Exploring peptide foldamer-membrane interactions using optical spectroscopic techniques

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<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>Aib</td>
<td>alpha aminoisobutyric acid</td>
</tr>
<tr>
<td>AMPs</td>
<td>antimicrobial peptides</td>
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<tr>
<td>Arg</td>
<td>Arginine</td>
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<tr>
<td>Asp</td>
<td>Aspartic acid</td>
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<tr>
<td>BOC</td>
<td>tert-Butyloxy carbonyl</td>
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<tr>
<td>Cbz</td>
<td>Carboxy benzyl</td>
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<tr>
<td>CCD</td>
<td>charge-coupled device</td>
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<tr>
<td>CID</td>
<td>circular intensity difference</td>
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<td>E</td>
<td>10</td>
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<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
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<tr>
<td>GUVs</td>
<td>giant unilamellar vesicles</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>IR</td>
<td>Infrared light</td>
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<td>Iva</td>
<td>isovaleric acid</td>
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<td>LUVs</td>
<td>unilamellare vesicles</td>
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<tr>
<td>LD</td>
<td>Linear Dichroism</td>
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<tr>
<td>mg</td>
<td>milligrams</td>
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<tr>
<td>mL</td>
<td>millilitre</td>
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<tr>
<td>MLVs</td>
<td>multilamellar vesicles</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
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<tr>
<td>NaI</td>
<td>sodium iodide</td>
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<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NIR</td>
<td>Near-infrared light</td>
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<tr>
<td>NR</td>
<td>Neutron Scattering</td>
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<tr>
<td>Nd:YAG</td>
<td>Neodymium-doped Yttrium Aluminum Garnet</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
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<tr>
<td>OtBu</td>
<td>tert-Butyl ester</td>
</tr>
<tr>
<td>peptaibol</td>
<td>peptides containing the alpha aminoisobutyric acid</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
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<tr>
<td>PPII</td>
<td>polyproline II helix</td>
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<tr>
<td>RGD</td>
<td>Arg-Gly-Asp</td>
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<tr>
<td>ROA</td>
<td>Raman Optical Activity</td>
</tr>
<tr>
<td>SUVs</td>
<td>small unilamellar vesicles</td>
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<tr>
<td>UV</td>
<td>Ultraviolet light</td>
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<tr>
<td>UVCD</td>
<td>UV Circular dichroism</td>
</tr>
<tr>
<td>VCD</td>
<td>Vibrational Circular Dichroism</td>
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Abstract

The University of Manchester

Maria Giovanna Lizio

Doctor of Philosophy

Exploring peptide foldamer-membrane interactions using optical spectroscopic techniques

27th September 2016

The evolution of drug resistant pathogens creates the need for the introduction of new antimicrobial drugs. Peptaibols, a class of naturally occurring peptides, contain large amounts of alpha aminoisobutyric acid (Aib). They are known to exhibit their antimicrobial activity by perturbing the membranes of pathogens. However, a comprehensive model of action for these peptides has not yet been identified. Aib residues support the formation of $3_{10}$-helix conformation and it is thought that this secondary structure is important for their antimicrobial activity. It is possible to design small synthetic peptides, known as foldamers, endowed with specific properties; in particular, Aib-rich foldamers are used as a model for the understanding of the folding and membrane interaction of the naturally occurring species. The aim of this thesis is to investigate the conformational preference of monodisperse Aib-oligomers as well as understanding their interaction with bilayer membranes.

A large set of spectroscopic techniques have been used to establish the conformation of Aib-rich foldamers both in solution and when bound to membranes. In particular: Raman, Raman Optical Activity (ROA), Infrared (IR), Vibrational Circular Dichroism (VCD), Linear Dichroism (LD) and Neutron Scattering (NR) were employed to provide new structural insights.

These vibrational analysis (VA) and vibrational optical analysis (VOA) investigations in solution were focused on the identification of spectral features for $3_{10}$-helix conformation, particularly with Raman and Raman Optical Activity spectroscopies. Spectroscopic markers for this conformation in the amide I region were successfully identified. Moreover, it is known that chiral Aib-rich peptides can show a right or left handed screw-preference based on the primary sequence. VOA studies successfully distinguished between peptides with opposite helicity.

VCD, ROA, LD and NR of Aib-foldamers bound to membranes were shown to be useful for identification of conformational preferences of the peptides within the membrane as well as for determining their orientation in the bilayer, and ultimately the effect of the peptides on the membrane structure.
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Recent publications relevant to this study:

**A modular self-assembly approach to functionalised [small beta]-sheet peptide hydrogel biomaterials**

Two complementary β-sheet-forming decapeptides have been created that form binary self-repairing hydrogels upon combination of the respective free-flowing peptide solutions at pH 7 and >0.28 wt%. The component peptides showed little structure separately but formed extended β-sheet fibres upon mixing, which became entangled to produce stiff hydrogels. Microscopy revealed two major structures; thin fibrils with a twisted or helical appearance and with widths comparable to the predicted lengths of the peptides within a β-sheet, and thicker, longer, interwoven fibres that appear to comprise laterally-packed fibrils. A range of gel stiffnesses (G’ from 0.05 to 100 kPa) could be attained in this system by altering the assembly conditions, stiffnesses that cover the rheological properties desirable for cell culture scaffolds. Doping in a RGD-tagged component peptide at 5 mol% improved 3T3 fibroblast attachment and viability compared to hydrogel fibres without RGD functionalisation.

**The Computational Prediction of Raman and ROA Spectra of Charged Histidine Tautomers in Aqueous Solution**

Histidine is a key component of a number of enzymatic mechanisms, and undertakes a myriad of functionalities in biochemical systems. Its computational modelling can be problematic, as its capacity to take on a number of distinct formal charge states, and tautomers thereof, is difficult to capture by conventional techniques. We demonstrate a means for recovering the experimental Raman optical activity (ROA) spectra of histidine to a high degree of accuracy. The resultant concordance between experiment and theory is of particular importance in characterising physically insightful quantities, such as band assignments. We introduce a novel conformer selection scheme that unambiguously parses snapshots from a molecular dynamics trajectory into a smaller conformational ensemble, suitable for reproducing experimental spectra. We show that the “dissimilarity” of the conformers within the resultant ensemble is maximised and representative of the physically relevant regions of molecular conformational space. In addition, we present a conformer optimisation strategy that significantly reduces the computational costs associated with alternative optimisation strategies. This conformer optimisation strategy yields spectra of equivalent quality to those of the aforementioned alternative optimisation strategies. Finally, we demonstrate that microsolvated models of small molecules yield spectra that are comparable in quality to those obtained from ab initio calculations involving a large number of solvent molecules.
«Dico, l' azzurro in che si mostra l' aria, non essere suo proprio colore, ma è causato da umidità calda, vaporata in minutissimi e insensibili atomi, la quale piglia dopo sé la percussion de' raggi solari e fassi luminosa sotto la oscurità delle immense tenebre della regione del fuoco che di sopra le fa coperchio; e questo vedrà, come vid' io, chi andrà sopra mon Boso, giogo dell' Alpi, che dividono la Francia dalla Italia, la qual montagna ha la sua base che parturisce li 4 fiumi, che rigan per 4 aspetti contrari tutta l' Europa; e nessuna montagna ha le sue base in simile altezza. Questa si leva in tanta altura, che quasi passa tutti li nuvoli, e rare volte vi cade neve, ma sol grandine di state, quando li nuvoli sono nella maggiore altezza; e questa grandine vi si conserva in modo, che, se non fusse la (ra)retà del cadervi e del montarvi nuvoli, che non accade 2 volte 'n una età, e' vi sarebbe altissima quantità di diaccio inalzato da li gradi della grandine, il quale di mezzo luglio vi trovai grossissimo; e vidi l' aria sopra di me tenebrosa, e 'l sole, che percotea la montagna, essere più luminoso quivi assai che nelle basse pianure, perché minor grossezza d' aria s' interponea infra la cima d' esso monte e 'l sole».

