopy (SPM) family, a class of instruments that investigate the properties of a sample at or near the surface. They can be classified according the nature of the interactions measured between a probe and a sample (electrical, like STM, mechanical, such as AFM, optical, or a combination of these, like SNOM, Scanning Near-field Optical Microscopy).

The AFM operates measuring the interaction between a sharp tip and a surface, using a flexible cantilever with a tip on the free end. The tip typically is fabricated of silicon–oxide and has a radius of curvature of 10 nm for standard
probes (Fig. 2.1a). Smaller radii of curvature allows reaching higher resolution (Fig. 2.1b). For this aim, carbon nanotubes (CNT) terminated tips have been recognized among alternative choice for AFM probe due to the small diameter (<10 nm), robust mechanical properties and chemical inertness (Fig. 2.1c).

![Figure 2.1. SEM images of AFM tips. a) Example of one standard AFM probe. b) High resolution AFM tip. c) CNT-terminated AFM probe.](image)

The soft microsized cantilever has a spring constant that may vary according to the sample and the application (from few tens of pN/nm to few tens of nN/nm), that is function of the elastic constant of the material and cantilever dimension. The force of the interaction is not measured directly, but calculated measuring the deflection of the cantilever by a laser beam focused on the top of the free end of the cantilever and reflected on a four-elements position-sensitive photodiode (Fig. 2.2).

Knowing the stiffness of the cantilever, in first approximation, the Hooke’s law gives the linearly relationship between the force required to deflect the cantilever and its deformation:

\[ F = -kz \]

Where \( F \) is the force, \( k \) is the stiffness of the cantilever, and \( z \) is the distance the lever is bent.

A piezoelectric actuator moves accurately the AFM probe and the sample in the X-Y plane in a raster manner. The number of lines scanned in a surface unit contributes to the resolution of the image. A Z-actuator, composed by piezoelectric material as well, controls the vertical movement of the tip.
2.1.1 Static mode: Contact mode (CM)

The contact mode AFM is usually considered a static operation mode and, as the name suggests, the tip is in constant contact with the surface of the sample. Repulsive forces dominate the interaction between the probe and the sample. In ideal conditions, when the tip starts approaching the surface, long-range Van der Waals interaction predominates; when the tip is near (some Angstroms) to the surface, short-range repulsive forces dominate. From the measure of the cantilever deflection, it is possible to calculate the force of the interaction between the probe and the surface, qualitatively described by the Lennard-Jones potential curve (Fig. 2.3).
During the scanning, the deflection signal gives the vertical deflection of the cantilever; the error signal is the difference between the deflection signal and the AFM feedback system’s setpoint value, and it is the input to the feedback. The output of the feedback guides the z-actuator, which moves the sample or the probe in the z-direction in order to maintain the interaction with the surface constant. In this way, it is possible to reconstruct the topographic profile of the sample (Fig. 2.4).

Figure 2.3. AFM force-distance curve. From this curve, it is possible to understand the role of the different interactions depending on the distance between the tip and the sample. The tip, approaching to the surface, starts to feel attractive long range forces (non-contact AFM region) until tip and surface electron orbitals are so close to start exerting repulsive forces (contact mode AFM region). Between these two regimes lies the intermittent-contact (or tapping mode) region.
CM-AFM gives the possibility to have a high lateral resolution, which in some conditions, depending on the characteristics of the tip and the sample damage, reaches the atomic resolution of the sample. One of the main advantages of the AFM is the possibility to perform measurement on conductive and non-conductive samples both in air and in liquid environment, particularly relevant in the case of study of biological samples, like DNA molecules, living cells or nanopatterned biomolecules. In this case, the set point is usually maintained below 100 pN in order to preserve the integrity of the sample\textsuperscript{128}.

\subsection*{2.1.2 Dynamic mode: Non-Contact and Tapping mode}

In dynamic mode, the AFM cantilever is driven to oscillate near its resonance frequency. The amplitude of this oscillation is used as feedback parameter for the imaging of the sample. Martin et al.\textsuperscript{129} proposed for the first time in 1987 the use of the atomic force microscope in oscillating mode with a small vibration amplitude (below 1 nm), to connect the variation of this oscillation with changes in the derivative of the force along the normal to the surface. Later, Zhong et al.\textsuperscript{130} proposed to increase the amplitude of the oscillation (up to 100 nm) in order to avoid the trapping of the tip by surface forces (in air) and the method was named tapping mode.
In **Non Contact-AFM (NC-AFM)** the tip is positioned at a certain constant distance from the surface (within few of nanometers) where attractive forces (electrostatic, magnetic, attractive van der Waals forces) predominate. Once the tip approaches the surface, it starts to feel it. Variations in the resonance frequency, the amplitude and the phase of the oscillation are linked to the characteristics of the surface and to the tip-sample interactions. All these parameters are monitored in order to reconstruct the topography of the sample.

In the case of **Intermittent Contact Mode (or Tapping Mode)**, the amplitude of the oscillation, influenced by long-range forces, controls the feedback system and the error signal (difference between the setpoint and the amplitude) is the input for the feedback system. The output, as in CM-AFM, controls the z-actuator and the tip movement. Moreover, during the scanning it is possible to record the variation in the phase of the oscillation. This signal is particularly useful in the case of a heterogeneous material because gives information about the chemical properties of the sample. Since the tip touch intermittently the surface, the damage of the sample is significantly reduced. Despite the difficulties of operating in tapping mode in liquid environment due to the significant dumping of oscillation frequencies by the viscosity of the aqueous medium, it is widely applied in biological field for the study of living cells, lipid membranes, DNA molecules and amyloid fibrils.

### 2.1.2.1 Force-modulation microscopy (FMM)

The force modulation microscopy is an AFM mode in which the sample is scanned in contact mode, maintaining a constant cantilever deflection. In addition, a periodic vertical oscillation is applied either to the tip or to the sample with a frequency typically on the order of hundreds of kHz. The tip motion is converted in an electric signal composed by two components: a DC voltage signal that represents the tip deflection during the contact imaging, used by the feedback system to maintain a constant force and generate the topographic image of the sample; an AC signal obtained from the tip response due to the oscillation. In particular, harder is the surface larger is the deflection of the cantilever and the amplitude response whereas softer is the surface smaller is amplitude response, due to the partial absorption of the oscillation. From a practical point of view, the amplitude value (pA) is proportional to the
substrate stiffness. FMM-AFM can, consequently, reconstruct a 2D image where colour contrast is representative of surface stiffness distribution: brighter colours means higher stiffness and vice versa. The amplitude of the AC signal (called ‘FMM Amplitude’) is sensitive to the elastic properties of the sample surface. Depending on the elastic properties of the sample, it is possible to record an FMM image that could be used as a map of the sample stiffness (Fig.2.5).

![Figure 2.5. FMM AFM-mode. During the scanning the AFM tip is in constant contact with the sample while the tip (or the sample) oscillate. The sample surface is periodically pushed by the tip, and the change in cantilever oscillation amplitude can be related to elasticity of the sample surface.](image)

Knowing the scanner vertical displacement ($D_z$), the probe tip vertical displacement ($D$) and the cantilever force constant ($K_c$), it is possible to determine the local elasticity of the sample ($K_s$):

$$K_s = K_c \left( \frac{D_z}{D} - 1 \right)$$

Moreover, knowing the local elasticity of the sample it is possible to calculate the Young’s modulus of the sample using for example the Hertzian model of the elasticity or comparing calibrating samples with known values. Force
modulation measurements are widely used in polymers, semiconductors, biological samples, especially for the study of composite materials.

2.2 AFM nanolithography: nanografting

The spontaneous self-assembly of thiols on a gold surface is well understood. Self-assembled monolayers (SAMs) are ordered, compact molecular structures driven by forces that span over different scale lengths: usually chemical interactions between molecular end-groups and the surface, and long-range dispersion forces (e.g. van der Waals forces) between the molecular tails. Alkanethiol SAMs on metals, and particularly on Au, are widely used in many different fields because of the easy preparation (Fig. 2.6), the stability and the strength of the S-Au bond.

![Figure 2.6. SAM preparation procedure.](image)

The substrate is immersed into a solution containing the molecules of interest having at one end a specific group capable to react with the surface of the substrate forming a bond. Finally, a packed ordered layer of molecules is spontaneously assembled.

The quality of the SAM could be affected by a series of factors that introduce defects in the organization of the layer. For example, crystallinity, roughness, cleanliness of the gold substrate, the temperature at which the sample is prepared, the immersion time, the concentration of molecules in solution are some of many parameters that affects the homogeneity of the SAM. SAMs prepared from solution are therefore far to be perfect. Still, they work as good passivation layers.
SPM and diffraction studies demonstrated that unconstrained self-assembly of alkanethiols on gold is composed of two main steps. In the first step, the process implies an initial physisorption, in which thiolated molecules adsorb on the surface through van der Waals interactions. This phase could also be described as a gas-like, highly disordered system, and is followed by the chemisorption of flat-lying molecules on Au through the S-heads (a process that takes seconds). This ordered phase is called lying-down phase or stripe phase, with the chain parallel on the surface. In this phase, alkanethiol molecules bind to the Au substrate through the S head by losing the mercaptan H atom, turning in alkanethiolates. Ordered flat-lying surface structures, known as striped phases are then formed. The increase in the surface coverage results in the nucleation of ordered domains (a process that can take some minutes or even hours depending of the molecules’ concentration) of standing-up molecules that finally cover the entire gold surface. In this phase, called also standing-up phase, the molecules start up to stand with their longitudinal axis tilted of 30° with respect to the surface normal, forming a compact layer as the result of lateral van der Waals interactions between the chains. In a normal SAM, grown at room temperature, the molecules follows the Langmuir (or modified Langmuir) absorption kinetics, with two time-scale characteristic of the two ordered phases, laying-down and standing-up (Fig. 2.7b).

Nanografting is a lithographic AFM mediated technique, developed by Liu et al in 1997. It requires as a starting step a SAM covered surface: it promotes the local exchange between molecules of the SAM and other thiolated molecules present in solution, forming confined nanostructures of one kind of molecules embedded in a SAM carpet of other molecules. The methodology is very simple: a self-assembled monolayer (SAM) of alkanethiols, typically oligo ethylene glicole terminated, is grown on an ultra-flat gold surface (template stripped gold, TSG) and is used as reference carpet for topographic measurement of the surface. The main property of this SAM is to be biorepellent, to prevent the aspecific adsorption of molecules on the surface. For this reason, it does not change during the experiment (as can be verified by AFM roughness measurements). The scan of the tip at high force (around 100 nN) permits the local displacement of the reference molecules that are replaced by thiolated molecules of interest dissolved in the liquid medium. The thiols in solution in fact, being highly concentrated with respect to the thiols displaced, bind to the exposed gold surface. In this way, it is possible to immobilize the
molecules in a well-oriented manner with high control and precision. The imaging at low force (<100 pN) gives topographic height data of our nano-confined self-assembled monolayer, from now on referred to as NAM, nanografted assembled monolayer (Fig. 2.7 a).

The spatial confinement of the free gold surface, inhibits the formation of lying-down phases in NAMs, favouring directly the standing up configuration and, under identical reaction conditions, self-assembly occurs much faster in NAMs than in unconstrained SAM. It was demonstrated moreover that the order and the packing of the nanografted molecules is much higher than the spontaneously assembled layer, as can be seen in Fig. 2.7b. Moreover, under identical reaction conditions, self-assembly occurs much faster during nanografting than in unconstrained self-assembly (Fig. 2.7b).

**Figure 2.7. Nanografting vs unconstrained self-assembly.** a) Nanografting procedure. The scanning of AFM tip at high force displace the molecules of a reference SAM of thiols promoting the replacement with the thiolated molecules of interest dissolved in solution. b) topographic images of C18 nanografted (right square) and self-assembled on bare gold (left square). Notably, self-assembly in spatially confined process like nanografting is much faster than the spontaneous unconstrained self-assembly. *Adapted from Liu et al, 2008*
The simplified adsorption kinetics in NAMs with respect to SAMs, implies a reduced amount of defects, which are in part connected to grain boundary formation in the laying-down to standing-up transition. In order to prove the increased order in a NAM compared to a SAM, the autografting approach was introduced. In this case, 1-octadecanethiol (C18) was nanografted inside a SAM of the same molecule. The CM-AFM topographic image of a 1-octadecanethiol (C18) patch enclosed into a C18 SAM was simultaneously acquired with friction image (Fig. 2.8). The tip is moved at fixed load and the cantilever torsion due to the tip/surface interaction is recorded (lateral force). The cantilever twisting is constant during scanning on a perfectly homogeneous surface, while it increases or decreases because of changes either in the gold surface morphology (boundary edges, surface steps or holes, but also gold surface roughness) or in the molecular film properties. In order to avoid the contribution of geometric characteristics of the sample, the net friction force image is obtained as the difference between the two lateral force signals (forward and backward trace). Friction signal is very sensitive to variation of the chemical properties of the sample that influences the proximity interactions with the tip, and give additional information for the sample characterization.

The topography of the patch is almost indistinguishable while, the friction image clearly displays the patch as a decreasing in contrast, suggesting that the tip moves more freely on the patched area. This experiment points out the better molecular packing and order of the molecules of a NAM respect to that of a SAM.

Figure 2.8. (A) Topographic image of an autografted C18 patch enclosed into a C18 matrix SAM and (B) the simultaneously acquired friction image. It is almost impossible to detect the patch in topography but there is an evident contrast reduction in the friction image. Darker areas mean lower cantilever torsion, so lower tip/surface lateral interaction. From D. Scaini, PhD thesis.
Conductive tip AFM (CT-AFM) measurement were also performed in order to confirm these speculations. Current flow is indeed very sensitive to the molecular order giving the possibility to appreciate defects more than AFM friction analysis. Topographic and current images of a 1-decanethiol (C10) autografted were simultaneously acquired (Fig. 2.9).

As observed in the previous experiment, also in this case the topography of the autografting is almost inappreciable, while it is possible to observe the grafted area in the current image. Analysing the current signal, it reveals the presence of current spikes at the edge of the patch and in the SAM area while no defects are present in the nanografted area. The main value of the current flow inside and outside the patch is the same but clearly more uniform in the NAM (unpublished data).
Another important advantage of the deposition of molecules through nanografting is the possibility to control the density of the molecules immobilized, tuning the fabrication parameters: the concentration of the molecules in solution, the force used during the displacement and the ratio between the scanned area and the total area of the confinement (S/A parameter) (Fig. 2.10).

![Figure 2.10. Surface density modulation via Nanografting. Molecules’ packing density tuned by changing the scan density ratio, that is the ratio between the actual area scanned by the tip and the total area of confinement. A visual cartoon is depicted in the specific case of a tip/surface contact area diameter of 10 nm, image size of 160×160 nm², and the number of line wrote by the tip on the fast scanning direction (pixels) are indicated in the three cases.]

### 2.3 Immobilization strategies

Proteins can be immobilized on a gold surface by exploiting different chemical approach. In the following sections, some of them are described.

#### 2.3.1 Non-covalent adsorption

This approach is widely used for the immobilization of proteins through non-selective interactions between the protein and the surface. The immobilization is due to physicochemical interactions such as hydrophobic adsorption or electrostatic interactions on charged materials. These interactions could be sufficient for the stable immobilization of the molecules but the orientation and the distribution of the molecules is extremely uncontrolled.
2.3.2 Covalent binding of proteins

2.3.2.1 Cysteine-tag

A good strategy for the immobilization of protein on a gold surface is the addition of a di-cysteine tag in a specific position of the protein (usually on the C or N-terminal of the protein). In this way, using the gold-sulphur chemistry, it is possible to immobilize a protein in a well-oriented manner by specific covalent bond.