Leonardo Da Vinci c.1506-1508
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My best friend Lory, who has supported me (e sopportato) during these 4 years and my ‘Lost in the world’ friends.
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1. **Spectroscopy Techniques**

1.1 **Introduction**

Light can be described as electromagnetic radiation resulting from the oscillation of an electric \((E)\) and magnetic \((B)\) field, propagating as independent waves perpendicular to one another, in a sinusoidal fashion. The polarization of the light is defined by the direction of the electric field, \(E\): when the electric field \(E\) oscillates in the same plane the light is named as plane or linearly polarized. On the contrary, radiation is circularly polarised when the electric field retains the same magnitude but not the same orientation propagating in a rotary manner.

![Figure 1.1: Representation of linearly and circularly polarized light.](image)

Quantum theory defines electromagnetic radiation as being formed by particles with zero mass of discrete energy named “photons”. Planck was the first to hypothesise and demonstrate the
The wave/particle duality of electromagnetic radiation.\textsuperscript{2-4} The relationship between the energy associated with the photons and the frequency of the radiation is defined by Planck’s equation (1):

\[ E = h \nu \]  

(1)

where \( h \) is the Planck constant, \( 6.626 \times 10^{-34} \text{J.s} \) and \( \nu \) is the frequency. Electromagnetic radiation can be described by three physical properties: frequency \((\nu)\), wavelength \((\lambda)\) and energy \((E)\) which are related to one another by the speed of light \( C \);

\[ \nu = \frac{c}{\lambda} \]  

(2)

\[ \lambda = \frac{c}{\nu} \]  

(3)

\[ \nu = \frac{\Delta E}{h} \]  

(4)

Increases in lightwave frequency correspond to decreases in wavelength and vice versa; the complete range of possible frequencies is called the electromagnetic spectrum (Figure 1.2). Radiation of different wavelengths is associated with different energies and will interact differently with matter, and so can be used to investigate the different properties of molecular species. The electronic cloud surrounding the nuclei of a molecule is influenced by the electromagnetic radiation and so interaction of a photon with a molecule can lead to the molecule being promoted to higher energy levels. The energy of a molecule can be considered in terms of different types that describe their behaviour; electronic, vibrational (oscillatory motion of the atoms within a molecule) and rotational (energy related to the tumbling motion of molecules). Vibrational spectroscopies use the interaction of electromagnetic radiation with the vibrational states of matter. A molecule can translate along the three Cartesian directions...
(x,y,z) which, therefore, allows three degrees of freedom for the translation. Molecules can also rotate in the three Cartesian directions, these are described by the three degrees of freedom for the rotation. For a molecule of \(N\) atoms there are \(3N\) vibrational modes and three rotational degrees of freedom (two for linear molecules). Taking into account both vibrational and rotational freedom (3 rotational degree of freedom, 2 for linear molecules), the number of vibrational modes are \(3N-6\) and \(3N-5\) for linear molecules.\(^2\,^5\)

Figure 1.2: Representation of the electromagnetic spectrum.

The spectroscopic techniques presented in this dissertation use UV-visible light (400-800 nm) and infra-red (700 nm\(^{-1}\) mm). This region of the electromagnetic spectrum can be used to investigate vibrational and rotational transitions of molecules, with techniques such as: Raman spectroscopy, Infra-red spectroscopy, Raman Optical Activity and Vibrational Circular Dichroism, as well as, in the case of UV or visible radiation, the excitation of the outer electrons, through UV spectroscopy, Circular Dichroism and Linear Dichroism. These techniques provide complementary information about peptide and protein structure and all have been used during research reported in this thesis.\(^2\,^3\)
1.1.1 **Linear Dichroism**

The word ‘dichroism’ is derived from the Greek word διχρωμα which means ‘two colours’. It is a physical phenomenon resulting from the ability of certain solutions or crystals to absorb or transmit preferentially one of two linearly-polarized lights. Amethyst is a good example of this particular phenomenon; it is a variety of quartz that contains metal ‘impurities’ which shows a violet colour for ordinary rays (light polarized perpendicular to the optic axis) and reddish violet for extraordinary rays (light polarized parallel to the optic axis). This optical phenomenon is fundamental to Circular Dichroism (CD) and Linear Dichroism (LD), both of these techniques are useful for the investigation of structure in biological molecules. In particular, LD reports information about the orientation of molecules in systems that are intrinsically oriented or that can be oriented, giving information on the orientation of subunits of proteins, or the overall orientation of proteins in membranes.

1.1.2 **Introduction to Linear Dichroism**

When light is absorbed by matter, the molecules are promoted to a higher energy level. The energy associated with UV-visible radiation is equal to the energy gap between specific orbitals. The radiation can interact with the electrons of the outer shell promoting them to an un-occupied orbital pushing the molecules from the ground state to an higher electronic energy level. Not all the orbitals in a molecule have the same energy gap and the part of a molecule that absorbs wavelengths in the UV-visible region is defined as the *chromophore*. The direction of net linear displacement of charge is defined as polarization of the transition. Each transition has its own maximum when the electric transition dipole moment $\mu$ and the electric field of the light are parallel. When the radiation is absorbed the intensity of the incident radiation, $I_\text{i}$, and transmitted radiation, $I$, are not equal:
A = \log_{10}\left(\frac{I_o}{I}\right) \quad (5)

The absorbance is defined according to the Beer-Lambert law as:

A = \varepsilon cl \quad (6)

l is the path-length, c is the concentration of the sample and \varepsilon is the excitation coefficient. LD is the result of the difference in absorption between parallel and perpendicularly polarized light. The intensity and the sign of the LD signal depend on which direction of the light is referred to as perpendicular and which as parallel which is generally determined by the alignment system as it will be explained in more details later in the text. In two extreme cases we have: the polarisation of the transition is completely parallel to the orientation of the sample, therefore

LD = A_\parallel - A_\perp = A_\parallel > 0 \quad (7)

Or the polarization of the transition is perpendicular to the orientation of the sample:

LD = A_\parallel - A_\perp = A_\perp < 0 \quad (8)

In all other cases the “reduced” LD is calculated which is done by dividing the LD by the isotropic absorbance, A.

LD^\prime = \frac{LD}{A} = \frac{A_\parallel - A_\perp}{A} = \frac{3}{2} S (3 \cos^2 \alpha - 1) \quad (9)

This value is independent of the path length and concentration and it is linked to the angle \alpha between the transition moment and the orientation axis (Figure 1.3). This relationship can be used to probe the orientation of molecules when the polarisation of the system is known.
Figure 1.3: The LD signal is the result of the different absorption between parallel and perpendicular polarized light. This figure is a representation of the event that leads to an LD signal. The light (sinusoidal lines) is shone on and oriented sample (represented the vertical blue line oriented with respect to the three Cartesian axes). The difference in absorption between parallel and perpendicular light result in a LD spectrum.

In order to record an LD spectrum a molecule’s dipole moment $\mu$ need to be preferentially oriented so as to interact with only one of the two polarization vectors of the light wave. If the system does not have an intrinsic orientation it can be superimposed using different orientation methods. For example, polymers such as DNA or hydrogels can be oriented by using a flow system.\textsuperscript{6}

1.1.3 Molecular alignment techniques

There are several methods that have been used for orienting molecules, though the description of each of these is beyond the scope of this dissertation and only the system that applies to proteins and peptides will be described in detail here. A general description of the other methods can be found elsewhere.\textsuperscript{1, 7}
The most commonly used method for the investigation of fibrous proteins or in general large proteins is a “flow system” in which the force applied from the flow macroscopically orients the big proteins. The first method for producing such force was a “linear flow-through” system using a HPLC-pump. Flaws of this method are the large amount of sample required for the analysis and the interference of air bubbles circulating in the system. In 1964 Wada introduced the Cuvette flow cell system in an attempt to overcome some of these issues. This Cuvette cell was composed of a quartz capillary and a quartz rod (Figure 1.4). The capillary rotates generating a flow in solution that is confined to the inner space between the rod and the capillary; this system eliminates both the problem of the air bubble interference and reduces the amount of solvent necessary to perform the experiment, and modern micro-cuvettes can use as little as 30 μL. The forces applied during the rotation are robust enough to cause orientation of macromolecules like DNA and fibrous proteins, but are not sufficient to orientate smaller molecules like peptides. When an object is too small to be oriented by applying an external force, it may be oriented by binding it to a bulky object that can be oriented by the external force. Taking this into account, it has been observed that liposomes were distorted in a Cuvette cell and aligned all in the same direction, therefore they were proposed as a good system for studying the orientation of small molecules that bound to liposomes. Small membrane peptides are too small to be oriented by the flow forces, however, once bound to the membrane they acquire an orientation and an LD spectrum may be used to probe the orientation of the peptides with respect to the lipids.