The main disadvantage of this approach is that the folding of the protein could be affected because of the formation of disulphide bond between the cysteine of tag and the cysteines along the sequence.

This strategy was successfully used for the direct immobilization, by nanografting, of the maltose binding protein (MBP) and combinatorial protein S-284-C (Fig. 2.11).

Figure 2.11. Cartoon depicting a direct nanografting of de novo polypeptides. From Hu et al., 2005.

2.3.2.2 Amine-coupling

Amine-coupling represents another strategy to immobilize proteins through a chemical reaction. In this case, a surface functionalized with carboxyl terminated molecules, and activated by 1,3-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS), is available to react with amine groups in lysine side chains of the protein (Fig. 2.12). The main advantage of this approach is the chance to immobilize protein in a stable manner without any
protein modification although this strategy does not usually provide any selective reactivity.

Figure 2.12. Amine-coupling. Activated carboxylic groups react with amine groups of the polypeptide chain. Adapted from Cooper et al, 2002.

2.3.3 Bioaffinity interactions

2.3.3.1 Histidine-NTA

The poly-histidine tag is the most common affinity tag genetically encoded, realized for the first time by Hochuli et al in 1988. This tag is usually composed by six histidines, which chelate metal ions such as Cu (II), Co (II), or Ni (II), although the latter is the most commonly used. A chelating moiety such as nitrilotriacetic acid (NTA), known for chelating Ni, is employed in order to produce a support presenting the metal ions. In this way, it is possible to immobilize the proteins through its histidine tag (Fig. 2.13). This method is widely used for the purification of recombinant proteins by immobilized metal affinity chromatography (IMAC) and for this reason protein histidine-tagged modified are easily commercially available. This tag is usually located at one of the two termini of the protein, in order to maintain the capability of the binding to metal ions independent from the secondary structure of the protein.
Figure 2.13. NTA-Ni(II)-His-tag Immobilization Strategy. NTA-functionalized support coordinates a Ni (II), which trap proteins through interaction with their histidines.

The main advantage of this technique is the possibility of the oriented immobilization of proteins on a surface. It was applied in microarray field and for nanofabricated SAMs as well\textsuperscript{142–144}. Moreover, it is interesting for the possibility to remove the protein and reuse the NTA-functionalized surface exploiting the imidazole affinity for NTA.

2.3.3.2 DNA-Directed protein Immobilization (DDI)

Proteins modified by the chemical addition of a short ssDNA strand can be immobilized via Watson-Crick base pairing onto ssDNA NAMs. In this way, ssDNA array can be easily transformed, with good efficiency, into a protein array (Fig. 2.14). The process is named DNA Directed Immobilization (DDI). The main advantage of the DDI strategy, is the possibility of multiplexing: different ssDNA sequences can be immobilized on the surface, and used to address different proteins conjugated with the respective complementary DNA tails. By nanografting, DDI immobilization of Streptavidin, Glucose oxidase\textsuperscript{145} and Gliar Fibrillary Acidic Protein (GFAP), a potential cancer biomarker for glioma, was performed successfully (see Appendix).
Figure 2.14 Schematic representation of DNA Directed Immobilization strategy. The hybridization of nanografted ssDNA with the complementary strand, conjugated with the protein of interest, lead to the conversion of DNA NAMs into a protein array.

2.4 AFM nanografting: a potential tool for the study of IDPs

Nanografting technique was successfully exploited for the immobilization of biomolecules and applied for different studies \(^{146-148}\). Through the immobilization of proteins was possible to prove the stability and activity of nanografted molecules \(^{149}\), naturally exploitable for biosensing studies \(^{145}\). The technique was indeed successfully employed for the study of binding affinity parameters \(^{137,150}\). For all these reason nanografting could be a solution for the study of IDPs, difficult to handle with other approaches. For example, the technique has been already exploited in our lab for the immobilization and study of the prion protein (PrP). The prion (PrP\(^{Sc}\)) is a conformationally altered isoform of the prion protein (PrP), a normal membrane-anchored protein.

Great concern arises in the scientific community when the transmission \(via\) meet consumption was assessed through blood transfusion to a recipient patient was disclosed. Therefore, as current detection techniques are unable to detect PrP\(^{Sc}\) at the suspected infectious concentration in blood, the development of a technique able to detect the presence of the protein is required.

\(Via\) precise interfacial nanostructure measurements, the characterization of antigen-antibody biorecognition phenomena was performed on the
nanostructured array with unprecedented control over PrP orientation (Fig. 2.15).

**Figure 2.15. Cartoon of the oriented immobilization of recombinant mouse PrP (recMoPrP).** After nanografting of NTA-EG3-C16-SH into a reference carpet of protein repellent EG3-C11-SH, histidine-tagged Fabs (CloneP, on the left, and D18, on the right) are immobilized. Afterward, the sample is incubated in a solution containing recMoPrP 300 nM. At each step of the assay, topographic measurements (CM-AFM profiles are reported here) are collected for each nanopatch. *From Sanavio et al., 2010*

**Figure 2.16. Dose-response curve.** The blue curve report the differential height of recombinant mouse PrP after its addition on Clone P patches. The gray line reports the roughness variation of the same patches. In both cases the dissociation constant has been calculated ($K_d=2.9$ nM and $1.5$ nM respectively). *From Sanavio et al., 2010*
In this case, in fact PrP was immobilized in two different orientation, through the interaction with two distinct antibodies (Fab) immobilized on the surface through NTA-histidine chemistry. Therefore, the relative titration curves have been obtained\textsuperscript{142} (Fig. 2.16).
3. Proteins at model membranes

The cell membrane represents one of the most complex components of the cell. It separates the internal cell environment from the external one, contributing to maintain the cell homeostasis. Moreover, it selectively allows the transition of ions and organic molecules, it is responsible of signalling processes and is constituted essentially by lipids and proteins. Inside eukaryotic cell, lipid membranes surround also cellular organelles, as endoplasmic reticulum (ER), Golgi body, mitochondria. In spite of their different functions, all biological membranes have in common a lipid bilayer structure. When different lipids are present, there is the possibility they segregate forming domains called lipid rafts, recently suggested as involved in signal transduction, membrane trafficking pathways and neurotransmission\(^{152}\). The proteins associated with the membrane are much less characterized than soluble ones, although they represent the 15-39% of the human proteins. Understanding functions and characteristics of proteins that bind lipid membranes could be fundamental to see through many cellular processes. Among them, there is AS, which characteristics and functions are still unclear in many aspects, as already described in the first chapter. The use of model membrane is widely used in literature in order to simplify a complex system and try to perform a fundamental study at the basis of these interactions. In the following section, informations about membrane composition, assembly and study are briefly described.

3.1 Membrane composition and phase separation

Essentially, three main classes of lipids compose living and artificial cell membranes:

a) diacylglycerol-derived lipids,

b) sphingosine-derived lipids

c) sterol lipids (cholesterol).
Figure 3.1. **Structure of membrane lipids**: diacylglycerolipids (a), sphingolipids (b), and sterol lipids (cholesterol, c).

Diacylglycerol-derived lipids include phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), diacylglycerols (DAGs) and glycolipids with a glycosidic bond between the carbohydrate and the head group.

Sphingolipids are derived from the binding of a sphingosine base and an acyl chain through an amide bond. Sphingomyelin (SM), having a phosphorylcholine head group, is one of the most represented sphingolipids in brain (Fig. 3.1). Respect to glycerolipids, they are characterized by much more higher melting temperature (Tm), which can be over 60 °C.

Lipids spontaneously self-assemble forming a bilayer. This assembly is tremendously dependent on the temperature. Every lipid has indeed a Tm that varies with the length and degree of saturation of the acyl chains. Below Tm, they can assembly into a solid (gel, solid ordered, S ○ or Lβ) state in which the alkyl chains are well ordered in a crystalline hexagonal lattice and the alkyl chains are all in *trans* conformation; above Tm, the fluid (liquid-crystalline, liquid disordered, Lα or Ld) phase is observed, in which one or more double bond of the alkyl chain are in the *gauche* conformation. In general, lipids with unsaturated acyl chains increase their propensity to be in a Lα phase, the packing is low and the thickness of the bilayer decreases.
Cholesterol is the main constituent among sterol. The structure of cholesterol is very different from the other lipids. It is characterized by a highly condensed hydrophobic rigid area represented by a series of planar fused rings. The 3-hydroxy moiety is the only hydrophilic part (Fig. 3.2a). After the insertion of cholesterol inside the lipid bilayer, the rigid body is located alongside the fatty-acid tails of phospholipids and can help the order of these chains.

![Figure 3.2. a) The structure of cholesterol. It is composed by a rigid part of fused rings, a polar hydrophilic group and a hydrocarbon hydrophobic chain. b) Cholesterol, like other membrane lipids, has both hydrophilic and hydrophobic poles that determine its positioning within the lipid bilayer. When the hydroxyl group is next to the phospholipid ester carbonyl, the rigid body of cholesterol is situated alongside the fatty-acid tails of neighbouring phospholipids and can help to order these tails. From Maxfield et al, 2005.](image)

The interaction of cholesterol with other lipids is preferential for certain lipids. In particular, complexes with lipids such as SM through hydrogen bonds are observed. Moreover, cholesterol, inserted perpendicularly into the lipid bilayer (Fig. 3.2b), reduces the bilayer permeability, while the effect on lateral diffusion of proteins and lipids in the plane of the bilayer is minimal. Cholesterol, filling the interstitial spaces, increases the rigidity of the membrane and the packing of the acyl chains. This has an effect on the lipid phase. This particular lipid organization is called liquid ordered phase (Lo) in which the molecules are more tightly ordered and packed than in the Ld phase. In fig. is reported the phase diagram of DPPC in presence of cholesterol. At low cholesterol concentrations, Lo phase is in equilibrium with the Ld phase up to about 30 mol %, while at high concentrations (more than 30 mol %), Lo phase only is observed and DPPC thermal transition disappears.
This explains the high cholesterol content of biological membranes (around 35 mol %) in which the phase transition diminishes. The $L_o$ phase is associated to lipid rafts domains in the real cell membrane. 

In vivo, phospholipids, cholesterol and sphingolipids are between the main components of the most common membrane, although the precise composition depends on the cell type and the membrane localization. In particular, the major component of synaptic vesicles membrane is phosphatidylcholine (PC, 40.9 mol%), then phosphatidylethanolamine (PE), sphingomyelin (SM), plasmenylethanolamine, phosphatidylserine (PS), and phosphatidylinositol (PI) (24.6, 12, 11.5, 7.3, and 3.7 mol% respectively). High level of cholesterol is present (38.5% of total lipid content) 157.

Moreover, it is important to consider that the composition of lipid membranes is asymmetric. For example, the outer leaflet is rich in PC and SM, while it is possible to find negatively charged lipids as PA, PG and PS in the inner leaflet of the plasma membrane 158.

### 3.1.1 Lipid rafts

“Lipid rafts” 159 were discovered as the fraction of the natural cell membrane resistant to treatment with non-ionic detergent (Triton X-100 at 4 °C) 160. This
portion of the membrane is rich in sphingolipids and cholesterol, which interact through the formation of hydrogen bonds between the OH groups of cholesterol and NH group of sphingosine or through the interaction between cholesterol and sphingolipids with long alkyl chains. Moreover, lipid rafts are rich in lipid-modified proteins containing saturated acyl chains such as GPI-anchored proteins or doubly acylated Src-type kinases and transmembrane proteins \(^{161}\) (Fig. 3.4).

![Figure 3.4. Schematic representation of lipid rafts in the cell membrane. Lipid rafts are rich in sphingolipids (brown head, blue tails) and cholesterol (in yellow). Moreover, glycolipids, GPI-anchored proteins and transmembrane proteins enrich these domains of the membrane.](image)

The distribution of lipid rafts on the cell membrane surface depends on the cell type. For example in vitro, lipids rafts accumulate in the apical plasma membrane in epithelial cells, while in the axonal region in neurons. Few rafts in basolateral and somato-dendritic membranes respectively are detected \(^{159}\). Lipid raft are mostly abundant in the plasma membrane but they are observed in biosynthetic and endocytic pathways too. Few is known about lipid rafts, and the main reason is the difficulty to visualize them on cells because too small to be characterized by conventional light microscopy. In fibroblast, for examples, rafts assembly into 50 nm diameter domains \(^{162}\). It is still unclear the mechanism of proteins insertion but up to 15 equal proteins were observed in the same raft \(^{163}\), supporting the hypothesis of a certain specificity of the binding.
3.1.2 Lipid rafts and disease

Recent experiments, focusing on the study of the cell membrane, have demonstrated an important role of lipid rafts in biological activities. In particular, proteins associated within those domains have been demonstrated to be fundamental in many cellular functions such as signal transduction, protein and lipid sorting, cell adhesion and motility. In particular, they are related to many neurodegenerative diseases\textsuperscript{164}. For example, a form of the prion protein (PrP), responsible of transmissible spongiform encephalopathies (TSEs) have a GPI anchor and seems to localize inside rafts. The mechanism of the pathogenesis require the conversion of PrP into the pathogenic form PrP\textsuperscript{sc} which seem to be inhibited by a decrease of cholesterol that lead to the detachment of the raft associated proteins\textsuperscript{165}. Alzheimer’s disease (AD) is characterized by the abnormal accumulation on Aβ (amyloid-β-peptide) produced by the precursor cleavage by β- and α-secretase, both localized on membrane rafts. A reduction of cholesterol decrease the formation of Aβ peptide\textsuperscript{166}. Parkinson’s disease is related to lipid rafts too. In particular, in the early stages of PD, the composition of lipid rafts in the frontal cortex of incidental PD (iPD) is altered and comparable to PD cases in advance stages. The concentration of long chain polyunsaturated fatty acids (LCPUFA), which plays a role in the stability of rafts, decreases dramatically, where SM, cholesterol and saturated fatty acids concentration increases in cortical lipid rafts. Lipid rafts becomes more ordered compared to the age matched control, leading to an indication of progressive neuronal dysfunction. Moreover, low concentration of LCPUFA facilitates the aggregation of AS in the early stages of the disease. For this reason, the study of lipid rafts could be fundamental for understanding the processes at the basis of pathogenic events\textsuperscript{164}.

3.2 Artificial lipid bilayers

Artificial lipid bilayers are widely used as model in membrane-protein binding studies \textit{in vitro}. Lipid molecules, composed by hydrophilic head group and hydrophobic tails, spontaneously self-assembly in liquid environment, in order to hide their hydrophobic core. Depending on the molecular characteristics, lipids could form:
A. micelles, spherical structures composed by a single layer of lipid, in which lipid hydrophobic chains are toward the center and the hydrophilic head groups are toward the external aqueous solution;

B. vesicles, spherical structures composed by two lipid layers in which lipid hydrophobic chains face the others, while hydrophilic head groups are exposed to the liquid internal and external environment;

C. planar lipid bilayers.

Lipid vesicles could be prepared as multilamellar (multiple layers) or as unilamellar (single lipid layer). Unilamellar vesicles are easy to prepare and to manipulate and are a good model for the study of biomembranes in vitro. They are classified according their dimensions as small (SUVs, 10–100 nm), large (LUVs, 100 nm) and giant unilamellar vesicles (GUVs, >1 μm) and are usually used as model system for small trafficking vesicles (SUVs) and cellular membranes (GUVs) (Fig. 3.5).

![Figure 3.5. Schematic representation of different model artificial lipid bilayer commonly used in biophysical research and their approximate size. Adapted from Pfefferkorn et al, 2012](image)

Supported lipid bilayers are robust and stable and give the possibility to use common surface analytical techniques. In order to obtain a good lipid layer, with few or no defects, the solid support should be hydrophilic, flat and
perfectly clean. For these reasons, mica\textsuperscript{168–170}, silicon\textsuperscript{171,172} and glass\textsuperscript{173} are widely exploited. Planar supported lipid bilayer (SLB) can be prepared using different methods. The most used are briefly described:

1. **Langmuir-Blodgett** (LB). The experimental apparatus consists in a Teflon bath with movable barriers. Lipids are deposited at air-water interface dissolved in solvents. The movement of the barriers is used in order to compress lipids and a pressure \textit{vs} area isotherm could be recorded, giving information about the lipid packing (Fig. 3.6-3.7a).

![Figure 3.6](image)

**Figure 3.6.** Theoretical pressure \textit{vs} area isotherm obtained compressing an insoluble lipid monolayer at air-water interface. At low pressure, the area covered by lipid molecules is high because of the lack of layer organization (gas phase). Increasing the lateral pressure, the molecules start to standing up (liquid phase) until the formation of a uniform monolayer (solid phase).

The lipid monolayer is then transferred on a solid support moved through the solution with constant lifting speed (Fig. 3.7b). At this point, it is possible to obtain a lipid bilayer transferring a second lipid layer (Fig. 3.7c).
Using LB, it is possible to obtain mainly three types of membranes (X, Y, Z). Repeating immersion (downstroke movement) and emersion (upstroke movement) of the substrate Y-type membranes are obtained; by downstroke and upstroke movement only, it is possible to obtain X and Z structures respectively (Fig. 3.8).

2. Langmuir-Schaefer (LS). LS was introduced in 1938 and consists in the preparation of a solid lipid layer on the air-water interface, as
already described for LB, but the deposition on the solid support is horizontal. In particular, the solid substrate is approached at low speed toward the lipid monolayer (2-4 mm min\(^{-1}\)) and, after contact, lifted up (1-2 mm min\(^{-1}\)). In this way, X-type films are prepared (Fig. 3.9).

![Figure 3.9. Schematic representation of Langmuir-Schaefer deposition. X-type lipid layers are easily prepared by subsequent approaches of the substrate to a lipid solid layer formed at air-water interface.]

3. **Fusion of vesicles.** It is one of the most popular method for the preparation of lipid bilayers on a solid support. After the preparation of SUVs in solution, they are put in contact with a flat surface where the rupture of the vesicle occurs. The fusion of vesicles on the surface is affected by the lipid composition, size, surface characteristics, pH, and ionic strength\(^{174}\). The mechanism is not completely understood but it is known that the process involves adsorption of the vesicles on the surface, deformation, flattening and rupture to form a continuous SLB (Fig. 3.10).
The main advantage of this technique is the simplicity of this approach, although compared to LB, it is impossible to control the lateral pressure and to prepare asymmetric bilayers.

4. **Spreading of organic solution.** A solution of lipids, dissolved in a solvent are directly pipetted on a solid support and let to evaporate favouring the self-assembly of the molecules (Fig. 3.11). The main advantage of this approach is that it does not require any instrumentation, is easy and fast to perform\textsuperscript{175,176}.

*Figure 3.11.* The picture depicts the deposition process of lipids by solution spreading. A syringe is used to deposit a drop of organic lipids’ solution at the center of a silicon wafer. *From Seul et al, 1990*\textsuperscript{175}.
It is used especially for structural studies by NMR or X-ray scattering because of the multilamellar characteristics of the membrane obtained, impossible to prepare with the previously described techniques but, at the same time, it is difficult to control the exact number of layers forming.

5. **Spin coating.** In this case, lipid dissolved in organic solution are deposited on a supported substrate (hydrophilic or hydrophobic) and accelerated to rotation using a spin coater 177 (Fig. 3.12). Thanks to the centrifugal forces, after the evaporation of the solvent a homogeneous film of well-oriented lipids is deposited on the surface.

![Figure 3.12. Sketch illustrating the spin-coating procedure showing a pipet with lipid solution above the rotating substrate. Adapted from Mennicke et al, 2002 177](image)

### 3.2.1 Artificial Lipid bilayers: a model for lipid rafts

Artificial membranes are simple systems that allow easy control of the experimental conditions and are extremely useful to investigate the principles at the basis of membrane organization. SLBs are commonly used as model system in order to study biological membranes. The main advantage of this approach is the stability of the membrane, the possibility to prepare membranes with different lipid composition and the availability of an easy protocol for the deposition. The alternative is the study of vesicles in solution, especially GUVs, having dimension from few to hundreds of micrometers, which can be studied with conventional optical microscopes. In presence of a lipid composition
mimicking rafts, a spontaneous organization into domains occurs. These domains are rich in sphingolipids and cholesterol (L_o phase), distinct from a more disordered phase (L_d). These model systems have been characterized with different techniques. Among them, Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Recovery After Photobleaching (FRAP) were used to distinguish the two phases, based on fluidity differences, in SLBs {178,179} and GUVs {180,181}. These approaches were used also to calculate the diffusion coefficient, the effect of saturation in the acyl chains, cholesterol concentration, and dynamics. From the other hand, SLBs were used to evaluate the effect of the presence of proteins on the lipid lateral mobility and phase transition temperature {182–185}. Recently, the combination of FCS with AFM measurements, allowed the characterization both of dynamic characteristics and morphological organization at the same time {186}. A combination of FCS and STimulated Emission Depletion microscopy (STED) was also used to investigate the dimension and the dynamic properties of raft-like domains {187}. AFM measurements have been used to characterize topology of rafts and the difference in height of ordered domains, which, depending on the cholesterol content, are around 0.5-1 nm higher {188,189}, giving a precise three dimensional characterization of these structures at nanometer scale. Wide-angle X-ray diffraction (WAXRD) was used to characterize the thickness of lipid domains in multilamellar vesicles composed by DOPC/sphyngeomielin, resulting in an increase in the height of 0.9-1.1 nm respect to DOPC only {190}. Moreover, Single-Particle Tracking (SPT) was used to analyse the lipid diffusion inside rafts. In this case, some lipid molecules are bound to a gold nanoparticle and, it is possible to follow the particle movements inside the layer using confocal laser microscopy. In this experiments the coefficient of lipids inside the L_o phase was demonstrated to be similar (0.38 x 10^{-8} cm^{-2} s^{-1} ) than in the L_d phase (1.1 x 10^{-8} cm^{-2} s^{-1} ) {179}. 
4. Results: AS immobilization on a gold surface

The unfolded characteristics of IDPs and their propensity to aggregate make difficult to study them with conventional, solution based techniques. In particular, the determination of binding affinity parameters is a challenge and no data are available in literature. In this context, techniques based on protein immobilization on surfaces can be a valid alternative to study biorecognition phenomena. In this section, preliminary experiments about the immobilization of AS on a gold surface by AFM nanografting and the study of the interaction with DA are described.

4.1 via cysteine-tag

Despite the known limitation of surface studies, they can represent a good mean for IDPs study. Surface techniques can provide indeed important information about the interaction with small molecules, impossible to be measured in bulk. In order to immobilize in a well oriented manner AS on a gold surface, we decided to engineer a specific tag at the N-terminal of the protein. In particular, we introduced via recombinant techniques two cysteines at the N-terminus through which grafting the protein on a gold film exploiting the Au-S chemistry (see Fig. 4.1). The N-terminal as grafting site has been chosen because in this way we expected to have the C-terminal of the protein free to interact with the solution. The acidic region of the protein contains in fact the 125YEMPS129 site (res 125-129) that is reported as the main binding site for DA. Scope of our work was to study the binding affinity of AS with this small molecule, DA, physiologically relevant but still unknown in literature. Exposing the 125YEMPS129 site towards the solution, we aimed at obtaining a homogeneous distribution of AS orientation relatively to its grafting site. Self-assembling assisted by the AFM tip, as in nanografting, can represent a good method for the study of IDPs. In particular, AS was immobilized into a self-assembled

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b In collaboration with dr. Barbara Sorce and dr. Stefania Sabella, IIT-Lecce, Italy
monolayer of alkanethiols terminated with six ethilenglycole units (EG6) that does not change during the experiment because of its bio-repellent properties and, for this reason, it is used as reference for topographic AFM measurements of AS functionalized patches.

Figure 4.1. Schematic representation of the nanografting experiment. A self-assembled monolayer of biorepellent thiols on gold is substituted locally by cysteine-tagged AS. The protein on the surface expose the C-terminal that contains the specific site for DA binding (125YEMPS129), which overlaps for one amino acid with the site for a AS mAb (121DNEAY125).

Because of the difficulty to handle unstructured proteins, the protocol of the immobilization required a careful review. The immobilization of AS was in fact never successful when it was dissolved in buffer solution where the protein is expected to be in the unfolded state. Besides the reduced diffusion, protein grafting was probably hindered by the structure of the unfolded protein, by which was hiding the cysteine tag engineered at the N-terminus, preventing the interaction between the cysteine sulfur group and the gold surface. Therefore, we exploited grafting buffers that favor either alpha-helical or beta-sheet conformation. When AS was dissolved in trifluoroethanol (TFE) to force alpha-helices 104, the increased rigidity of the protein in correspondence of a long segment at the N-terminal 191 (similarly to membrane-associated AS conformation) did not leave the cysteine tag free to react, and the grafting was unsuccessful.
Successful immobilization was obtained only in solvents that promoted secondary β-sheet structure and amorphous oligomers for AS. We nanografted AS in pure methanol, with an addition of a reducing agent (TCEP) in millimolar concentration: the latter keeps the di-cysteine tag reduced and the thiols freely available to interact with gold. This result was totally unexpected, since in principle disulfides adsorb on gold more efficiently with respect to thiols. Again, we explained this result in terms of protein conformation, which could hinder somehow the disulfide-Au interaction. In addition, in presence of TCEP, the N-terminus is negatively charged, and possible electrostatical repulsions with the acidic C-terminus can be another reason for the thiolated end staying out of the β-sheet secondary structure of the protein.

Figure 4.2. The AS was nanografted in a reference thiolated EG6 SAM (a,c). The presence of the protein was confirmed by the loading of the mAb and a variation in height, compatible with the size of the mAb, was recorded (b,d). Scale bar 1 µm.
AS nanografting was performed in methanol scanning 1 µm x 1 µm area of the gold surfaces with force around 100 nN. The sample was imaged in contact mode at minimum force (below 100 pN) in buffer solution (Fig. 4.2). The AS patch appeared as a hole of height of -1.5 nm, with respect to the EG6 carpet. To calculate the “true” height of AS molecules in the patch, we measured the height EG6 carpet with respect to the gold film surface, creating a hole in the EG6 SAM via “nanoshaving” (i.e. nanografting in a solvent for EG6, methanol in our case, without any other thiolated molecules in solution). From the topographic profile of the hole, a height of 2.6 nm was calculated, compatible with EG6 dimension (Fig. 4.3).

![AFM topography of a hole into the EG6 SAM.](image)

**Figure 4.3. Nanoshaving into EG6 SAM.** a) AFM topography of a hole into the EG6 SAM. b) Topographic line profile of the surface suggests a height of about 2.6 nm.

As a consequence, AS average height in the nanostructure was about 1.3 nm. This is in agreement with previous reports measured the effective radius of AS, assuming a spherical structure, to be 1.7-2.1 nm.
Moreover, the fact that we obtained a hole and not a protruding AS nanostructure may be related to the low density of the grafted molecules, due to the reduced mobility of AS in solution, and to the compressing effect of the tip during imaging, despite the extremely low load applied.

In the experiment shown in Fig. 4.2 (from now on recalled as exp.1), the presence of AS grafted on the surface was also confirmed by exposing the surface to a specific mAb (8 nM, clone syn 211) able to recognize an epitope at the C-terminal of the protein (Fig. 4.2 b,d). We chose a mAb whose recognized epitope spans residues 121-125 (W121DNEAY125) and overlaps for one amino acid the region of residues 125-129 (Y125EMPS129), which is known to be a target for DA, as shown schematically in Fig. 4.1.

The differential increase in height after mAb binding is compatible with the average dimension of an immunoglobulin G molecule (IgG ca 15-18 nm). Specificity of the binding was also confirmed with a mock nanografting experiment. An area was “nanoshaved”, removing the preexisting SAM and exposing the bare gold surface. The sample was then incubated for 2 hours with mAb that did not bind to the selected area, except few particles, not comparable with the specific protein recognition (Fig. 4.4).

![Figure 4.4. AFM nanoshaving was performed into an EG6 thiols carpet. A hole was measured by contact mode AFm in liquid (left). The sample was incubated in presence of mAb (clone syn 211) for two hour. AFM imaging suggest no relevant variation in the shaved surface, meaning that no aspecific interaction with gold occurred.](image)

In order to prove further the success of AS nanografting, we observed that both surface roughness and friction within the nanografted area were different from the surrounding carpet, which is compatible with a modified surface (Fig. 4.5).
Figure 4.5. The image report the friction signal (difference between lateral force trace and retrace) for a nanopatch obtained after nanoshaving of the surface (left) and after AS nanografting (right). Clearly, in the first case, the friction signal is very weak and the nanoshaved area is almost indistinguishable while the surface functionalization is evident by an increase of friction.

4.1.2 AS-DA affinity

Having proved that nanografting is an effective way to immobilize AS in confined areas, we tested such micropatterns as sensor to study the binding affinity with DA. This neurotransmitter is known to interact with residues 120-125 in the C-term tail of the protein, and simultaneously with residues in the NAC region as long range contact that may stabilize the bound conformation.\textsuperscript{115,116}

4.1.2.1 AS nanostructures to study DA-AS affinity

In order to estimate DA-AS binding affinity, we build a binding assay, measuring the topographical variations of AS nanografted patches incubated with increasing concentration of DA.
Figure 4.6 AS-DA binding experiment. AS change morphologically and some particles appear after increasing DA binding, scale bar 500 nm. The loading of DA at increasing concentration lead an evident morphological modification of the protein.

An average increase in height, though very moderate, could be recorded. More interestingly, the roughness of the nanografted area showed an increase, related to DA binding (Fig. 4.6).

Inspection of the AFM images shows the presence of particles that do not increase in number (they are the same throughout the experiment) upon DA dosing. We analyzed the binding of DA to AS through a sigmoidal dose response of the patch height, obtaining an apparent dissociation constant in the micromolar range (Kd= 1.66 ±1.03 µM) (Fig. 4.7a). The same fitting was performed on the roughness variation data, confirming the same trend (Kd=1.35 ±1.57 µM) (Fig. 4.7b).
Figure 4.7. AS nanografted patches were incubated in presence of increasing concentrations of DA. The binding was recorded by an average increase in the height of the patches. Fitting with a Langmuir isotherm gave a $K_d = 1.66 \pm 1.03 \, \mu M$ (b). The same trend is showed by roughness variation analysis. The $K_d$ calculated is $1.35 \pm 1.57 \, \mu M$ (b). The error is the standard deviation of the average height calculated on five different patches.

After loading of DA, the sample was treated with nanomolar concentration of mAb syn211 that recognize epitope^{121}DNEAY^{125}, which overlap for one amino acid (Y125) to the DA binding site. The height of the patches upon mAb loading increases, suggesting that the surface is still covered by the protein with the binding site exposed to the liquid-solid interface, in agreement with
previous studies. The measured height (3.3 nm over the AS nanografted patch) is however lower than expected (see exp. 1, Fig. 4.2 b,e) suggesting that only a portion of the C-terminal is exposed, probably because, upon the formation of AS-DA adducts, only a part of AS molecules expose the C-terminal to the solution (Figure 4.8).