The linearly polarised light is oriented parallel and perpendicular to the orientation of the flow which is also the orientation of the liposome. Equation (24), describing the origin of the quantity LD', can be modified to take into account the symmetry of the liposome:

\[
LD' = \frac{3}{4} S (1 - 3 \cos^2 \beta)
\]  

(10)
In this new notation $\beta$ represents the angle between the transition moment and the normal to the liposome surface which is parallel to the lipid long axis (Figure 1.4). $\delta$ is a value that denotes the fraction of liposome oriented completely parallel to the flow orientation.

Figure 1.4: a) Representation of the shear effect on the lipid induced by the flow couette. In this figure the lipids are represented as small red dots (head of the lipids) with two red lines (the tail of the lipids). The vesicles undergo to a deformation under the influence of the flow assuming an elliptic shape. The ellipsoid obtained in approximate to as show on figure with the normal perpendicular to the lipid. b) picture of the microvolume Cuvette flow cell with 250 $\mu$m annular gap, outer quartz capillary of 3 mm inner diameter and inner quartz rod of 2.5 mm (outer diameter).c) schematic diagram of a Cuvette flow cell. The polarized light enters laterally and it is perpendicular to the rotational axes of the cuvette. The light that passes through the sample is then measure by a detector.
1.1.4 LD of proteins and peptides

In proteins and peptides a chromophore consist of σ-bonding electrons, non-bonding electrons on the oxygen and nitrogen and π-electrons delocalized over carbons, oxygen and nitrogen. These chromophores absorb in the region between 180 nm and 240 nm with the lowest energy transition given by the non-bonding \( n \) electrons to the anti-bonding \( \pi^* \) orbital (\( n \rightarrow \pi^* \)), absorbing between 210-230 nm. The next lowest energy absorbance is due to the \( \pi \rightarrow \pi^* \) transition and is centred at \( \sim 190 \) nm. Amino acid side chains can also have electronic transitions that may overlap with the backbone chromophore, for example, phenylalanine has an electronic transition around 218 nm. Usually the side chain transitions are stronger compared to the peptide \( \pi \rightarrow \pi^* \) transition, however in long peptides and proteins the amide bonds are in excess compared to the number of overlapping side chain absorptions. The \( \pi \rightarrow \pi^* \) transition is polarized along the carbonyl bond; therefore, different secondary structures present different orientations of this transition. For an α-helix, \( \pi \rightarrow \pi^* \) transitions of neighbouring residues couple together and the transition around \( \sim 195 \) nm is split into two transitions, one perpendicular to the axis of the helix at \( \sim 190 \) nm, and one parallel to it at \( \sim 210 \) nm. The \( n \rightarrow \pi^* \) transition is polarized perpendicularly to the helix axis and absorbs at \( \sim 222 \) nm. β-sheets present \( \pi \rightarrow \pi^* \) transitions polarized parallel to the long axis of a single strand at 195 nm. The \( n \rightarrow \pi^* \) transition does split into two transitions, one perpendicular to the axis of a single strand at 221 nm and one parallel to the axis at 219 nm.

Taking into account the polarization of the transition and the sign of the LD spectrum it is possible to determine the orientation of the proteins or peptides once embedded into the liposome. When single unilamellar vesicles (SUVs) are subjected to the rotary flow in the flow cuvette they distort and align to the shear flow (Figure 1.4), therefore the molecules that are interacting with the SUVs will be oriented, giving an LD spectrum. Molecules that do not
interact with the SUV will not be preferentially oriented and will not produce an LD signal. Figure 1.5 and Figure 1.6 present the expected pattern of LD spectra for α-helix and β-sheet peptides. If molecules preferentially adopt a helical conformation, we can observe three main bands in the LD spectrum, at 195, 208 and 222 nm. If they align on the surface of the SUVs it will lead to a negative signal at 195 nm, a positive signal at 208 nm and a negative at 222 nm. Conversely, if the molecules insert parallel to the axes of the lipid the expected LD spectra will have a positive signal at 195 nm, a strong negative at 208 nm and a positive band at 222 nm. For β-sheet secondary structures, the only signal recorded in LD is the transition at 195 nm which will be positive if the peptide aligns on the surface and negative if it inserts.

![Diagram](image)

**Figure 1.5:** Illustration of the expected LD signal. The intensity and the sign of the signal is represented by the position and the diminution of rectangles. a) Representation of the expected LD signal for α-helix inserted or oriented on the surface of the vesicles. b) Expected LD signal for β-sheet on the surface or inserted in the vesicles.
Figure 1.6: UV transition polarizations of α-helix, b) β-sheet c) PII-helix.

Taking this orientation prediction, LD can be used to probe the insertion, orientation and kinetics of membrane peptides and proteins. As an example, this technique has been successfully used for studying the orientation of Gramicidin into SUVs.\textsuperscript{12} Esbjörner \textit{et al.} have successfully investigated the conformational changes that Gramicidin undergoes during the insertion and intermembrane refolding from non-channel to channel form Gramicidin. By analysing the tryptophan transition moments and the relative LD sign, it was possible to evaluate the average orientation of this residue and therefore of the Gramicidin itself within the membrane. It has been noted the tryptophan adopts a well-defined orientation at the membrane interface acting as an anchor at the membrane interphase. Moreover, it was previously experimentally observed that the ECD spectra from the non-channel and the channel form are significantly different suggesting that the structure must have been very different. LD experiments proved that the orientation of the tryptophan between the two different forms is very similar.
1.3 **Raman Spectroscopy**

Raman spectroscopy is a vibrational spectroscopic technique that is increasingly used for the investigation of biological molecules, because it is non-disruptive, it is compatible with studies in aqueous solution as well as being applicable to samples in solid state, in organic solutions or in complex samples such as living cells and tissues. Raman is widely used in many different fields; including: interstellar studies, archaeology, pharmacy, cosmetics and life sciences. By analysing the Raman shift, the height, and the width of the Raman bands it is possible to perform quantification analysis and learn about the relative composition of materials in a sample, layer thickness, crystallinity and so on. Raman spectroscopy is also sensitive to small changes in the chemistry and the structure of the sample, and so it has been successfully used for the investigation of protein-protein interactions, conformation and aggregation analysis.13, 14

1.3.1 **Raman scattering**

Light interacts with matter in different ways. It can be transmitted through the material, reflected, absorbed or scattered and in some particular cases it can also stimulate the emission of energy (Figure 1.7).2

Figure 1.7: Representation of the interaction of light with matter. Photons are represented by the black arrows.
The scattering of light is a natural phenomenon which affects day-to-day physical observations; a classic example of this phenomenon is the scattering of solar rays by the particles in the air to give the sky a blue colour when perceived by the human eye, known as the Tyndall effect.\textsuperscript{15} Moreover, the colours of some particular gems or metal sols are an optical phenomenon resulting from a combination of scattering and absorption of specific wavelengths by the particles suspended within them. In nature almost every material scatters light with the exception of metallic elements that reflect light completely.\textsuperscript{13, 16} The scattering of light is related to the refractive index of the material, the dimension of the particles and the wavelength of the incident radiation. In the case of monochromatic radiation, if the particles are similar in size or larger than the wavelength of light, they give raise to a Mie scattering phenomenon.\textsuperscript{14} In the case of molecules smaller that the incident radiation, the monochromatic light can be elastically scattered, this is the dominating event and is known as Rayleigh scattering, or inelastically scattered which is referred as Raman scattering. For example, if light with a specific frequency is shone onto a sample, the scattered photons can have either the same (Rayleigh scattering) or different frequency because of the interaction with the molecular. Unlike elastic scattering, Raman scattering is a very rare event; only around 1 in 10 million photons is scattered with a shift in the frequency.\textsuperscript{14, 17}

The inelastic scattering of light was first hypothesized by Smekal in 1923 and first experimentally observed in 1928 by Raman and Krishnan.\textsuperscript{18} In the original experiment, Raman used sunlight focused by a telescope onto a sample. He won the Nobel Prize in 1930 for this work and the optical phenomenon was named after him.