**Figure 4.8 AS-DA patches recognize the mAb.** The AS nanografted patches were treated with 10 mM DA (a). The patches were then incubated with nM concentration of the mAb (b). The topography of the surface increases but it is less than expected (c).

### 4.1.2.2 mAb-DA non-competitive binding

AS nanografted patches are recognized by mAb syn211 that binds epitope DNEAY, which overlap for one amino acid (Y125) to the binding site of DA. This specific mAb was chosen among many for its capacity of binding the
C-term chain of AS and because of the vicinity of the epitope to the DA binding site. In fact, though the DA binding site overlap for only one aa, there is a chance that the conformational change due to the short and long range interaction of DA with $^{125}$YEMPS$^{129}$ and residues in the NAC region destabilizes the binding site of the mAb, as is indeed observed experimentally. The overlapping of only one amino acid of the bindings sites is not sufficient to consider the binding of DA and mAb competitive. For this reason, we called it as “non-competitive”.

**Figure 4.9 mAb-DA non-competitive binding.** AS patches recognized by the mAb are incubated with increasing concentration of DA. The plot reports DA response as height variation over AS-mAb nanopatterns ($H/H_0$). The displacement of the mAb is monitored as a decrease in height. The plot report the height variation as a function of DA concentration. Fitting with an inverted Langmuir isotherm gave a $K_d = 3.92 \pm 1.64 \, \mu M$.

Since small molecules, like DA, need very precise instrument in order to register their binding, the non-competitive binding assay can confirm more confidently the data obtained in the direct binding assay. In this case, bigger height variations, relatively easy to be measured, can give more precisely the estimation of the binding constant. In this assay, immobilized AS was incubated in presence of the mAb and the saturated surface was analyzed by AFM topography variations.
After DA addition at increasing concentration, the patches show a decrease in height due to the partial displacement of mAb molecules over the patch (Fig. 4.9).

Plotting the height signal as measured height over saturated height vs DA concentration, we obtain an inverted Langmuir isotherm from which it is possible to calculate the Kd value that represents the concentration of DA that displays half-maximal displacement of the mAb. The value obtained is around 4 µM and in good agreement with the experimental Kd calculated in the direct assay previously described. As observed by the data plot, at saturation the mAb is just partially displaced by the surface. This phenomenon suggests that, during the conformational change that affects AS molecules, probably a part of them continues to expose the C-terminal to the solid-liquid interface (see previous experiment, Fig. 4.8).

4.1.1 AS nanostructures to promote the formation of supramolecular assembly and to study the effect of DA on them.

Finally, I present preliminary results about the potential use of this assay to study the early stages of the AS aggregation. In this case, AS nanografted patches (Fig. 4.10a) were incubated with a solution containing recombinant AS (histidine-tagged) to promote AS aggregation. We observed a time-dependent topographic height increase over the AS patches versus incubation time (24-48-72 hours, Fig. 4.10b, c, d) suggesting a local interaction between AS molecules. The addition of highly concentrated DA (20 µM, close to the saturation point) disrupts these aggregates after 30 minutes incubation, probably stabilizing small oligomers, observable as round spheres by AFM imaging (Fig. 4.10e). Further addition of AS histidine-tagged molecules plus DA after DA treatment does not show any increase in patch height after 24 hours incubation, confirming the stable binding of DA on the protein (Fig. 4.10f). The analysis of the roughness of the patches confirms the formation of aggregates on the surface (Fig. 4.11b).
Figure 4.10. AS patches were incubated in presence of AS histidine tagged. The surface was monitored over time by topographic AFM measurement (a, b, c, d). Finally, the effect of DA was evaluated. DA interacts with the AS self-association site disrupting the aggregates formed in 72 hours, stabilizing small oligomers (e). Such binding is stable and prevents the self-association in presence of AS molecules also after 24 hours of incubation (f). Scale bar 500nm.
Figure. 4.11. The aggregation process and the disaggregation are reflected by the topography (a) and the roughness (b) of the surface.

These data support the hypothesis that DA and its oxidation products, such as dopaminochrome, interacting with the $^{125}$YEMPS$^{129}$ region, inhibit the formation of AS aggregates. This suggest that DA binding probably causes a conformational rearrangement in the AS molecules, due to the interaction with distal residues at the N-terminal, leading to an off-pathway intermediate, unable to produce aggregates.
4.2 Other strategies for AS immobilization

In order to immobilize a protein we tried also other strategies but they revealed unsuccessful. Some of these tests are described.

4.2.1 Via NTA-Ni-Histidine chemistry

Another approach we used in order to immobilize the AS on a gold surface was via NTA chemistry. The main advantage of this approach is the possibility to use a histidine-tagged protein (commercially available). In this case, three NTA molecules are covalently bound to a DNA sequence 24 bases long (Fig. 4.11).

![Figure 4.11. Schematic representation of ssDNA sequence modified with three NTA residues.](image)

In this way, the hybridization of ssDNA NAM gives a functionalized surface available for the protein immobilization. Another advantage of this approach is to have the protein far away from the surface and freely available to fold in the proper manner. Moreover, it is possible to have an assay that could be regenerated, used many times, and naturally prone to the multiplexing via DNA base-pairing.

For this reason, we performed the nanografting of a thiolated ssDNA sequence, 24 bases long (HS-F5) on a gold surface into a self-assembled monolayer of alkane-thiols, terminated with 3 ethilen-glicole units (HS-(CH$_2$)$_{11}$-(OCH$_2$CH$_2$)$_3$OH) used as reference carpet. The nanografting of the ss-DNA, at different densities (S/A from to 5.12 to 0.64) was done (Fig. 4.12).

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4 In collaboration with Dr. Lijljana Fruk, DFG- Centre for Functional Nanostructures, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany.
As expected, the height of the patches increases as the density of the molecule increases. This is because crowded standing up molecules are much more affected by repulsive van der Waal forces and/or electrostatic repulsion which let them to stretch in the unconstrained vertical direction. At this point, in the easier and fast 2 step-approach (Fig. 4.13), the conjugate between cDNA-NTA+NiCl$_2$+Protein was prepared in solution and then let to conjugate with the immobilized ssDNA. In order to optimize the technique for the deposition of the protein, we decided to start with the deposition of D18, a Fab (50kDa) of the prion protein, already used in our laboratory for the deposition via histidine-tag on thiolated-NTA-terminated molecules. The complex was prepared was prepared in solution (600 nM F5-NTA, 1.8 µM NiCl$_2$, 1.8 µM D18 in PBS) and incubated for one hour on the surface.
Figure 4.13. Schematic representation of protein immobilization via NTA-Ni(II)-Histidine in two steps. After the nanografting of ssDNA sequence, the hybridization of the complex (cDNA-NTA +Ni(II) +Protein) can be performed.

The measurement of the topography of the surface suggests a successful hybridization of the complex, which, as expected is strongly dependent on the initial ssDNA (Fig. 4.14, red bars). In fact, it occurs with higher efficiency when the crowing is lower while higher is the covering of the molecules, less is the probability the complementary strand interacts. As already mentioned, the surface could be easily regenerated by imidazole, which, competing with the histidine tag of the protein, is able to displace it. In this way, the surface expose the dsDNA –NTA-terminated, measuring height profiles compatible with the hybridized DNA (Fig. 4.14, green bars).
Figure 4.14. Height of the patches for D18 Fab immobilization on a gold surface with NTA strategy. Nanografting of ssDNA was performed at different S/A (from 5.12 to 0.64) and the height recorded reflects the density of the molecules (blue bars). The hybridization of the D18 complex was more efficient when DNA molecules are less dense (S/A=1.28 and 0.64). Finally, imidazole, competing with histidine, removed the protein (green bar).

The same approach was tried for the immobilization of AS, but no hybridization occurs. We speculated that DNA sequences in solution interact aspecifically with the protein an hence is not able to hybridize the complementary DNA.
For this reason, a second strategy was used. In this case, the reaction was performed step by step (Fig. 4.15).
First, the nanografted thiolaed ssDNA was let to hybridize with its complementary strand NTA-conjugated and the variation in the topography of the patches was measured. Then, the surface was activated by the Ni$^{2+}$ chelation and finally AS was added (Fig. 4.16).

Unfortunately, also in this case the immobilization did not occur. Probably, as observed for the direct nanograf ting also (see chapter 4.1), the conformation of AS in acqueous solution, expected to be unfolded, easily could hide the tag at the N-terminal (histidine-tag in this case) decreasing the efficiency of the immobilization.
4.2.2 Amine-coupling

The immobilization of proteins through the covalent binding to a carboxyl-functionalized gold surface was also exploited. For example, Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) was immobilized with this strategy. First, a self-assembled monolayer (SAM) of a thiolated carboxylic acid (16-mercaptoadecanoic acid, HS-(CH₂)₁₅-COOH) was growth on a flat gold surface and the formation of the SAM was confirmed by “nanoshaving” AFM measurements. The height of the layer on gold is around 1.8 nm, compatible with the dimension of the molecule. Second, carboxylic groups were activated through the reaction with DCC and NHS, as described in chapter 2, and the protein was incubated for 1 hour. Also in this case “nanoshaving” was used in order to confirm the reaction with the protein. The variation in this case is of few nanometers and some particles are observed,

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Figure 4.16. NTA-Histinе tagged AS immobilization by nanografting. Nanografting of thiolated ss DNA 24 bases was performed into an EG3 SAM. The sample was then hybridized by the complementary DNA strand modified with NTA. Finally, after Ni (II) loading, histidine tagged AS was added but no variation in height was found.

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In collaboration with Prof. Vincent Torre, SISSA, Trieste.
compatible with the partial aggregation of the protein and the formation of the tetramer (Fig. 4.17).

Figure 4.17. AFM imaging in contact mode and relative topographic profile of Amine-coupling process for the immobilization of HCN4. A SAM of a 16-mercaptoesadecanoic acid has been formed on a flat gold surface. The nanoshaving of the SAM gave the height of the layer (upper images). Applying the amine-coupling protocol the immobilization of the protein was measured as a height increase (bottom images).

The same protocol was used for the immobilization of AS. In this case, nanografting of the thiolated carboxylic acid was performed and topography of the patches measured (Fig. 4.18). The height of the NAM in this case is around 2 nm, higher than in the SAM of the previous experiment, meaning a major order and packing of the molecules.
Following the same protocol, unfortunately no AS immobilization occurred also in this case. This result strengthens the hypothesis that the unfolded conformation of AS, expected in aqueous solutions, and its high flexibility hide the binding site, decreasing the efficiency of the immobilization.

### 4.3 Discussion and conclusions

In this work, we demonstrated that nanografting is a useful tool to immobilize AS and study the interactions that affect this protein. First, we exploited different approaches in order to immobilize AS. We tried to immobilize histidine tagged protein on a DNA-NTA functionalised surface by nanografting but although we proved that our protocol was efficient for the immobilization of other proteins (e.g. D18, PrP Fab), it was unsuccessful for AS. Moreover, amine-coupling was exploited with the same unsuccessful rate. Finally, the direct immobilization of AS through a cysteine tag engineered at the N-terminus was investigated. Also in this case the immobilization was really tricky, probably because, due to the unfolded state of the protein, the binding site could not be exposed to bind efficiently the gold surface. The only case in which the immobilization was successful was forcing the beta sheet conformation of the protein in which probably the C-terminus is free to react. Unfortunately, it is difficult by only AFM imaging to understand the precise conformation and state of AS but, referring to data in literature, small beta enriched oligomers are expected in solutions containing methanol. In order
to study the binding affinity, the nanografted protein was then incubated with increasing concentrations of DA and imaged in liquid. The orientation of the molecules on a confined surface and the high precision of AFM allowed the measurement of height and roughness variations that can be attributed to a conformation rearrangement of AS molecules after DA binding. This matter is still debated in literature. Previous CD studies suggested that DA-treated AS in fact changes its structure toward mostly random coiled with little alpha helices or beta pleated sheets structure, while FTIR spectrometry showed beta pleated sheets structure only \(^{114}\). Therefore, further investigation are needed. Anyhow from our assay, exploiting these supposed conformational changes, we were able to calculate for the first time the dissociation constant between AS and DA, that is in the micromolar range. Such result was obtained both from a direct assay, i.e. DA binding over AS nanostructures, and from an indirect assay, what we called a “non-competitive” binding of DA over mAb covered AS patches. We showed in fact that DA was able to partially displace the mAb (syn clone 211). The overlapping of the binding sites for one amino acid only and the higher affinity for the antibody (typically nM for IgG) in principle could not be sufficient to displace the antibody. For this reason, we called this assay non-competitive. Our observation validated the hypothesis of a strong rearrangement in the three dimensional structure of the protein due to effective interaction of DA on the expected binding site \(^{125}\)YEMPL\(^{129}\) and to long-range interactions. Such structural variation led to the partial displacement of the mAb from its binding site. At saturation, a portion of mAb was still bound to the protein, probably because some C-terminals, not involved into the conformational change due to DA binding, were still exposed to the solid-liquid interface because not involved into the conformational change due to DA binding (data confirmed by the inverse experiment). This non-competitive binding assay gave us the possibility to measure the displacement of the mAb after loading of DA with more precision with respect to the direct assay, because of the quite large height variation signal, relatively easy to detect. This assay confirmed the micromolar dissociation constant calculated in the direct assay, supporting the validity and the precision of this technique for binding studies of small molecules. Finally we presented preliminary results about the use of our assay to study the supramolecular assembly of AS. The incubation of nanografted AS in presence of AS-histidine tagged molecules allowed to follow the growth vs. time of aggregates that are then dissolved by DA. The use of histidine-tagged AS, cheaper and easier available than wild type, leaves some
doubts. It is in fact possible that the histidine tag interacts with the acidic end of the AS through attractive electrostatic interactions. Our speculation, as suggested by previous experiments, is that the histidine tag is hidden by the protein structure in buffer solution and therefore not available for binding. However, further investigation are needed to clarify this aspect.
5. Results: supported lipid bilayers, a method to study AS interactions

In the second part of my project, I focused on the study of supported lipid bilayer and in particular, I was interested in the development of a model membrane able to mimic the lipid rafts domains of the cell. The existence of lipid microdomains that differ from the other areas of the membrane for their lipid composition is object of strong research and debate. The enrichment of these domains of sphingolipids and cholesterol was demonstrated. The first evidence comes from the analysis of the membrane domains resistant to cold non-ionic detergent extraction from MDCK cells, which suggested the presence of Sphingolipids/Chol/glycerophospholipids in molar ratio close to 1:1:1. This cholesterol concentration induces a phase separation into Sphingolipids/Chol enriched domains in a Lo phase, surrounded by a Ld matrix formed by unsaturated species. Among sphingolipids, natural sphingomyelin (SM) is the most abundant and offers the peculiarity to undergo very broad gel to liquid crystal phase transitions.

The human brain is particularly enriched in cholesterol and cholesterol derivates (>25% of total lipid content). For this reason, the function of lipid rafts in the central nervous system (CNS) could be critical. Lipid rafts has been identified in both neuronal and glial cells and a role in the neuronal signalling, neuronal cell adhesion and axon guidance has been suggested. Moreover, the presence of ionotropic receptors and neurotransmitter transporters at lipid rafts level suggests a role in neurotransmission.

For these reasons, we used a three components system as the simplest method for the reconstruction of the phase separation of biological membranes: an unsaturated phospholipid represented by dioleoylphosphatidylcholine (DOPC), a saturated phospholipid represented by sphingomyelin (SM), and Chol (Fig. 5.1). At room temperature, SM is in the gel Sα phase, whereas DOPC is already in the fluid Ld phase at temperatures above -20 °C.