When light is shone onto a sample, the interaction between the molecule and the photons results in a distortion of the electronic cloud around the nuclei (polarisation). During this interaction the molecule and the photon form a short-living “complex” and the molecule is promoted to a
higher energy state called the “virtual state” (Figure 1.8). This virtual state is created by the interaction of the photon with the molecule and its energy depends on the frequency of the photon itself. During Rayleigh scattering, the photon interacts only with the electron cloud and the change in frequency is not detectable as the electrons are relatively light and the energy exchanged can be ignored. In contrast, when the photons interact simultaneously with the electron cloud and the nuclei motion, since these are much heavier than electrons, the energy transfer is appreciable and therefore it can be detected. The exchange of energy must follow quantum mechanical laws and the energy lost or gained by the photons needs to match the difference in energy between two different molecular states. During a scattering event, the mechanism of energy exchange is concerted, i.e. instantaneous. The Jablonski diagram of the vibrational energy states for molecules in the ground vibrational state is presented in Figure 1.8. Rayleigh scattering does not involve any energy transfer; therefore the incident and scattered photons have the same frequency (purple arrows). In contrast, during a Raman scattering event the molecule may gain energy and be promoted from the ground state to a higher energy level, this is referred to as “Stokes scattering” (green arrows). However, some molecules will already be in an excited vibrational state, and for these molecules the energy can be transferred from the molecule to the photon. This is referred to as “Anti-Stokes scattering” (blue arrows). The relative intensity of the Stokes and Anti-Stokes scattering depends upon the temperature of the sample and the energy level of the modes; consequently, for molecules at room temperature the Stokes scattering is typically more intense. The distribution of molecules between different energy states can be calculated based on the Boltzmann equation reported in Equation 1.5. \( N_b \) is the number of species in the excited vibrational state, \( N_a \) is the number of species in the ground vibrational state, \( g_b \) is the degeneracy in the excited state and \( g_a \) is the degeneracy in the ground state, \( (E_b - E_a) \) represents
the difference in energy between the two different states; k is defined as Boltzmann constant \((1.3806503 \times 10^{-23} \text{ m}^2 \text{ kg s}^{-2} \text{ K}^{-1})\), \(T\) is the temperature (K).

\[
\frac{N_b}{N_a} = \left( \frac{g_b}{g_a} \right) e^{-(E_b-E_a/kT)}
\] (11)

Rayleigh scattering is the more frequent event compared to the Raman scattering and it is observed that only one in every \(10^6\) to \(10^8\) photons is scattered in an inelastic manner.\(^2\)\(^{14}\)

Figure 1.8: Diagram of Rayleigh and Raman scattering processes and Infrared absorption. The Jablonski diagram shows vibrational energy states of a molecule and the transitions between them.

The intensities of Stokes and anti-Stokes Raman bands depends upon the population distribution. At room temperature, the majority of the molecules will typically occupy the ground state, whereas only a small percentage will be distributed in the higher energy state.
1.3.2 Selection rules

The interaction of linearly polarized light with the molecules distorts the electron cloud, and the molecule is “polarized”. The polarization describes how easily the electron cloud oscillates in response to an electric field. The oscillation of the electron can happen in all three directions of the Cartesian plane, x, y, and z. When the electrons are polarized an induced dipole moment μ is created as defined by the Equation 1.6

\[ \mu = \alpha E \]  

(12)

With \( \mu \) is the induced dipole, \( \alpha \) is polarization and \( E \) is the electric field charge.

\[
\begin{bmatrix}
\mu_x \\
\mu_y \\
\mu_z \\
\end{bmatrix} =
\begin{bmatrix}
\alpha_{xx} & \alpha_{xy} & \alpha_{xz} \\
\alpha_{yx} & \alpha_{yy} & \alpha_{yz} \\
\alpha_{zx} & \alpha_{zy} & \alpha_{zz} \\
\end{bmatrix}
\begin{bmatrix}
E_x \\
E_y \\
E_z \\
\end{bmatrix}
\]  

(13)

In the notation \( \alpha_{ss} \) the subscript is the direction of the polarizability and of the incident light respectively. Vibrations, also defined as normal modes, are Raman active if they cause a change in the polarizability, i.e. when the molecules vibrate the vibration must cause a change in the electron cloud along one of the Cartesian axis directions.\(^{13}\)

This is in contrast to Infrared spectroscopy, a related vibrational spectroscopic technique, in which the vibration must cause a change in the dipole moment of the molecule. Symmetric vibrations are usually responsible for changes in the polarizability of molecules.

1.3.3 Fluorescence interference

Fluorescence emission and Raman scattering can compete during the analysis of a sample because of the similarity in energy required to observe these two different phenomena. When
a molecule is electronically excited and not involved in a chemical reaction, in order to return to the ground state it has to release the energy gained during the excitation. In solution, most of the excess energy is given back as vibrations are absorbed by the solvent. When the lower vibrational state is reached but the molecule is still electronically excited, the energy can be emitted as a photon. The energy relaxation can happen by two different mechanisms: radiative and non-radiative processes. The emission of light is called phosphorescence when the excited state is of a different multiplicity ($2S+1$, where $S$ is the total spin angular momentum) than the ground state. This is called fluorescence when the excited state is of the same multiplicity as the ground state (Figure 1.9).

When the energy of the laser used is sufficient to promote the transition of the energy between two electronic states fluorescence can compete with the Raman scattering. Experimentally, the higher the energy associated with the photons, the higher the possibility of fluorescence interference in the Raman spectrum. This interference represents a limitation for biological

![Diagram of fluorescence and phosphorescence emission.](image)

Figure 1.9: Schematic representation of fluorescence and phosphorescence emission.
compounds such as proteins and peptides, because impurities or intrinsic sources can lead to fluorescence phenomena. In order to overcome these interferences alternative light sources can be used such as UV and NIR lasers.\textsuperscript{20} In the case of NIR lasers, the energy associated with the radiation is not sufficient to promote electronic excitations. In the case of UV Raman, even if the energy of the laser could stimulate a fluorescence emission, the energy gap between the Raman scattering and the fluorescence emission is too high for fluorescence to interfere with the spectrum. If the instrument is fitted with a visible laser, like the instrumentation used in the work presented in this thesis, the sample may be treated with graphitic carbon in order to absorb the impurity or a fluorescence quencher such as NaI or acrylamide may be used.
1.4 Vibrational Optical Analysis (VOA)

VCD as well as ROA are vibrational optical active spectroscopy techniques. The definition of VOA can be generally described as the properties of molecules to interact differently with a right and left circularly polarized light. Many techniques agree with this definition, the more representative of which are the VCD and ROA here presented.

1.4.1 Raman Optical Activity (ROA)

Optical activity is a property exhibited by chiral molecules, and is given by two effects: optical rotation and optical rotary dispersion. The optical rotation is described as the ability of matter to rotate the plane of linearly polarized light, whereas the optical rotary dispersion is the unequal rotation of the plane of polarization of light of different wavelengths. Raman optical activity (ROA) combines the Raman scattering and the optical properties of chiral molecules. It is a vibrational technique that measures small differences in the scattering of incident left and right circularly polarized light (ICP-ROA) or conversely, the difference in the circular components in the scattering of un-polarized or linearly polarised incident light (SCP-ROA).