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A tentative phase diagram at room temperature of this ternary mixture is present in literature 204. Unfortunately the boundaries that divide the different phase states are not accurate and are used as a rough guide for the determination of the phase separation. In our case, equimolar concentration of DOPC and SM have been used, while the concentration of cholesterol has been modulated in order to enhance the phase transition. Therefore, moving along the vertical axis of the triangular DOPC/SM/Chol phase diagram (straight blue line on the tertiary phase diagram, Fig. 5.2), it is possible to observe as at room temperature and low cholesterol concentration, a phase separation between Ld and S0 phase occurs. Increasing gradually the concentration, there is a transition state in which three phases coexists (Ld-S0-L0). At cholesterol concentrations between roughly 8-35% a phase separation Ld-L0 has been observed whereas at concentrations higher than 35% the only L0 phase is present. The solubility of cholesterol has been found to be 66-67 % mol, therefore above this concentration (Fig. 5.2, hatched region of the graph) cholesterol precipitates.
In order to elucidate the characteristics of the different lipid phases as a function of cholesterol concentration further investigations were needed. First, I produced supported lipid bilayers by solvent spreading, a very simple method extremely useful in the preparation of multi-component membranes. Second, I characterized the effect of cholesterol and water (hydration) on the organization of the lipid phases by both AFM imaging and GISAXS measurements. Finally, I present preliminary results about the interaction of AS with this flat supported lipid bilayers in order to understand the effect of the lipid phase without the influence of the membrane curvature and the lipid acid charge.

5.1 Supported lipid bilayers: characterization

5.1.1 Method of deposition

As described in the previous chapter, the easiest way to produce SLBs is the spreading of an organic solution of lipids on a solid support, leading the evaporation of solvent and the self-assembly of lipid bilayer when samples are
exposed to ambient humidity. This method is strongly affected by the conditions in which the evaporation occurs and, therefore, extremely high attention is needed during the deposition procedure. For example, if the temperature and the humidity were not controlled thoroughly, their variation might influence the evaporation rate of the solvent and, as a consequence, the assembly of the lipid layer. In particular, if the evaporation occurs too rapidly, the molecules do not have time to assemble correctly and the bilayer does not form, as verified experimentally. To give an instance, if a solution of DOPC dissolved in chloroform is immediately dried under a stream of nitrogen, the surface appears dirty and covered by molecules adhering aspecifically (Fig. 5.3, left). To avoid this problem, the use of high boiling point solvents (e.g. decane BP ~173°C) could help the self-assembly, decreasing the evaporation rate (Fig. 5.3, right).

Figure 5.3. AFM non-contact imaging of DOPC on mica surface in air. a) DOPC dissolved in chloroform was immediately dried under a stream of nitrogen and the surface appears as dirty. b) DOPC dissolved in chloroform:decane 2:1 was left to evaporate slowly at 30 °C in high humidity chamber (80% humidity).

Another important factor is the humidity, which helps the assembly and packing of the molecules. Phospholipids are in fact composed by a hydrophilic head group and hydrophobic chains, which spontaneously organize into bilayers in order to hide the hydrophobic core and expose the hydrophilic groups to the surrounding aqueous layer. Therefore, hydration in high humidity chamber (~80% humidity) improves the quality of the layer (Fig.5.3). Further evidences are described in the following sections.
5.1.2 Role of water

After a first characterization of the membrane in air, we moved to image lipid bilayers in a buffer solution at physiological pH (10 mM HEPES at pH 7.4). As soon as the evaporation of the solvent was completed (T~30 °C), with no hydration step, the sample was mounted in a liquid cell and characterized by AFM imaging in buffer solution. The behaviour of the lipid phases was followed in real-time collecting topographic measurements every 10 minutes. When DOPC/SM (1:1) bilayers, supported on a freshly cleaved mica foil, were imaged in tapping mode, protruding flat domains of varying size have been visualized (Fig. 5.4).

In particular, immediately after the preparation, the sample shows, unexpectedly, the clear separation into three phases, represented by domains characterized by three different height, measured over the mica substrate (Fig. 5.4):

A. one about 4.2 nm that increase in height during the experiment until about 5.4 nm;
B. another of about 3.6 nm that increase in height during the experiment until 4 nm;
C. one of 1.8 nm that increase in height till becoming visible as small defects and disappearing after 30 minutes of imaging

Regarding phase B and C, we speculated that some lipid molecules immediately after the deposition were not well packed.
Figure 5.4. AFM topography of DOPC/SM 1:1 performed in tapping mode in Hepes 10 mM, pH 7.4. The sample, prepared without hydration step was immediately immersed in liquid cell and imaged during time. Clearly, lipid phases characterized by different height have been observed (A,B,C). During time, smaller domains fuse together forming larger ones.
This is confirmed by the increase in the height of the phases during the experiment, until they merge into one single phase (around 4 nm), compatible with the height of Lα phase, represented by DOPC molecules (Fig. 5.5, blue circles). Same behaviour for the highest phase (A), which increases in height of around 1 nm during the experiment till reaching 5.4 nm, value compatible with the S0 phase (Fig. 5.5, red circles). The water has therefore a strong role in regulating the correct self-organization of the bilayer driving lipid order.

**Figure 5.5. DOPC/SM 1:1.** Freshly prepared samples were incubated in liquid (Hepes 10 mM) and real time imaged. The membrane showed phase separation and the height, calculated respect to the mica support, is reported.

Moreover, the gradual disappearance of domains smaller in diameter has been observed over time by AFM. It is clear that the smaller domains shrink and disappear, probably because they merge into larger domains during time. For this reason, all the samples were prepared into a chamber at 30 °C and high humidity (80% humidity). The overnight hydration in fact avoids the presence of defects favouring the order of the molecules (Fig. 5.6)
Figure 5.6. DOPC/SM after overnight hydration in humidity chamber. The phase separation into two domain occurs without defects. AFM tapping mode imaging was performed in Hepes 10 mM immediately after the immersion of the sample in liquid. The detail in figure shows $S_o$ domains higher 1.2 nm higher than the surrounding $L_d$ bilayer.

5.1.3 Role of cholesterol

Cholesterol is an important component of the cell membrane. As reported above, it is fundamental for the stability of the membrane and is one of the main components of the cell membrane. After the optimization of the method for the deposition of supported lipid bilayers, we characterized our system by Force Modulation AFM measurements. With this approach, it is possible to characterize both the topography and the rigidity/stiffness of the system under investigation.

In particular, we produced two samples one composed by DOPC/SM in the same molar ratio and the other in which cholesterol has been added with the same concentration as the other two components in order to obtain a $L_o$ phase. Images obtained for these two systems are reported in the following figure (Fig. 5.7).

Topographic AFM image showed phase separation in both the experiments (Fig. 5.7 a,b). Overlapping FMM-amplitude signal, representative of substrate stiffness (see chapter 2.1.2.1) to topography, it is possible to correlate that the higher is the domain, the higher is the stiffness. In other words, $L_d$ domains, having a higher structural disorder, are lower in height than $S_o$ and $L_o$ phases.
Figure 5.7. AFM FMM have been performed on DOPC/SM (left) and DOPC/SM/Chol (right) in the same molar ratio in air. The figure reports the topographic AFM image and the amplitude FMM signal in both cases. Overlapping the two signal is clear that with and without cholesterol, higher is the phase higher is the Amplitude signal and therefore the stiffness of the domain. Scale bar 1 µm.

In the first sample, composed by DOPC/SM only, we expect a phase separation between Ld phase represented by DOPC and So phase by SM (see ternary phase diagram, Fig. 5.2). In this case, the height of the So phase is higher that the surrounding Ld phase. In presence of cholesterol instead, the separation between a Ld and a Lo phase is expected. The information about the rigidity of the domains of the bilayer have been extrapolated as current variation (higher signal, higher is the stiffness) normalized on a reference substrate, in our case mica, recorded by a scratch on the surface. The following plot has been obtained (Fig. 5.8).
Figure 5.8. AFM-FMM amplitude signals normalized over the mica substrate for DOPC/SM and DOPC/SM/Chol in the same molar ratio. In both cases, the first signal represents the shorter domain, the second to the higher. According to the three components phase diagram, possible corresponding phase is reported.

From the graph, it is possible to observe qualitatively as in absence of cholesterol the two phases (Ld-S0) had a slight difference in the stiffness, that is moderately larger for the highest S0 phase. In presence of cholesterol, both phases show a stiffness increase compared to the samples without cholesterol. Therefore, at this concentration cholesterol has a preferential accumulation in the L0 phase although a portion of molecules enriches also the fluid phase increasing its rigidity.

Moreover, AFM measurements (tapping mode, air) have been used in order to characterize the height of the domains of DOPC/SM plus different cholesterol concentrations. The following graph, reporting the difference in height between the higher and the shorter phases, summarizes the results (Fig. 5.9).
Figure 5.9. Difference in height between the lipid phases obtained from DOPC/SM plus various percentage of cholesterol. The trend is that increasing cholesterol concentration, a decrease in the difference in height is observed. These data support the previous experiment and a cholesterol effect on the L_d phase could be speculated. At 5% cholesterol three phase have been observed. Height values were calculated as difference referring to the higher phase.

These data supports the previous FMM measurements. In fact, we can observe as the difference in height between the domains of the membrane has a slight decrease increasing the cholesterol content. Our speculation is that at high concentration cholesterol stabilizes also the L_d phase, increasing the packing of molecules and, as a consequence, the height of the layer and its rigidity. Notably, at 5% cholesterol, the coexistence of three phases have been observed, probably because in this regime we are exploiting the transition region in the phase diagram. Summarizing, the position of the measures, cholesterol enriched lipid phases in DOPC/SM/Chol phase diagram has been speculated and reported in Fig. 5.10.
Figure 5.10. The phase diagram reports the experimental points characterized by AFM topography of DOPC:SM 1:1 varying cholesterol concentration. When cholesterol is not present in the lipid mixture, Ld-So phase separation has been speculated whereas at 5% cholesterol three phases have been observed. At higher concentrations (10-20-33% mol Chol) only Lo-Ld phases are present.

5.1.4 AFM characterization in liquid

Although we demonstrated that the careful control of hydration allows for the formation of well ordered lipid phases, we moved toward the characterization of our model system in liquid with the final aim to study the system more close to the physiological environment. Supported lipid bilayers with different lipid composition were produced and characterized in topography, in liquid (buffer Hepes 10 mM, pH 7.4), with the aim to confidently assigning each domain to a certain height value. The values obtained are summarized in table 5.1.

<table>
<thead>
<tr>
<th>Composition (molar ratio)</th>
<th>Phase A Height, nm</th>
<th>Phase B Height, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM</td>
<td>-</td>
<td>5.6 ±0.4</td>
</tr>
<tr>
<td>SM/CHOL 1:1</td>
<td>-</td>
<td>6.35 ±0.2</td>
</tr>
<tr>
<td>DOPC/SM 1:1</td>
<td>4.5 ±0.5</td>
<td>5.6 ±0.2</td>
</tr>
<tr>
<td>DOPC/SM/CHOL 1:1:1</td>
<td>4.92 ±0.4</td>
<td>6.10 ±0.3</td>
</tr>
</tbody>
</table>

Table 5.1. AFM height of lipid domains with different composition. The data are calculated from tapping-mode images performed in liquid (Hepes 10 mM). The values have been obtained measuring the height of lipid domain of membranes with different composition over the mica support. The error was calculated as the standard deviation of average value obtained from five different samples.
From the values reported in the table, we can see for example that in the case of the presence of SM only, when simple $S_0$ phase is expected, we obtain a value of height, around 5.7 nm. In the case of DOPC/SM sample, we have two phases, one with the same $S_0$ phase height, attributable then to phase-separated SM domains, the other, lower in height (4.5 ±0.5), assignable to the DOPC domains. Similarly, in the case of SM/Chol we measure a single domain of height ~6.3 nm, corresponding to a $L_0$ phase. In the case of the DOPC/SM/Chol sample we measure two phases, one with similar height of the SM/Chol phase, the other slightly higher (4.9 nm) than the DOPC phase measured in DOPC/SM lipid mixtures (4.5 nm). Such height increase, also observed from analogous measurements in air, can be attributed to the presence of cholesterol also in the DOPC phase.

5.1.5 GISAXS measurements and humidity-cholesterol effect

Grazing Incidence Small-angle X-ray scattering (GISAXS) is a versatile tool emerged in the last years for the study of micro and nano-structured thin film and surfaces able to give information about shape and electron density of the sample. For this reason, it is an excellent complement to AFM measurements for the study of lipid bilayers.

The geometry of a typical GISAXS experiment is illustrated in Figure. A monochromatic x-ray beam (in our case with an energy of 8keV) with wavevector $k_i$ is directed on a surface with a very small incident angle $\alpha_i$ with respect to the surface. The Cartesian $z$-axis is the normal to the surface plane, the $x$-axis is the direction along the surface parallel to the beam and the $y$-axis perpendicular to it (Fig. 5.11). After the interaction with the sample, the scattered x-rays, with outgoing wavevector $k_f$, are collected by a detector. The dimension of the latter determines the maximum measurable angle of the scattered beam. The difference between $k_i$ and $k_f$ defines the so called scattering vector $\vec{q}$:

$$\vec{q} = \vec{k_f} - \vec{k_i}$$
In the case under study, lipid bilayers, the GISAXS signature is formed by stripes at regular spacing along the qz direction. In Langmuir-Blodgett films, such stripes in the diffuse reflectivity are referred to as Bragg sheets.

In order to better understand the role of cholesterol and humidity on our model system, we performed GISAXS measurements at the SAXS-beamline at ELETTRA-Synchrotron light source.

In particular, we used this method to investigate the structural transition of DOPC/SM multi-bilayer in presence of a different percentage of cholesterol (here we present the results relative to 10% cholesterol) at different bilayer’s hydration levels by changing the relative humidity (from 5% to 85% to 5%). For each experiment, we performed 60 independent measurements, 15 seconds reading, with a time interval of 2.5 minutes. Supported lipid bilayers have been prepared on silicon wafers by solvent spreading at 30 °C and 80% humidity chamber. After the complete solvent evaporation and hydration in order to enhance the order and packing of the molecules, the samples have been put in a vacuum chamber in order to remove completely the solvent residues, which could interfere with the GISAXS measurements.

Here the example of DOPC/SM +10% cholesterol. The following plot reports, in a colour scale, the variation of the scattered intensity as a function of the scattering vector q and the relative humidity (Fig. 5.12).
Figure 5.12. The following plot reports, in a colour scale, the variation of the scattered intensity as a function of the scattering vector \( q \) and the relative humidity. A and B represent the Bragg’s peaks of \( L_o \) and \( L_d \) phases respectively.

Bragg’s peaks typical of a parallel lamellar structures were observed. In general, we observe two different series of Bragg’s peaks. In general, we observe two different series of Bragg’s peaks corresponding to two distinct ordered phases (A1-A4, \( L_o \) and B1-B4, \( L_d \)). As a function of the humidity, the two phases change height and internal order thus changing the GISAXS spectra (Fig. 5.11). Specifically, the number of orders of diffraction is indication of the packing and the order of the lipid bilayer. We note here that the spectra are reversible as a function of humidity although after a complete cycle the A lines at orders higher than 1 split into two main components. At the moment, this behaviour has not been completely understood and further investigations are required.

In order to better understand the behaviour of lipid bilayers as a function of the humidity we plot two different scattering profiles vs \( q \) at high and low humidity (Fig. 5.13). At high humidity the fourth order is evident for the two phases (Fig. 5.12, A1-A4 and B1-B4 peaks, blue curve), whereas at low humidity, the \( L_o \) phase only appears at the fourth order (A1-A4 peaks, red curve).
Figure 5.13. The plot reports the scattered intensity vs q, recorded for DOPC/SM + 10% chol. The red plot has been recorded at low humidity (~5%) whereas the blue curve at high humidity (~85%). A and B represent the Bragg’s peaks of L₀ and L₄ phases respectively.