This technique, combined with the Raman spectrum can be extremely useful in detecting the dynamic behaviour of biological molecules in their biological environment and it has been successfully used for the structural determination of proteins, carbohydrates nucleotides and viruses. The ROA phenomenon was first hypothesized by Atkins and Barron in 1969 and the first experimental measurement of an ROA spectrum in the liquid phase was published in 1973.\textsuperscript{21} The original instruments were based on the ICP-ROA setup, however commercially available spectrometers are based on the SCP-ROA.\textsuperscript{22} The advantage of using vibrational optical spectroscopies, such as ROA and Vibrational Circular Dichroism (VCD), lies in the ability of this optical phenomenon to obtain information about the stereochemistry of a
protein’s backbone directly from its vibrations. In contrast, more widely adopted techniques, such as CD, measure the stereochemistry indirectly by measuring the electronic transitions of chromophores in the molecule.

1.4.2 ROA theory

ROA is generated by the interference between the radiation waves scattered via the molecular polarizability and the optical activity tensors of the molecules. The resulting variation in the scattered light, i.e. the intensity of the scattered photons, depends upon the degree of circular polarisation of the circular component of the scattered light. This first measurement was subsequently improved and summarised in the following equation:

$$\Delta = \frac{(I_R - I_L)}{(I_R + I_L)}$$

(14)

This equation represents the circular intensity difference (CID) $\Delta$, $I_R$ and $I_L$ represent the intensity of scattered left and right circular polarized incident light. ROA intensity results zero in forward scattering and optimal at the backscattering. This can be explained with the polarizability of the molecules. By considering the electric-dipole-electric dipole polarizability tensor $\alpha_{\alpha\beta}$ and the electric dipole-magnetic dipole and electric dipole-electric quadrupole optical activity tensors $G'_{\alpha\beta}$ and $A_{\alpha\beta\gamma}$, the CID for collection at $0^\circ$ (forward) and at $180^\circ$ (backwards) are as follows:

$$\Delta (0^\circ) = \frac{4[45\alpha G' (I_R - I_L)]}{(I_R + I_L)}$$

(15)

$$\Delta (0^\circ) = \frac{4[45\alpha G' + \beta (G')^2 - \beta (A)^2]}{c[45\alpha^2 + 7\beta (\alpha)^2]}$$

(16)
\[ \Delta(180^\circ) = \frac{24[\beta(G')^2 + \frac{1}{2}\beta(A)^2]}{c[45\alpha^2 + 7\beta(\alpha)^2]} \] 

(18)

\[ \alpha = \frac{1}{3} \alpha_{aa} G' = \frac{1}{3} G'_{aa} \] 

(18)

\[ \beta(\alpha)^2 = \frac{1}{2} (3\alpha_{a\beta} \alpha_{a\beta} - \alpha_{aa} \alpha_{\beta\beta}) \] 

(19)

\[ \beta(G)^2 = \frac{1}{2} (3\alpha_{a\beta} G'_{a\beta} - \alpha_{aa} G'_{\beta\beta}) \] 

(20)

\[ \beta(A)^2 = \frac{1}{2} \delta_{a\beta} \epsilon_{a\gamma\delta} A_{\gamma\beta} \] 

(21)

\[ \Delta(0^\circ) = 0 \] 

(22)

\[ \Delta(180^\circ) = \frac{32\beta(G')^2}{c[45\alpha^2 + 7\beta(\alpha)^2]} \] 

(23)

In this notation, we refer to the Cartesian tensor over the three directional components. In the idealised case of a molecule being formed by axially symmetric bonds, \( \beta(G')^2 = \beta(A)^2 \) and \( \alpha G' = 0 \), therefore by resolving the equation 8 and based on the simple bond polarizability theory, the maximized ROA signal is generated by anisotropic scattering, i.e. at 180° and zero in the forward direction. For this reason both ICP-ROA and SCP-ROA experimental designs measure the backscattered light from the sample.

### 1.4.3 SCP-ROA spectrometer

In the original set-up used, ICP-ROA, the incident light was circularly polarized and the final ROA spectrum was calculated by subtracting electronically the Raman intensities measured from all polarization states of scattered photons. In the SCP-setup, however, the incident light is
linearly polarized, and SCP-ROA measures the difference in the intensities of circularly polarized components of the scattered light. The instrumentation used in this thesis was SCP-ROA, which is the only commercially available setting produced by BioTools. It should be emphasised that in the absence of electronic enhancement, i.e. under non-resonant conditions, ICP- and SCP-ROA generate equivalent spectra.

The SCP-ROA spectrometer was first designed by W. Hug with a backscattered geometry which is essential for measuring ROA in aqueous solution. The major improvement offered by the SCP strategy, compared to the ICP setting, is the removal of the interference of the “flicker noise” arising from particles travelling through the laser beam, as well as laser power fluctuations, density fluctuations in the sample and so on. The flicker noise is cancelled out because in the SCP setting the intensity difference measurements required to extract the circularly polarized components of the scattered beam are taken between two orthogonal components of the scattered light measured during the same acquisition period.23-25 This improvement results in a better signal-to-noise compared to the ICP setting, and faster spectral acquisition time, from 10-20 h (for an ICP setting) to ~2-5 h for samples at the same concentration. Figure 1.10 shows a schematic representation of the ChiralRaman SCP ROA spectrometer; the laser source is a 532 nm frequency-doubled Nd/YAG laser. At the source, the light is linearly polarized, or can be unpolarized; the polarization is “scrambled” by a fast rotation of the azimuth and deflected into the sample by very small prisms coated to provide "paired" s- and p-reflection compensation. The backscattered light from the sample is collimated onto a liquid crystal retarder set that converts the right and left-circular polarisation components of the scattered beam into linear polarization states with the azimuth perpendicular and parallel, respectively, to the plane of the instrument. The scattered light then passes through an edge filter to remove the Rayleigh line. The beam is then split by a beam-splitting cube that diverts the perpendicular component at 90° whereas the parallel component retains its direction.
Using this splitting the left and right circularly polarized components of the backscattered light are collected separately via two different fibre optics. The fibre optics converge into a spectrograph containing a highly efficient single volume-holographic transmission grating that allows the Raman spectra generated by the right and left circularly polarized components to be dispersed concurrently on the chip of a back-thinned CCD detector.

Figure 1.10: Schematic diagram of the BioTools Chiral Raman-2x™ layout. The path of the laser is represented by the red arrowed line; the path of the Raman scattered light is represented by the dotted blue line. Labels A-Z identify the components of the instrument. (Adapted from the BioTool Health&Safety brochure).
In Figure 1.10 the schematic layout of the Chiral Raman-2x is reported. The instrument is designed based on a backscattered SCP strategy earlier discussed. The sample is transferred into a customized quartz cell. It is placed in the sample holder (position O), the incident laser (excitation wavelength of 532.5 nm) passes through a series of components and finally focuses on the sample. Among the components, the quarter wave plate (position H) acts as a circularity converter supported by two synchronised, counter-rotating half-wave plates (positions I and J). This setting removes the artefact, induced by linear polarization in the incident laser beam. The time of illumination of the sample is regulated by an incident shutter (position L). The light passing through the sample is absorbed by a beam dump (position P). The backscattered beam (180°) passes through an holographic notch filter (position S) that removes the Rayleigh scattering before being split into the left- and right- components by the beam splitter (position U). The split beams are guided along fibre optics and converted into linear light before being projected onto the chip of the CCD camera.

1.4.4 Raman Optical Activity of protein biomolecules

An advantage of ROA spectroscopy lies in its compatibility, with water allowing structural analysis of solvated biomolecules in their native environments. Both Raman and ROA spectra can be routinely measured from a sample in water without interference from the solvent vibrations, unlike other vibrational techniques such as IR. Moreover, unlike the parent Raman spectra of proteins in which amino acid side chains dominate the spectra, ROA features are most sensitive to the geometry of the chiral α-carbons of the polyamide backbone defined by the dihedral angles φ and ψ of the skeletal chain, and therefore they represent a signature of the backbone secondary and tertiary structures. Raman scattering events are faster than conformational fluctuations, therefore, ROA spectra are considered a “snapshot” of all the
conformations of the sample in the equilibrium state in solution. The observable SCP-ROA ultimately depends upon the chirality of the bond conformations; therefore in structures with “opposite chirality” there are cancellations between mirror contributions. This characteristic makes ROA spectroscopy an excellent tool for probing the conformational dynamicity of biomolecules in solution as the intensity and the broadness of the bands depends upon the structural transitions. In stark contrast, Raman spectra are mostly additive of the different contributions, and so are less sensitive to the dynamic aspect of conformations in solution. The main disadvantage of the ROA technique compared to Raman spectroscopy is the low intensity of the signal; Raman scattering events happen to 1 in every $10^6 - 10^7$ photons, ROA signals are three orders of magnitude weaker. This results in the need for a high concentration of the sample and long collection times.