In order to better understand these changes, we try to fit the single spectra (Fig 5.13) with Lorentian’s function:

$$f(q) = I_0 + \frac{I}{(q - q_0)^2 + \gamma}$$

Where $I_0$ is the background, $I$ is the maximum intensity of the peak, $q_0$ is the center of the peak and $\gamma$ the half-width of the peak at half-maximum.

From $q_0$ we can easily compute the corresponding height of the lipid bilayer using the following equation:

$$h_0 = \frac{2\pi n}{q_0}$$

where $n$ is the order of the maximum.
In each experiment, the fitting of the GISAXS spectra was performed on the first order of diffraction and the height values have been calculated. The following plot reports the height values of the sample with 10% cholesterol and the humidity variation during the experiment (Fig. 5.14, green curve). In this case, two heights are present, corresponding to the two lipid phases. In particular, \( \text{L}_o \) phase and \( \text{L}_d \) phase is expected, as discussed above. The height values at the maximum humidity value are 5.7 nm for \( \text{L}_o \) phase (Fig. 5.14 red points) and 5.1 for \( \text{L}_d \) (Fig. 5.14 blue points), in good agreement with previous AFM measurements.

**Figure 5.14.** The plot reports the height variation of the two phases of DOPC/SM +10% Chol (red and blue crosses) as a function of the humidity variation during the experiment (green line).

From these results, it is interesting to observe the effect of humidity on the height of the two main phases of the membrane, which reflects the structure and the packing of the molecules. Moreover, the effect seems to affect much more the shorter (\( \text{L}_d \)) than the higher phase (\( \text{L}_o \)), probably because the disordered
fluid phase has more defects, a lower content of cholesterol and rigidity than the ordered one; the combination of these effects probably favours the insertion of water molecules. It is clear from the graph that high humidity enhances the packing of the disordered layer while the height of the ordered one is almost unchanged. Notably, the effect of the humidity is reversible and the height of the $L_d$ phase return perfectly to the initial values after the reduction of the humidity.

Moreover, the electron density distribution of the bilayer has been calculated. The electron density distribution of a bilayer has maxima in the headgroup regions and a minimum at the methyl terminus of the hydrocarbon-chains (Fig. 5.15a). The electron density function can be computed only if the measurements present four or more orders of diffraction. In this way, using an inverse Fourier-transformation, one can distinguish among the different parts of the lipid bilayer. In particular, it is possible to decompose the lipid bilayer d-spacing into the different structural components such as the phosphate to phosphate distance ($d_{pp}$), the water layer dimension ($d_w$) and the monolayer thickness ($d/2$) (Fig. 5.15b).

**Figure 5.15. GISAXS electron density function.** a) the electron density function of a supported lipid bilayer shows maxima in the headgroup regions and a minimum at the methyl terminus of the hydrocarbon-chains. b) therefore, from the graph obtained, it is possible to calculate the dimension of the lipid bilayer ($d$), phosphate to phosphate distance ($d_{pp}$) and the water layer thickness ($d_w$). Adapted from Rappolt et al, 2003.

As discussed above, the electron density function of the $L_d$ phase was not calculated because of the lack of the sufficient number of order of diffraction, indicative of a low packing and order in the organization of lipids in the formation of the layer. However, at high humidity, four orders of diffraction
have been identified, suggesting a relevant role of water in the organization of the Ld phase. The Lo phase instead is not affected too much of humidity effect, showing a good organization of the layer also at low humidity. In the following graph the electron densities of Lo phase at low humidity (Fig. 5.16 red curve) and Lo-Ld phases at high humidity are reported (Fig. 5.16 dashed lines).

![Figure 5.16](image)

**Figure. 5.16** Electron density profile obtained from the sample composed by DOPC/SM +10% cholesterol. The graph has been obtained overlapping the higher phase (corresponding to the Lo phase) at low (hum ~5%, red line) and high humidity (hum ~85%, dashed red line). The blue dashed line corresponds to the Ld phase at high humidity. The lack of order for Ld phase at low humidity did not provide sufficient data for the calculation of the electron density map.

For each curve, d_{pp}, d/2 and d_{w} have been calculated and reported in the following plot (Fig. 5.17)
Figure 5.17. From the electron density map it was possible to calculate the phosphate to phosphate distance ($d_{pp}$), half of the bilayer thickness ($d/2$) and the thickness of the water layer ($d_w$) for Lo phase at low (red blocks) and high (pink blocks) humidity and for Ld phase at high humidity only (blue blocks).

If we compare the values calculated for Lo phase at low and high humidity, it is possible to observe the increase of the thickness of all the $d$ parameters in the case of high hydration. In particular, $d_{pp}$, i.e. the thickness of the bilayer, increases (variation about 0.16 nm) meaning that water has a role in the organization of the lipid phase order. Moreover, the same trend is observed for $d/2$ (variation about 0.14 nm) and $d_w$ (variation about 0.1 nm) parameters, suggesting, as expected, an increase of the water layer thickness that covers the hydrophilic head groups of the layers. Unfortunately, the lack of sufficient data for the calculation of the electron density function for Ld phase also at low humidity does not allow the comparison with the high humidity values. However, comparing the $d_w$ of Lo and Ld phases at high humidity, we can observe that it is higher for Lo phase, but we do not have access to the information about the effect on the lipids’ structure.
5.2 AS-lipid membrane interactions

Finally, the effect of AS binding on a lipid raft mimicking membrane was evaluated by both AFM and GISAXS measurements. In this section, preliminary results are described.

5.2.1 AFM-characterization

In order to evaluate the effect of AS binding to a model lipid bilayer, artificial lipid membrane with different lipid phases have been incubated in presence of AS in solution (AS 3 μM dissolved in Hepes 10 mM, pH 7.4) and the binding was monitored in real-time by tapping-mode AFM imaging in liquid. This approach permits us to visualize and study submicrometric lipidic domains.

In the first experiment, a membrane composed by sphingomyelin only was prepared on a mica substrate. In this case, a So phase was expected. After a first characterization of the sample, a solution containing AS was incubated and the surface has been imaged for some hours by AFM (Fig. 5.18). The height of the membrane, after an initial decrease (around 1 nm after 1 hour AS incubation), does not show any variation and also the roughness of the surface of the membrane remains unchanged.

The decrease in height (~1.1 nm), after the addition of the protein solution, is probably due to the aspecific adsorption of AS on the mica surface visible as spots on the background (Fig. 5.18). The lack of AS binding on the membrane surface is probably due to the organization of lipids in the bilayer. In fact for So phase, we have a crystalline phase that is characterized by high packing of SM molecules.
Figure 5.18. AFM topography of SM in presence of 3 µM AS. The images, collected during time in tapping mode, show no evident morphological variation of the membrane, while some molecules seems to attach on the mica substrate.
In the second experiment, a sample DOPC/SM 1:1 sample, has been prepared in absence of cholesterol, and a phase separation \( S_{\alpha}-L_d \) occurred. In this case, tubular structures have been observed after 4 hours AS incubation. Initially these structures are 100-200 nm in length but during time the number of fibers and their length increase dramatically, until became difficult to determine their dimension. The height over the lipid bilayer is around 0.2 nm. Finally, after 7 hours of incubation, the number of these tubular structures is so high that is almost impossible to distinguish the single tubule (Fig. 5.20).
Figure 5.20. AFM topography of DOPC/SM 1:1 in presence of 3 µM AS. The images, collected during time in tapping mode, show the formation of tubular structure after 4 hours only on the surface of the membrane.
Moreover, it is interesting to consider that the formation of the tubules affects only the membrane whereas on the mica substrate we do not observe any particular structure meaning that the phenomenon is specific for the lipid bilayer (Fig. 5.21c).

Figure 5.21. AFM topography of DOPC/SM 1:1 after 1 hour AS incubation. a) The colour scale underline the presence of filamentous structures on the membrane surface. b) The AFM topographic profile shows the height of the membrane and the dimension of the protruding fibers.

An effect on the height of the lipid membrane has also been detected. In the previous experiment a decrease in the height around 1.1 nm after 7 hours was observed. In this case instead, a decrease of 3.3 nm has been noticed after the addition of AS solution. During time, the decrease is more evident until the shorter phase disappears and only one phase of around 3.3 nm is detectable (Fig. 5.22).
Figure 5.22. AFM height variation of DOPC/SM 1:1 membrane incubated in presence of 3 µM AS. The plot report the height variations of the two domains measured during the experiment. The shorter phase is not appreciable after 5 hours of incubation.

Finally, another experiment has been performed in the same way on DOPC/SM/CHOL 1:1:1. Also in this case, a L\textsubscript{d}-L\textsubscript{o} occurs. Same concentration of protein (AS 3 µM dissolved in Hepes 10 mM, pH 7.4) was incubated and the sample imaged for some hours.
Figure 5.23. AFM topography of DOPC/SM/Chol 1:1:1 in presence of 3 µM AS. The images, collected during time in tapping mode, show the formation of tubular structure only on the surface of the membrane after 4 hours of incubation.
As in the previous case, filamentous structures start to associate after 4 hours of AS incubation and became more evident after 5 hours. Unlike the previous experiment, these structures do not affect all the lipid bilayer islands, their length does not change during time and the number also is really low. This phenomenon could be addressed to the presence of cholesterol that, increasing the rigidity and the packing of lipids, decreases the number of defects and probably opposes to the lodging of AS. Also in this case an effect on the lipid phase separation and lipid bilayer structure is evident. As in the previous experiment it is possible to observe a decrease in the height of the lipid bilayer after the addition of the protein but the progressive disappearance of the shorter phase until only one domain (h =2.7 nm) has been observed.

![Figure 5.24. AFM height measurements variation during the time of the two domain in DOPC/SM/CHOL 1:1:1 after the addition of 3 µM AS. The shorter domain, visible at the beginning of the experiments disappears after 1 hour of incubation.](image)
The comprehension of the nature of these tubular structures and the real effect on the lipid phase separation is difficult to assign only based on AFM observation. For this reason, the use of other techniques could help in highlighting the phenomena.

### 5.2.2 GISAXS measurements

GISAXS measurements have been performed on a DOPC/SM/CHOL 1:1:1 after incubation in presence of AS. In this case, after the preparation of the sample by solvent evaporation of the lipid solution, the sample was covered by AS 3 µM in water solution until complete evaporation. Before GISAXS analysis, the sample was placed in vacuum overnight in order to remove completely solvents residues that could interfere with the measurement. GISAXS is a useful tool in order to support AFM measurements because allows the detection of the effects on the bilayer structure and the phase separation without the contribution of unbound molecules that, binding aspecifically the mica surface, could affect the AFM height measurements.

![Figure 5.25](image.png)

**Figure 5.25.** DOPC/SM/CHOL 1:1:1 after the incubation in presence of AS 3 µM. The plot reports the height of the two domains obtained by GISAXS measurements as a function of the humidity.
The results support previous AFM measurements confirming an effect on the structure of the bilayer and the lipid phase separation. Initially, at low humidity content, two main phases were observed, the higher ($L_0$) of about 5.28 nm and another of 4.12 nm ($L_d$). These heights, less than expected, support AFM findings. During the experiments and increasing the humidity, the height becomes higher for the shorter phase until it reaches the value of the higher one that is more stable and does not vary too much from the initial value. Finally, the height values become too similar (5.49-5.38 nm) to be appreciated by AFM topography measurements. Notably, the phenomenon is strongly dependent on the humidity content because the increase of the humidity favours the reorganization of the membrane into one phase only.

5.3 Discussion and conclusions

The cell membrane is one of the most important machinery of the cell and, at the same time extremely complex. The large number of components constituting a real membrane makes difficult its detailed study. In particular, lipid rafts, functional domains of the cell membrane, are widely studied because of their potential relevance in many cellular processes. For this reason, many biophysical studies are performed on artificial lipid bilayers. This approach gives the possibility to study selectively the behavior of lipid molecules, the basic interactions governing the formation of the membrane, and most importantly, protein-membrane interactions.

In our case, we optimized a simple protocol for the formation of stable supported lipid bilayer by solution spreading and we studied the lipid phase separation. In particular, we focused on the effect of water and cholesterol in the lipid phase separation of DOPC and SM, among the most abundant phospholipids of the brain and chosen as model in order to mimic lipid rafts composition. First, the membranes were analyzed by AFM imaging. AFM in fact is a great means for the characterization of this system in the three dimensions with nanometer resolution both in air and in liquid environment. It gave the opportunity, measuring height variation, to study the behavior of the lipid phases and allowed to assign a specific lipid phase to each domain height in the different experimental conditions. My results underline the central role of water in the proper lipid organization to form a compact and ordered bilayer. In particular, we studied the effect on the two lipid phases ($L_0$ and $L_d$), whose order and fluidity increases with hydration. Moreover we studied the effect of
cholesterol, one of the main constituents of the cell membrane, present naturally at high amount (>25% of the total lipid amount). Its function is to stabilize the membrane; it prefers the accumulation in the ordered phase allowing the development of the \( \text{L}_\text{o} \) phase characteristic of lipid rafts. AFM-FMM measurements gave us information about the contribution of cholesterol on the membrane stiffness. The results confirmed an increase in the stiffness of the \( \text{L}_\text{o} \) phase although, at 33 mol\%, cholesterol increases the rigidity of the \( \text{L}_\text{d} \) phase, meaning that a part of the molecules contributes to the stabilization also of the fluid phase. Moreover, changing the composition of the membrane, we were able to assign confidently a characteristic height value to each lipid phase. In this way, we allotted our experimental point on previously reported lipid phase diagram confirming our speculations. Finally, we characterized by GISAXS measurements our system. Preliminary results on supported lipid bilayers composed by DOPC/SM + 10 mol\% cholesterol have been presented. By GISAXS, it was possible to calculate the height variation of the two lipid phases changing the humidity (from 5\% to 85\% and again to 5\%). The results show a strong water effect on the \( \text{L}_\text{d} \) phase that increases its height as the humidity increases whereas the ordered phase is much less affected by this variation. This means that \( \text{L}_\text{d} \) phase, having more structural defects is much more available for water molecules insertion and the phenomenon is reversible. Water has a relevant role in the packing and order of the membrane, as observed by AFM topography too. The electron density map gave the possibility to understand better the effects on the \( \text{L}_\text{o} \) phase. The results show, as expected, a weak effect of the already well ordered phase with the only expected increase in the water layer with negligible effect on lipid structure. Unfortunately, the lack of order in the \( \text{L}_\text{d} \) phase at low humidity, did not allow the same calculations for this phase. Therefore, these preliminary results highlighted the potential use of this technique, in combination with AFM, in the precise characterization of supported lipid bilayer, which could be used to deepen the knowledge in this field.