ROA spectra of biological molecules can be very complex because of the number of vibrational modes possible ($3N-6$); for this reason ROA spectral analysis is often focused on regions bearing key information about molecular structure. In the case of proteins and peptides, these regions are represented by the amide I and amide III regions revealing key information about the conformation.\textsuperscript{26} Bands in the Amide III region ($\sim$1230-1310 cm\textsuperscript{-1}) arise from the in-phase combination of the in-plane N-H deformation and C-N stretching vibrations. However, it has also been acknowledged that in the Amide III region the N-H and C\textsubscript{\textalpha}-H deformations also contribute, and this region has been extended to at least 1340 cm\textsuperscript{-1}.\textsuperscript{27} The Amide I ($\sim$1630-1700 cm\textsuperscript{-1}) region is generated mainly by C=O stretching, while the Amide II region ($\sim$1510-1570 cm\textsuperscript{-1}) originates from vibrations from the out-of-phase combination of the in-plane N-H deformation and the C\textsubscript{\textalpha}-N stretch.
1.5 Vibrational Circular Dichroism

Vibrational circular dichroism is a powerful vibrational technique sensitive to the absolute configuration of molecules in solution. It has been successfully used for the investigation of biological molecules, transient metal complexes and natural products.\textsuperscript{28} VCD is sensitive and non-invasive and allows for absolute configuration. NMR and X-Ray, although routinely employed for structural determination, can hardly distinguish between individual conformers because of their intrinsically slow response to structural changes. In contrast, since molecular vibrations occur at the sub-picosecond time, vibrational spectroscopy are sensitive to individual conformers. VCD measures differences in the absorbance of a right and left circularly similarly to ECD. These two named techniques are closely related, however, the former uses light in the UV-vis region while VCD employs IR, NIR lights. Despite that these two techniques are closely related, VCD presents some specific characteristics. In particular, there are no electronic transitions involved; therefore there is no necessity for a chromophore in the sequence. Moreover, thanks to the high resolution of the spectra, it is possible to evaluate the configuration and the conformation from the same spectrum. The first VCD measurements goes back to 1974 when George Holzwarth recorded the VCD spectrum of (+), (-) and the racemic mixture of 2,2,2-trifluoromethyl-phenyl ethanol.\textsuperscript{29} Since then numerous improvement have made this technique a well-established chiroptical spectroscopy. Remarkable for the development and commercialisation of VCD has been the work of Laurence A. Nafie and Rina Dukor, cofounder of the BioTools, the first company to produce a commercially available VCD spectrometer as well as ROA.
1.5.1 **VCD theory**

As already stated, VCD measures the difference in absorption between two polarized lights of opposite circularity. Although the VCD signal is usually about $10^4$ – $10^6$ times lower than the intensity of ECD, the absorption can be expressed as following:

$$\Delta \varepsilon = \varepsilon_L - \varepsilon_R$$ (24)

The interaction of a photon with molecules in the ground state $g_0$ promotes them to a higher energy level $g_1$ which in the case of VCD is induced by a left or right circularly polarized radiation. Ideally, $\Delta A$ is expected to be 0 in case of achiral molecules, as they will interact in the same way with the two circular components. By contrast, chiral molecules will absorb one polarized light more than the other, and it will result in a positive or negative sign. For a given pathlength and concentration the same equation can be expressed in terms of difference in absorptivity:

$$\Delta A = A_L - A_R$$

Figure 1.7: On the left there is a schematic representation of the promotion of the molecules from the ground vibrational state ($g_0$) to the excited vibrational state ($g_1$). Chiral molecules will interact preferentially with right or left circularly polarized light. The different in the absorbance of the two components is responsible for the VCD signal.

The IR band intensity is proportional to a quantity called dipole strength which is defined as the absolute square of the electric dipole transition moment, while VCD intensities and sign
depend upon the rotational strength of the molecules. The rotatory strength is an imaginary component of the scalar product of the electric dipole transition moment onto the magnetic dipole transition moment. They can be noted as follow:

\[
D_{ge} = \langle \psi_g | \mu | \psi_e \rangle \cdot \langle \psi_{ge} | \mu | \psi_g \rangle
\]  
\[R_{ge} = -\text{Im} \langle \psi_g | \mu | \psi_e \rangle \cdot \langle \psi_{ge} | m | \psi_g \rangle
\]

From the equation (18) and (19) it is apparent that the dipole strength will always be positive; whereas the rotatory strength can be either positive or negative, based on the relative orientation of the electric and magnetic dipole transition moments. VCD is generated by the combination of linear (electric-dipole) and circular (magnetic-dipole) oscillation of charges during vibrational motion. By contrast, the parental IR absorption is influenced only by the linear oscillation of the charges.

1.5.2 **VCD instrumentation**

The source for VCD spectrometer is usually thermal, such as a SiC glower, or an electric arc as in a xenon lamp.

![Schematic representation of a VCD spectrometer.](image)

Figure 1.8: Schematic representation of a VCD spectrometer.
The light coming from the source is passed through a interferometer. The light passes through an IR optical filter, a polarizer and a photo-elastic modulator (PEM) in order to select left-circularly polarized light (LCP) and right-circularly polarized light (RCP). The photo-elastic modulator is a modulating wave plate operating between 35 and 60 kHz. The infrared radiation that passes through the sample is subsequently focused on a liquid-nitrogen-cooled detector, the instrument adopted in the InSb or HgCdTe (MCT).

1.5.3 **ROA vs VCD**

Both ROA and VCD are types of Vibrational Optical Active techniques. These two techniques share similar differences as the parent IR and Raman. Unlike these techniques, however, both VCD and ROA are sensitive to chirality, therefore there is to consider not only the wavenumber shift or the shape of the bands but also the sign. The definition of the sign conversion in VCD and ROA is very different. In the first case, there are influences from the electronic mechanism, the dipole moment derivatives with respect to the nuclear motion. In case of ROA, the polarizability derivatives have a greater influence on the final sign of the bands. Due to the different phenomenon involved there are several advantages and/or disadvantage for these techniques.

Firstly, the frequency range for ROA can be extended to a region below 500 cm\(^{-1}\), whereas, in case of VCD it has not been observed. However, the ROA intensities coming from hydrogen stretching in the high frequency range are difficult to measure compare to VCD that has proven to be much more sensitive to these vibrations.

Both techniques are sensitive to vibrational coupling in the species because of the specific distribution of the vibrational modes over the framework of the molecules. Since VCD is influenced also by the electronic dipole moment and these can couple *via* radiative mechanism,
VCD results are sensitive to long range coupling compared to the ROA which is sensitive to the local chirality of the vibration modes. As a result, the ROA is much more sensitive to the secondary structure in the amide III region that is given by localized hydrogen bending vibrations near the chiral centre, which varies with the secondary structure. In contrast, for VCD, the amide I region bears the information for the protein structure. VCD spectra are much easier to measure compared to ROA because the measurement of a ROA spectra can be drawback from a phenomenon known as the depolarization ratio, which is the consequence of variation of the Raman spectrum influenced by different states of the relative polarization between the incident and scattered light. A great advantage of ROA over VCD is the sensitivity for the dynamic and the conformations of biological molecules. Overall these two spectroscopy techniques are often considered complementary and using a combination of both it is possible to highlight different structural information.

1.6 Neutron Scattering (NR)

Neutron scattering is the process of interaction of neutrons with matter. It is generally used to refer to a series of techniques where neutrons are used as probes to investigate different aspects of matter. To date a large number of techniques are available and a full discussion is beyond the scope of this dissertation. Neutron reflectometry is an optical techniques that has been successfully applied to the investigation of material interfaces, polymer films, surfactants and lipid bilayers in biological membranes, owing to the ability of neutrons to examine buried interfaces. The importance of the properties of the neutron in interaction with matter was noted first by Hater et al. in 1981, and since then has been gaining more and more attention from the scientific community, as testified by the increased installation of reflectometers in neutron sources. This technique is well suited to the study of soft-matter, thanks to the peculiar property of the neutron, which is that it is strongly scattered by light atoms such H, C, O and N, highly
abundant in biological molecules. Moreover, different isotopes have different scattering densities, therefore isotopic substitution allows contrast variation for the analysis. It should be noted that H/D substitution is particularly useful for soft-matter investigation. By tuning the ratio of these two isotopes it is possible to modulate the refractive index distribution at the interface, or at the surface, to highlight or mask certain features of the sample.

There will be a brief introduction to this complicated technique presented subsequently in this thesis. Neutron reflectivity was the only technique used in the study and its basics are discussed in this section. For a more comprehensive description of the technique, detailed information can be found in the literature. 31
1.6.1 **NR theory**

Neutrons are sub-atomic particles predicted by Rutherford and discovered by Chadwick in 1932; they bear no charge and they can easily penetrate far into matter. Furthermore, whereas electrons or photons interact with the atomic electron cloud, neutrons interact only with atomic nuclei. Nuclear forces are very short range (on the order of a few femtometers), therefore they do not perceive solid matter as “very dense” because the size of a scattering centre (the atomic nucleus) is typically several orders of magnitude smaller than the distance between centres. This means that most solid samples are effectively semi-transparent to neutrons, enabling the study of adsorption at buried interfaces. Thanks to their high penetrating nature, the interaction with matter is with the nuclei of the molecule, with no interference from the electron shell. This is the reason why different isotopes of the same element lead to a different neutron scattering.

Neutron beams interact with surfaces in the same way light does, therefore, when an incident beam hits the surface it can be reflected and part transmitted through the media. The reflected and transmitted parts are determined by the nature of the surface.

![Graphic representation of the reflection and transmission on a neutron beam at a single interface. \( \phi_i \) is the incident angle and \( \phi_t \) is the transmitted angle.](image)

Figure 1.9: Graphic representation of the reflection and transmission on a neutron beam at a single interface. \( \phi_i \) is the incident angle and \( \phi_t \) is the transmitted angle.
In a neutron reflectivity experiment, a highly collimated beam of neutrons is shone onto a very flat surface. The beam is then reflected and the measure of the intensity of the reflected radiation is recorded as a function of the momentum transfer perpendicular to the interface, $Q$, which is defined by the beam incident grazing angle $\theta$ and the neutron wavelength $\lambda$:

$$Q = \frac{4\pi \sin \theta}{\lambda} \quad (27)$$

The intensity of the reflected beam is related to the refractive index of the interface, therefore it provides information about the structure of the surface and its interfaces, including the density profiles, thickness, and roughness of the films layered on the substrate.\(^{32}\)

Since neutrons are highly penetrating and only weakly interact with matter, reflection only occurs at sufficiently small $\theta$, typically a fraction a degree. The reflected intensity is related to the refractive index profile normal to the interface; therefore the technique yields information regarding the composition and density profiles of interfaces. The variation of reflectivity with $Q$ (the so-called reflectivity profile) depends on the neutron refractive index, which is defined as:

$$n = 1 - \rho \lambda^2 / 2\pi \quad (28)$$

The neutron scattering length density is very close to 1, therefore the scattering length density $\rho$ is often conveniently used instead. $\rho$ is given as:

$$\rho = \sum_i N_i b_i \quad (29)$$

Where $b$ is the nuclear scattering length and $N$ is the number density. One unique feature offered by neutron scattering is that the scattering length $b$ changes not only between different atoms, but also between different isotopes. Particularly, $b$ is numerically different and opposite in sign for hydrogen and deuterium ($b=-3.739$ fm for H, $b=6.671$ fm for D). This is at the basis
of the methodology called *contrast variation* where the \( \rho \) of selected parts of a sample can be fine-tuned by carefully adjusting the H/D ratio. This is commonly used to highlight or match out selected components in the sample. In particular, for the *contrast variation* methodology, by changing the ration of H\(_2\)O/D\(_2\)O to match the other components of the system, it is possible to highlight different parts of system in analysis.

A scheme and photographs of the Target Station 1 facility and ISIS-SURF instrument is reported in Figure 1.14. High speed protons (dotted pink line) reach the heavy metal cylinder (red dot) where the spallation process takes place. The protons break and the atoms and neutrons are shot in all directions at high speed. Before each station (instrument) receives the neutrons they pass through a moderator that slows them down. The collimated neutron beam enters into the station trough ‘slit 1’ (a) and is reflected by a mirror (b) that selects only neutrons of a specific speed before passing through a second slit 2 (c) and then shone onto the sample. The relative aperture of both slits regulates the dimension of the area of the sample illuminated by the beam. Neutrons then pass through a vacuum tube (e), thereby reducing the interaction with the air, with reductions of 10 % of the intensity of the signal at the detector (f) which measures indirectly the neutrons. Since neutrons are not charged, they do not interact with electrons; however, they can interact with nuclei and induce the loss of charged subatomic particles. The detector at the ISIS-SURF facility is a gas-filled detector with Helium-3 which interacts with neutrons according the following nuclear reaction:

\[
\text{^3He} + \text{n} \rightarrow \text{^3H} + \text{^1H} + 765\text{keV}
\]  

(30)

The results of this interaction is the release of charged particles that are easily detectable.
Figure 1.10: Schematic representation of the target station 1 facility (as found on the website, http://www.isis.stfc.ac.uk/instruments/instruments2105.html) and photographs showing the ISIS-SURF neutron reflectometer. a) Primary collimation slit of the neutron beam. The neutrons are bounced by a mirror (b) to select neutrons of specific speed and then selected through a second slit (c) before hitting the sample of the tray (d). The neutrons reflected by the sample pass through a vacuum tube (e) and finally they reach the detector (f).
1.6.2 Data analysis

The most widespread and versatile approach to data analysis is the optical matrix method. With this approach, a model of the interfacial region is proposed where the interfacial region is divided into a finite number of discrete layers, each characterised by a given thickness $d$ and scattering length density $\rho$. A Gaussian roughness $\sigma$ is often used to simulate interpenetration between layers. The reflectivity is calculated from the model and compared to the experimental data in an iterative approach until a good match (fit) is obtained.

One major drawback of neutron reflectivity is that several models, sometimes very different from each other, can produce the same reflectivity profile. This is commonly avoided by using prior knowledge of the system (i.e. overall sample thickness, volume fraction of known components) and with the use of contrast variation (i.e. to change the $\rho$ of the aqueous sub-phase or use compounds in their deuterated form instead of hydrogenated). This way, a set of reflectivity profiles is obtained that must be fitted by a unique model.

For the experiments presented in this thesis it was used a series of six contrasts were used to over-constrain the model and produce a true representation of the sample.
1.7 **Proteins and amino acids**

Proteins and peptides are polymers of amino acids linked by the amide bond (peptide bond) between the carboxyl group of one amino acid and the amino group of the following one. The difference between proteins and peptides is mainly in the length of the polymer. According to the IUPAC classification, sequences of 15 amino acids or less are defined as “oligopeptides”, sequences of up to 50 amino acids are named as “polypeptides” and longer molecules (more than 50 residues) are defined as “proteins”.

The biological function of these polymers depends on the nature of the building blocks, the length of the molecule and its spatial organisation. For this reason, it is important to understand the nature of the amino acids in order to understand their influence on the conformation and ultimately on the biological activity of a peptide or protein.