In the second part of the chapter, we presented preliminary results about the application of our model system in order to study the AS binding to lipid membrane. It is known indeed that the protein interacts with lipid cell membrane and synaptic vesicles membrane. Previous reports described the preferential binding to anionic lipids in artificial membranes and the formation of tubules on negatively charged lipids but many aspects are still under debate. For example it is known that acidic phospholipids amount in synaptic vesicles
is around 10-18% whereas AS binds synthetic membrane when this amount is around 30%. In literature, it has also been supposed a preferential interaction with lipid rafts but also preferential interaction with disordered phase has been reported. Moreover, AS binds with higher affinity small radius SUVs suggesting a role of membrane defects in the protein insertion. For all these reasons, we tried to clarify some of these aspects using our supported lipid membranes systems. In particular, the use of lipids with no net charge can help in the comprehension of lipid charge effect in the binding. Moreover, the use of membrane with different phases can give further indication about the presumed interaction with lipid rafts. First, we analyzed by real-time AFM measurements the binding of the protein to the lipid bilayer in liquid. What we observed was that AS does not bind the membrane if S₀ phase only is present, meaning that probably the highly ordered structure does not allow the insertion of the protein. In the presence of Ld-S₀ phase separation instead, AS binds the membrane and forms tubular structures. A similar but slower behavior on Ld-L₀ phases has been observed. Moreover, a strong effect on the lipid phase separation has been ascertained in both cases. Preliminary GISAXS measurement, performed on Ld-L₀ phase, confirmed AFM measurements showing an initial decrease in the height of the two phases. The formation of one single phase at high hydration has also been speculated. Due to techniques limitations, it is difficult to understand structural AS changes, although referring to previous literature data some speculations can be done. For example, the formation membrane-bound aggregates potentially neurotoxic could be possible in our system. The height of these structures is in fact compatible with the partial insertion of the protein into the membrane. From the other hand, it has been reported the formation of tubular structures upon AS binding to lipid bilayers due to the membrane deformation ⁸⁹,⁹¹. Moreover, a possible effect on the membrane curvature and loss of membrane integrity has been suggested. Therefore, the decrease of the height of the layer, observed by both GISAXS and AFM, could be addressed to the insertion of the protein into the bilayer. In other words, the decrease in the radius of curvature of lipid vesicles reported in literature, could be translated in the decrease in the height of the lipid bilayer, due to the insertion of defects in the membrane layer that alter the integrity and the order, leading to one phase only.

For these reasons, among our perspectives, we plan to complete our measurements by AFM and GISAXS in order to understand the parameters at which the formation of these tubular structures occurs, for example modulating
cholesterol concentrations. Moreover, the use of complementary techniques and theoretical modeling could allow a better comprehension of the phenomenon and the protein structural changes at the basis of the binding.
6. Materials and methods

6.1 AS on gold surface

6.1.1 Materials and instrumentation

EG6-thiols ((1-mercaptoundec-11-yl)tri(ethyleneglycol), HS-(CH$_2$)$_{11}$-(OCH$_2$CH$_2$)$_3$-OH), and EG3-thiols ((1-mercaptoundec-11-yl)hexa(ethyleneglycol), HS-(CH$_2$)$_{11}$-(OCH$_2$CH$_2$)$_6$-OH), were purchased from Prochimia Surfaces (Poland). KCl, TRIS (2-Amino-2-hydroxymethyl-propane-1,3-diol), TCEP (tris(2-carboxyethyl)phosphine), sodium acetate, methanol and ethanol (99.8% purity) were all provided by Fluka and Sigma Aldrich, (Milan, Italy). All the solutions were prepared in ultrapure 18.2 MΩ·cm water (Milli-Q, Millipore SpA, Milan, Italy), and filtered with a sterile syringe-filter (0.22 μm) prior to use. All other reagents were of analytical grade. All AFM experiments were carried out with conventional AFMs using a XE-100 (Park System, former PSIA, Korea) and a MFP3D Stand Alone AFM, (Asylum Research, Santa Barbara, CA) working in contact mode. For nanografting commercially available silicon cantilevers (NSC19, Mikro-Masch, Poland, nominal spring constant 0.6 nN/nm, tip radius <10 nm) were chosen. For CM-AFM imaging purposes, a soft cantilever (CSC38B, MikroMasch, Poland, nominal spring constant, 0.03 nN/nm, tip radius <10 nm) was used.

6.1.2 Substrate and monolayer preparation

An ultra-flat gold surface was prepared following the Template Stripped Gold (TSG) procedure described by Wagner et al. Briefly, a layer of gold was evaporated on freshly cleaved mica sheets (clear ruby muscovite, Goodfellow Cambridge Limited, Huntingdon, England) or on a Si(100) wafer using an electron beam gold evaporator. Gold films were deposited at a rate of ~0.1 nm/s and a chamber pressure of about 10$^{-6}$ mbar until a thickness of 100 nm was reached. These films were fixed to Si(100) wafer pieces with a drop of SU8-100 (MicroChem Corp., MA), and the polymer was then cured (baked 5h at 95°C, exposed 20 min under a 70 μW/ cm$^2$, 462 nm UV lamp, and baked at least three hours at 95 °C). They were then separated at the gold–mica (or gold-Si) interface by peeling immediately before functionalization. This procedure made gold substrates with a flat surface morphology, reproducing the atomically flat
mica surface. Samples are then soaked in a freshly prepared 300 μM solution of EG6-terminated thiols in ethanol. The substrates were incubated overnight in the dark at room temperature. In this way, a self-assembled monolayer (SAM) of thiolated molecules covers the ultra-flat gold surface. AFM test measurements confirmed a roughness of the substrate in the range of 0.3-0.4 nm.

6.1.3 Recombinant Di-Cysteine AS expression and purification

In order to remove most tags at the end of the purification process, we used Proximity eXact™ fusion-tag system (Bio-Rad Laboratories, Hercules, Calif.) which uses an immobilized subtilisin protease to carry out affinity binding and tag cleavage to produce Di-Cysteine AS. The human AS cDNA was isolated by pUC57-α-syn vector purchased by GenScript. The following primers were used: 5’ CGAAAGCTTTGACTTCTTGCTGCGATGTATTCATGAAAGG 3’ containing nucleotides coding for Thr-Ser linker to obtain an optimal cleavage during the purification step, nucleotides coding for two Cys and restriction site for Hind III and 3’ TACCCATGGTAGCTCTTGCAGTTAGCTTT 5’ containing the restriction site for NcoI. The α-Syn cDNA was cloned into the Hind III and NcoI restriction sites of the bacterial expression vector pPAL7. BL21(DE3) E. coli were transfected with pPAL7/Cys-α-syn plasmid, and expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG). Cells were harvested, resuspended in 10 mM Tris, pH 8, 1 mM EDTA, 1 mM PMSF (1/10 culture volume), and lysed by freezing in liquid nitrogen followed by thawing and probe sonication. The di-Cyst AS protein was purified by size-exclusion chromatography (Superdex 200 10/300 GL, Amersham Bioscences) with 50mM sodium phosphate buffer pH 7.4, 300 mM NaCl. After the collected fraction was purified by using Proximity exact resin (Bio-Rad Laboratories, Hercules, Calif.). The protein purified was verified by SDS PAGE 2-15% and then lyophilized. Di-Cyst AS protein was determinated to be ca. 97% pure by SDS-PAGE.

6.1.4 Cysteine-tagged AS AFM Nanografting and imaging

Nanografting experiments were performed as follows: a freshly SAM passivated gold substrate was rinsed with ethanol and fixed in an AFM closed liquid cell. The gold surface was scratched with a worn out AFM tip to have a
recognition point onto the surface to facilitate finding back the nanostructures. The liquid cell was filled with the nanografting solution that contains 3 mM cysteine-tagged AS in methanol with the addition of 1 mM TCEP dissolved in methanol. The patches were obtained in contact mode scanning the desired area at high load (about 100 nN) and at a scan rate about 2 Hz. In this way local exchange between the EG6-thiolated molecules and the protein in solution is promoted. During nanografting procedure, patches of about 1 μm² (512x512 pixels) were fabricated.

The liquid cell was abundantly washed with 10 mM TRIS, 150 mM KCl, pH 7.4 after the incubation time of every step of the experiments. The imaging was performed in the same buffer at minimum load forces (below 100pN).

6.1.5 Dopamine (DA) Binding

Dopamine (Sigma Aldrich) was freshly dissolved and incubated with increasing concentration (from 10nM to 10mM) in buffer 20 mM sodium acetate, 150 mM KCl pH 5.5, to minimize auto-oxidation. The differential height was used as the response signal and fitted to a Langmuir isotherm, that describes the binding of a ligand to a macromolecule, in Igor Pro (Wavemetrics, Inc.). The topographic measurements were performed after 30 minutes incubation, time sufficient for the diffusion and the binding of the DA. A mAb (8 nM, syn clone 211, Sigma Aldrich) was used to recognize AS patches. The antibody was diluted in buffer 10 mM TRIS, 150 mM KCl, pH 7.4 and incubated for 2 hours.

6.1.6 Non-competitive binding

Nanografted AS patches were incubated in presence of a solution of the mAb (syn clone 211, Sigma Aldrich) 8 nM, dissolved in 10 mM TRIS, 150 mM KCl, pH 7.4 and incubated for two hours. The topographic measurements were performed after 30 minutes incubation, time sufficient for the diffusion and the binding of the DA. The differential height was used as the response signal and fitted to an inverted Langmuir isotherm, that describes the non-competitive binding of a second ligand to a macromolecule, in Igor Pro (Wavemetrics, Inc.).
6.1.7 AS self-association

Recombinant human AS histine-tagged (Sigma Aldrich) was freshly dissolved at 3.5 µM concentration in buffer 10 mM TRIS, 150 mM KCl, pH 7.4. AS nanografted patch were incubated in presence of the histidine tagged AS for different times. 20 µM DA was added and incubated for 30 minutes over the self-associated AS patches. The further addition of 3.5 µM AS in presence of DA for 24 hours did not increase the height of the patches.

6.1.8 Images analysis

For each experiment were fabricated multiple patches that contribute to the experiment statistic. For every patch the average height was calculated from the average profile and together with the standard deviation. Topographic images of the patches are then analyzed in term of pixel distribution (region analysis) in order to get the height value related to the patch at each single step of the experiments. The AFM instrument proprietary software (XEI, Park System, and MFP3D, Asylum Research) as well as free open source software, Gwyddion (www.gwyddion.net), were used.

6.1.9 Histidine-NTA coupling

Multiple Nanografting Assembled Monolayers (NAM) of thiol modified single strand DNA (ssDNA) were prepared by serial AFM-based nanografting inside a Self Assembled Monolayer of a Top oligo ethylene glycol terminated alkanethiol, ((1-mercaptoundec-11-yl)tri(ethyleneglycol), HS-(CH2)11-(OCH2CH2)3-OH from Sigma Aldrich) on ultraflat gold surfaces following standard protocol reported earlier. The DNA patches were obtained promoting the replacement of the TOEG molecules with the oligonucleotides by scanning an area of 1µm x 1µm with the AFM tip applying a large force (about 100 nN) in presence of a solution of thiolated 24 bases ssDNA sequences (5 µM in TE buffer 1 M NaCl). The imaging was performed in Phosphate saline buffer (PBS) modified with the addition of 1M NaCl.

The complex in solution was prepared in PBS plus 10 µM NTA-cDNA, 5 µM NiCl2, 5.5 µM D18 (or AS), vortexed for 30 minutes. The complex was then incubated on the nanografted ssDNA for 1 hour at room temperature. Finally, the sample was carefully washed with PBS and imaged in PBS modified.
6.1.10 Amino-coupling

A 16-mercaptoesadecanoic acid SAM was prepared by immersing a ultraflat gold substrate into a 100 µM solution of the thiol dissolved in ethanol overnight. After a careful wash of the surface, nanoshaving has been performed in ethanol following the grafting procedure described above. After that, a solution containing DCC 100 mM and NHS 100 mM, dissolved in Phosphate buffer 10 mM+ NaCl 150 mM, was mixed and incubated on the sample for 15 minutes. After abundant wash with Tris-buffered saline (TBS) a solution containing 0.5 mg/mL of Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) has been incubated for 45 minutes. In this case, nanoshaving has been performed in TBS/ethanol 1:1.

6.2 AS on lipid bilayers

6.2.1 Materials and instrumentation

18:1 (Δ9-Cis) PC (DOPC) 1,2-dioleoyl-sn-glycero-3-phosphocholine, Sphingomyelin (d18:1/12:0) N-(dodecanoyl)-sphing-4-enine-1-phosphocholine and cholesterol were purchased by Avanti polar lipids (Alabaster, USA). Chloroform, decane were all provided by Fluka and Sigma Aldrich, (Milan, Italy). All the solutions were prepared in ultrapure 18.2 MΩ·cm water (Milli-Q, Millipore SpA, Milan, Italy), and filtered with a sterile syringe-filter (0.22 µm) prior to use.

6.2.2 Supported lipid bilayers preparation

DOPC, SM and cholesterol has been dissolved in chloroform:decane 2:1 in 1mM concentration at various molar ratio. For AFM measurements 50 µL of the lipid solution has been deposited on freshly cleaved mica sheets (clear ruby muscovite, Goodfellow Cambridge Limited, Huntingdon, England) of around 1 cm² and lead for solvent evaporation in incubator (SANYO MCO-18AC incubator) at 30°C and 80% humidity. Wild-type human AS (Sigma Aldrich) 3 µM was dissolved in Hepes 10 mM and added to the liquid cell for AFM imaging. For GISAXS measurements Si wafers, after careful cleaning process (wash with acetone, isopropanol, and ethanol), 1 cm², have been used as substrate for
the deposition of 200 µL of lipid solution, leaved in incubator and put in vacuum chamber overnight in order to remove solvent residues. For AS GISAXS experiments, after the preparation of the membrane, AS solution (3µM dissolved in milliQ) was incubated for 4 hours and then removed, the sample washed and dried before measurements

6.2.3 AFM imaging

All AFM images were acquired using a commercially available microscope (MFP-3D Stand Alone AFM from Asylum Research, Santa Barbara, CA). Measurements were carried out at room temperature working in dynamic tapping mode. For imaging, commercially available silicon cantilevers (NSC19, Mikro- Masch, Poland, nominal spring constant 0.6 nN/nm and Olympus OMLC 800 PSA-1, nominal spring costant 0.76 nN/nm,) have been chosen for imaging in air and liquid respectively. In both cases cantilevers were used working at low oscillation amplitudes with half free amplitude set-point. High-resolution images (512 x512 pixels frames) were acquired at 0.6-1 lines/s scan speed.

6.2.4 FMM

Force modulation measurements were performed with a commercially available microscope (NT-MDT SolverPro AFM, Moscow, Russia) in air. A cantilever characterized by a resonant frequency of about 12 kHz (NSC21 A, Mikro- Masch, Poland, nominal spring constant 0.12 nN/nm) was used. The imaging was performed in the same buffer at minimum load forces (below 100pN) and the amplitude of the sample oscillation was fixed at 0.5 V. the images has been recorded with a resolution of 512 x 512 lines and a scan rate of 0.2 Hz.
Appendix

Other nanografting applications: single cell proteomics for tumor biomarkers detection

Based upon the latest genetic and proteomic insights into cancer biology, which opened new avenues for novel applied clinical research, trends in oncology highlight that molecular characterization of the tumorigenesis process will be essential in tomorrow’s clinical practice both to predict prognosis and to guide therapy. Noteworthy, the promise of individualized molecular medicine, that is particularly relevant to oncology, where even similarly classified tumors can follow quite different clinical outcomes, could be realized by identifying molecular targets for therapy and by measuring tangible response or regression in clinical trials.

Specific patterns of protein expression in tumors and matched normal tissues can now be reliably analysed using quantitative proteomic techniques. Nevertheless, when it comes to the analysis of small cell’s volumes (μl to pl), only miniaturized arrays of proteins may boost the detection of key biomolecules, eventually responsible for cellular-cellular or surface-cellular interactions. In fact, one of the main limits to knowledge advancement in oncology is the high sensitivity required to identify specific tumor (stem) cells protein patterns and to correlate them with cell behaviour. Such caveat suggests that new quantitative approaches, as the one based on nano-immunoarrays technology, could be highly effective in enabling a precise, and ultimately, low cost analysis of tumor cells’ protein content (down to the few cells level). However nanotechnology-based approaches could be (and also are currently being) explored to discover, identify and quantify clinically useful molecular signatures for early detection, diagnosis and prognosis of several tumors.

Focusing our attention to neuro-oncology, we identified the remarkable need for more sophisticated diagnostic tools with potential capability of unprecedented pathological screening and subtyping of glial tumors. These

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latter are the most common primary malignant brain tumors, and, among them, the most aggressive ones, namely Glioblastomas (WHO Grade IV) and Anaplastic Astrocytomas (WHO Grade III), present an incidence of 3.5-2.8 and 0.3-1.2 new cases per 100,000 per year respectively 218.

Gliomas are characterized by rapid growth, high level of cellular heterogeneity due to genetic alteration, and invasive behaviour. Complete microscopic excision followed by adjuvant radio- or chemotherapy represents the standard of care; nevertheless tumor recurrence generally occurs within few months due to the widespread neoplastic infiltration 219. In fact, primary brain tumors invade widely spreading single cells anywhere within the brain parenchyma, through infiltration of blood vessel walls, subpial glial spaces, or white matter tracts. These mechanisms lead to the development of tumor satellites, probably sustained by glial stem cells escaping resection and treatment, eventually serving as reservoirs for tumor recurrences. Despite continuous refinements in therapeutic strategies, the prognosis for patients with high-grade gliomas remains dismal, and less than 5% survive more than 5 years despite aggressive therapies 219.

There are several challenges to overcome in the successful treatment of glioblastoma. Among others, these include the characteristic tumor heterogeneity of glioblastoma, and the possible difficulty in overcoming resistant cancer stem cells (CSCs) 220. In fact, despite some controversy concerning their exact role and characterization, CSCs are believed to play a crucial role in malignant glioma tumor initiation, progression, angiogenesis and both drug- and radio-resistance 221. Therefore, the characterization of the protein content of glioma CSCs can help in developing drugs specifically targeting this extremely rare tumor-subpopulation and may improve the molecular classification of brain tumors 220. As mentioned, this possibility would be made possible only by the development of innovative nano-immuno-arrays.

A systematic review of multiple independent proteomic analyses of gliomas published on PubMed since 2008 has demonstrated alterations of almost 100 different proteins; among them, we have chosen the Glial Fibrillary Acidic Protein (GFAP), a biomarker belonging to the family of intermediate filaments for our proof of concept. GFAP is expressed by numerous cell types of the Central Nervous System (CNS), especially astrocytes and is crucial in cell’s
differentiation, so that only well differentiated cells retain the ability to express it, while the most aggressive ones lose this typical feature at a certain point in their dedifferentiation. GFAP is therefore functional to grade classification of gliomas.

During the first period of my PhD, I performed preliminary tests about developed a quantitative approach to enable precise and multiplexing protein analysis of very small systems, down to a single or a few cells. The proof of concept was realized for GFAP detection. In particular, we designed specific nano-immunoarrays capable to detect the presence of GFAP expression in small volumes and envisage the possibility to integrate it to a facing array of micro-fabricated wells (see Fig A1) hosting living astrocytes (ideally, one cell per micro-well).

Figure A1: Nano-immuno detection platform for few cells proteomic screening. A) Cartoon showing the hosting of one cell per well. B) Biosensor topped well containing a single cell secreting a protein of interest (Antigen) C) Topographic image of the area containing of the DNA-based-nano-immuno-sensors D) Cartoon showing the elements of one nanoimmuno sensor (patch). E) Scheme for the realization of the nanoimmunopatch.
To realize the nano-immunoassay for the detection of GFAP we used a combination of DNA barcoding, one of the leading technology for multiplexing bioassays and DNA Directed Immobilization (DDI) of DNA-protein conjugates. Using nanografting, we confined DNA molecules at the nanoscale in a nanoarray format, within a self-assembled monolayer (SAM) of biorepellent alkanethiols.

**Preparation of nanopatches via DDI of biotinylated antibodies and recognition of GFAP w/wo cell lysate**

The preparation of nano-immunoarrays was carried out with the aim to optimize protocols for future proteomics multiplexing analyses. As already mentioned, nanografting has unique capabilities for controlling and optimize density \(^{146}\) and conformation \(^{142}\) of patterned biomolecules at the nanometer scale. Moreover, the intrinsic characteristics of DDI, such as high efficiency of adsorption, site-selectivity and reversibility, allow for selective immobilization of a specific protein of interest on the generated DNA patterns using semisynthetic protein-DNA conjugates \(^{145}\).

The suitability of our nano-immunoarrays to selectively recognize the protein of interest not only in presence of recombinant GFAP alone but also in the whole cell extracts, which contains several different proteins potentially prone to bind unspecifically over the NAMs and/or the surrounding SAM, was finally tested by incubating nanopatches with 100μl of cell lysate (obtained by lysing 10⁶ glioblastoma U87 cells in 100μl of RIPA buffer) with and without GFAP at a known concentration of 40nM.

The three preliminary steps (grafting of ssDNA, hybridization of DNA-STV conjugate, immobilization of biotinylated GFAP Ab) needed to realize the nano-immunoarrays above described, along with the subsequent immobilization of GFAP in standard conditions and in the cell lysate were confirmed by topographic analysis performed by AFM.

On average the DNA’s NAM’s height increased of 7.5±2.5 nm upon DNA-STV conjugate immobilization (n=18, p<<0.01) and 6.5±2.3 nm after GFAP Abs (n=17, p<<0.01). This progressive NAMs’ height increase indicates that stepwise addition of the ligands (first DNA-STV, then GFAP Ab) occurs.
The roughness of both NAMs and SAMs, as showed in Table A1, did not change significantly before and after incubation with a GFAP-free cell lysate (p≪0.01 in all comparison) meaning that the biomolecules contained in the lysate do not attach to the surface of the nanopatches and onto the surrounding biorepellent SAM.

<table>
<thead>
<tr>
<th></th>
<th>Before incubation (Time 0)</th>
<th>After incubation (1 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cDNA+STV</td>
<td>0.2 ± 0.01 nm</td>
<td>0.2 ± 0.01 nm</td>
</tr>
<tr>
<td>cDNA+GFAP Ab</td>
<td>0.28 ± 0.01 nm</td>
<td>0.28 ± 0.01 nm</td>
</tr>
<tr>
<td>NAM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cDNA+STV</td>
<td>1.3 ± 0.5 nm</td>
<td>1.3 ± 0.7 nm</td>
</tr>
<tr>
<td>cDNA+GFAP Ab</td>
<td>1.9 ± 0.5 nm</td>
<td>1.9 ± 0.8 nm</td>
</tr>
</tbody>
</table>

**Table A1**: Roughness Analysis of the nanopatches before and after incubation with the cell lysate

In fact, the analysis of the topographic images showed that only in the presence of GFAP, significant increment (p<0.05) in the height of the nanopatches (Table A2, Fig. A2) could be observed.

**Figure A2**: (A) Height profiles of a nanopatch before (black) and after (red) incubation with a BB solution containing 40 nM of recombinant GFAP; (B) Height profile of a patch before (black) and after (red) incubation with a cell lysate containing 40 nM of recombinant GFAP.
Table A2: Height variation of nanopatches topped with GFAP antibodies after 1 hour incubation with BB solution and cell lysate w/wo recombinant GFAP (40 nM). BB=Blocking Buffer.

<table>
<thead>
<tr>
<th>Incubation with BB solution</th>
<th>Height variation (nm)</th>
<th>p =</th>
<th>N =</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without GFAP</td>
<td>-0.8 ± 0.9 nm</td>
<td>0.13</td>
<td>11</td>
</tr>
<tr>
<td>With GFAP (40nM)</td>
<td>3.9 ± 1.1 nm</td>
<td>0.02</td>
<td>7*</td>
</tr>
<tr>
<td>Incubation with Cell Lysate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without GFAP</td>
<td>0.4 ± 0.5 nm</td>
<td>0.14</td>
<td>6</td>
</tr>
<tr>
<td>With GFAP (40nM)</td>
<td>2.9 ± 1.5 nm</td>
<td>0.03</td>
<td>4*</td>
</tr>
</tbody>
</table>

A 3D image of the nanopatches after the recognition of GFAP in cell lysate is performed, also confirms the absence of non-specifically bound proteins to the area surrounding each NAM (Figure A3).

Figure A3: AFM topographic 3D representation of successful recognition and immobilization of GFAP added to cell lysate (white patches) and the absence of non-specific binding of protein cell extract proteins (surrounding area).
The Elisa benchmark

Once GFAP detection within a cell lysate was optimized, we wanted to investigate whether it is possible to quantitatively determine the concentration of GFAP in the sample. For this reason we obtained the affinity calibration curve for our nanobiosensor assay by measuring the variation in height of the nanopatches functionalized with the antibodies for GFAP after the incubation with blocking buffer containing a pre-determined concentration of GFAP ranging from 200 pM to 100 nM. As shown in Fig. A34, the changes in height detected for increase of GFAP concentration showed a sigmoidal behaviour, as expected for the binding of an antigen to an antibody. In order to quantitatively describe the recognition of GFAP via our nanobiosensor assay, we used the Hill equation. From the fit, we obtained a dissociation constant in the nanomolar range (Kd= 6.6±3.8 nM) and a slightly cooperative effect (n >1). However, the large error in the Hill coefficient could mask the fact that the system also has a negative cooperative effect meaning that once an antigen binds to an Antibody (Ab), then the affinity of the Ab for other antigens decreases. In fact, in case of our biosensor, we expect that the steric hindrance between proximal Ab on the patches could contribute to a decrease of cooperativity.

![Figure A4: Binding curves obtained from the height variation (a) and absorbance obtained using ELISA (b) vs GFAP concentration. Experimental data are shown in black and the fitting curves obtained by Hill equation in red. Values of h_min=0.78±0.28 nm, h_max=5.07±0.75 nm, n=1.43±0.82 and K_D=6.6±3.8 nM were obtained for nanoassay and A_min=0.00 ±0.01, A_max=1.55±0.10, n=1.51±0.26 and K_D=8.30±1.04 nM for ELISA assay.](#)

In order to compare the binding affinity curve of our nanobiosensor assay with a standard technique, we also obtained an ELISA assay for GFAP.
measurements using the same antibodies and we analogously fitted the values of absorbance with Hill equation. The value of n=1.51±0.26 indicated that a cooperative effect of the recognition event is present and a dissociation constant equal to Kd =8.30±1.04 nM is perfectly in agreement with the value found for the nanosensor assay showing a potential of our nanodevice to be an alternative to ELISA.

**Microwell development**

In parallel to the development of the quantitative nanoimmunoassay, I focused on the development of a microwell array able to host single or few cells.

**Microwell fabrication**

Arrays of microwells with different geometries (lateral dimension 50-100 µm, depth 25 µm) were produced in three steps. First, a silicon-SU8 master was produced by standard photolithography obtaining a pillar like structure. Second, a polydimethylsiloxane (PDMS, Sylgard 184, Silicon elastomer, Dow Corning) mold was created casting a drop of PDMS (1:10 curing agent to prepolymer, as suggested by manufacturer) on the master and treated at 60°C for 1 hour to promote the polymerization. Third, the membrane was peeled off and stuck on a glass slide.

The main advantage of the PDMS substrates is that they allow to produce microwells of the desired diameters, according to the specific dimension of the hosted cells. For astrocyte analysis, our microwells were fabricated with a size of 100 µm in diameter x 25 µm in depth.

**Surface functionalization**

PDMS surface alone is not able to enhance the adhesion and grow of neurons as observed experimentally. For this reason, a functionalization with adhesion molecules such as polyornithine or fibronectine was needed (Fig. A5).
Figure A5. Hypocampal cell culture requires PDMS treatment with polyornithine. PDMS without any treatment is not suitable for cell growth, while functionalization with polyornithine show good cell adhesion and grow after 5 days incubation. The image shows fixed cells stained with Coomassie blue.

In order to permit the neuron adhesion and growth on PDMS surface of the microwells bottom only, we functionalized them with an inverted microcontact printing method (Fig. A6). First of all the PDMS membrane was exposed to air plasma for 5 minutes to create negative charges promoting hydrophilicity of the surface and the absorption of positively charged molecule. A polyornithine solution (0.01% polyornithine plus 5% Lucifer yellow for the visualization, Sigma Aldrich) was incubated on the surface for 1 hour and then dried under a nitrogen stream. The excess of polyornithine outside each microwell was removed inverting immediately the sample on a glass slide overnight and a weight was placed on top during the stamping period.

Figure A6: Functionalization of PDMS microwells with polyornithine stained in green by lucifer yellow. Scalebar: 50 μm
Cell viability and grow

In order to test the biocompatibility of our micropatterned PDMS wells, we decided to grow a human glioblastoma commercial cell line (U251-MG) as well as CD133 positive stem cells isolated from glioblastoma biopsies on a fibronectin-coated PDMS membrane. Cells entered into the PDMS microwells and adhered to the floor, where they survived for few days. Both U251 and glioma-isolated stem cells express high levels of GFAP, so their presence and viability within the microwells was revealed and confirmed by anti-GFAP staining (Fig A7).

![Figure A7](image)

Figure A7: Glioblastoma cells cultured into functionalized PDMS microwells and stained for GFAP. Tumoral cells isolated from a glioblastoma biopsy (left) and U251 cells (right) were seeded on a micropatterned PDMS membrane. GFAP expression was revealed by immunofluorescence (red fluorescence) and nuclei were depicted by the blue fluorescence of the DAPI staining. Fluorescence and phase contrast images were overlaid by using Adobe Photoshop software.

In conclusion, we demonstrated the feasibility of our approach, that combines nanotechnological principles such as nanografting and DDI and capitalizes on the chemical robustness of DNA oligomer strands and on the reliable assembly of DNA labeled molecular binders via complementary hybridization, to produce nano-immunoarrays for biomarkers detection. Our DNA immunoassays are miniaturized reaching an active area of about 1 μm² per spot. Noteworthy, this strategy for realizing nano-immunoarrays is particularly advantageous in terms of multiplexing analysis since the limiting factor for the number of protein to be tested is only the number of different DNA strands and protein-binding antibody used.

The results herein discussed represent an initial step to test the sensitivity and specificity of nano-immunoarrays designed for the detection of a pivotal glioma
biomarker, GFAP. We are at present working to push DDI strategy for the concomitant detection of several proteins of interest, making this technique a valid alternative for the current benchmark of ELISA-based proteomics.

Although further studies are warranted to better characterize this technique of label-free nano-immuno detection, the promising results obtained in a cell lysate confirm the suitability of this diagnostic method to effectively work in a complex environment without being influenced by the unspecific bindings of many elements included in the cell’s proteome.

On the other side, we produced PDMS microwell arrays for hosting cells, to eventually be sorted via standard biochemical techniques, and demonstrated cell viability, upon properly functionalizing the bottom of the wells.

As a last step, we are working at aligning the microwells array onto the nano-immunoarray, engineered with the same repetition of active nano-areas in correspondence of each well, to obtain biomarkers profiling from the secretome/lysate of a pool of single sorted cells.

Another potential application of our device can be the study of protein expression of cells induced by the interaction with the substrate. It is known indeed that the substrate can modulate the cell shape, proliferation, differentiation or induce cell apoptosis. I did obtain some preliminary results about the growth of cells on PDMS substrates with different stiffness. In particular, I observed different levels of differentiation of adipose stem cells grown on PDMS with different Young’s modulus (data not shown). The morphological variation due to substrates’ stiffness deserve for sure further investigations. I plan to continue working along this direction, since I am confident of the potential implications in tissue engineering and regenerative medicine.
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