In nature there are two distinguishable classes of amino acids: “proteinogenic amino acids” and “non-proteogenic amino acids”. Proteogenic amino acids are found in all living organisms; they are encoded by DNA and are inserted in the protein or peptide via ribosomal synthesis. On the contrary, non-proteogenic amino acids are rarely found in nature, where they are found mainly in prokaryotic cells and simple eukaryotic organisms such as sponges, fungi and algae. These non-proteinogenic amino acids are synthesized and inserted in the sequence via enzymatic reaction. There are twenty different proteinogenic amino acids, all of which are \(\alpha\)-amino acids. They comprise an amino group (-\(\text{NH}_2\)), a carboxyl group (-\(\text{COOH}\)), a hydrogen atom and a further substituent (\(R\)) covalently attached to the same carbon (the \(\alpha\)-carbon, \(C_\alpha\)). All amino acids, except glycine, contain a chiral centre on the \(\alpha\)-carbon, therefore, for each of those there are two optical isomers: L-enantiomers and D-enantiomers. The chief building blocks of proteins and peptides are the L-amino acids, whereas, D-enantiomers are rarely found in nature. Indeed, there is evidence of D-enantiomers in some
ribosomal peptides in eukaryotes, but mainly D-enantiomers are found in prokaryotic cells such as in the cell walls of bacteria or the amino acid ornithine found in the sequence of the antibiotic bacitracin A.\(^\text{35}\)

The identity of the substituent on the \(\alpha\)-carbon dictates the chemistry of each amino acid. They are grouped based on the R substituent into several classes (in which there are some overlaps): hydrophobic, hydrophilic, acid-containing, base-containing, sulfur-containing, and aromatic amino acids. Proline is classed separately as it has a secondary amine.\(^\text{36}\) The 20 standard amino acids can also be grouped into two broad clusters: polar and non-polar amino acids.

![Amino acids diagram](image)

Figure 1.11: Amino acids, different amino acids have been classified based on the main characteristic of the side chain.

The amino acids listed in Figure 1.11 are encoded by DNA and the main components of proteins; however, often they can undergo post-transcriptional modification. Examples are
hydroxyproline and hydroxylsine found in collagen or the gamma-amino butyric acid (GABA) which is an important neurotransmitter inhibitor and anti-hypertensive agent.

1.8 **Protein and peptide conformation**

Linderstøm-Lang classified protein and peptide structures into four hierarchical levels of increasing structural complexity. The primary structure of a protein or peptide is the “sequence” of amino acids linked together by the peptide bond. Proteins and peptides, unlike other organic polymers, such as polysaccharides, are formed in a linear, unbranched chain.

![Image of protein and peptide structures](image)

Figure 1.12: Starting from the left are represented the four hierarchical level of protein folding. a) The primary structure is the sequence of amino acids. b) Representation of secondary structures resulting from the intra-molecular hydrogen bonding. c) The tertiary structure is the twisting and folding of the peptide arranged in its secondary structure. d) Quaternary structure, results from inter-molecular interactions.

The peptide bond is planar and the repeated unit of N, Cα, and C of each residue represents the “backbone” of the polypeptide with the different side chains projecting from it.

The secondary structure is represented by the spatial organization of the peptide chain resulting from the rotation around the torsion angles (φ and Ψ, Figure 1.12) along the peptide backbone. These are stabilized by hydrogen bonds, and will be discussed in more detail in the next section.
The next level of structural organisation is the “tertiary structure” which refers to the three-dimensional arrangements of the folded polypeptide chain. This special organisation is stabilized by interactions between the side chains via hydrophobic effects, electrostatic interactions and in some cases by disulfide bonds. From this spatial organisation emerges a protein size and shape that ultimately depends on the number of residues, the size of the residues and the secondary structure. In most cases the tertiary structure is responsible for biological activity. Different factors can alter this super-molecular organization, and as a consequence, the protein activity. For instance, changes in temperature, pH or ionic strength can induce changes, in the protein, termed ‘denaturation’.

The quaternary structure is used to describe the number and position of different units of multimeric proteins such as haemoglobin or G protein.\(^\text{37}\)

1.9 **Protein secondary structures**

In biochemistry the “protein native state” is defined as the final folded and/or assembled form which determines the biological activity of a protein or polypeptide. The event that results in the mis-folding of the protein and consequent loss of activity is defined as “denaturation”. When denaturation occurs, secondary and tertiary structures break down. Due to that effect, understanding the folding pathways of proteins and peptides is important in physiology and for drug discovery.

As mentioned in the previous section, proteins and peptides are polymers of amino acids linked together via the peptide bond (amide bond). Crystal structure analysis reveals that the length of the peptide bond is shorter than common C-N single bonds with a length of 1.32 Å, which is placed between the length of single C-N (1.49 Å) and the one of a double bond, C=\(\text{N}\) (1.27 Å).
Å). This is attributable to the mesomeric nature of the peptide bond that results in rotational restriction around the bond. The amide bond has a planar geometry with six atoms laying on the same plane: the C$_\alpha$, the carboxyl group C=O on one amino acid and the nitrogen and hydrogen of the NH and C$_\alpha$ of the next amino acid. (Figure 1.13) This conformational restriction allows only two possible conformations around the peptide bond: cis (with the two C$_\alpha$ on the same side of the peptide bond) and trans (with the two C$_\alpha$ on opposite sides). In native peptides the trans conformation is most common and more stable whilst the cis-peptide bonds can be found in X-Pro linkage. This is due to the fact that the nitrogen in proline is bonded to two carbons, reducing the energy differences between the trans and cis forms.

The peptide bond has very restricted rotational freedom, however, the bond between the C$_\alpha$ and the nitrogen and between the C$_\alpha$ and the carbonyl are free to rotate with the rotation influenced mainly by the side chain. Figure 1.13 reports the characteristic dihedral angles of a polypeptide chain.

Figure 1.13: Representation of the torsion angles $\Psi$ (C$_\alpha$-C), $\varphi$ (N- C$_\alpha$) of a peptide bond.
The torsion angle values determine the lowest energy conformation: \( \Psi \) represents the rotation angle of the C\( \alpha \)-C bond while \( \phi \) is the rotation angle around the N- C\( \alpha \), exploring values from -180° to 180°. The free rotational around \( \Psi \) and \( \phi \) is controlled by the steric repulsion of the methylene group on the C\( \alpha \). Ramachandran was the first to report this property and propose a model for structural prediction based on the dihedral angles around each residue in the sequence.\(^{34, 37}\) Based on the steric hindrance around each C\( \alpha \) certain regions of the plot are allowed or disallowed (Figure 1.14).

![Figure 1.14: Schematic representation of the Ramachandran plot. In this figure are outlined the positions of \( \alpha \)-helix, \( \beta \)-structure, PPII and the rare left-handed helical and 3\( _{10} \)-helix.\(^{38} \)](image)

1.9.1 **Helices**

Helical conformations are a common motif in protein secondary structures. The primary sequence, as result of intramolecular hydrogen bonding wraps around a central axis. The repetitive pattern is therefore a turn of helix that is stabilized by the hydrogen bonding between the oxygen atom of the carbonyl group of the amino acid in position \( i \) and the hydrogen of the amino group in position \( i + n \). Each helix is characterized by a different ‘\( n \)’ value and specific dihedral angles.
1.9.2  α-helix

This secondary structure is indeed the most common in nature, with torsion angle values usually around $\phi \sim -63^\circ$ and $\psi \sim -42^\circ$. This causes the peptide chain to coil in a right-handed sense with 3.6 amino acids per turn and a length per turn of 1.5 Å along the helix axis. Each C=O group is H-bonded to the N-H group of the amino acids four residues earlier. Helices are identified by the symbol $N_m$, where $N$ is the number of residues per turn and $m$ is the number of atoms included in the ring formed by the C=O and H-N hydrogen bonds. Using this system the α-helix should be referred to as 3.613-helix.

![Figure 1.11: Axial view (on the left) and side view (on the right) of alpha helix secondary structure. The representation on the right also reports the number of amino acids and the intramolecular hydrogen bonding (dotted line).](image)

1.9.3  310-helix

This secondary structure is not common in nature and it is generally found at the N- or C-terminus of an α-helix in big globular proteins. The 310-helix and α-helix differ in the number of residues per turn, the dihedral angles and the intermolecular hydrogen bond pattern between C=O and H-N. With the 310-helix being formed by 3.2 amino acids per turn ($n=3$) the ring for this helical conformation is much tighter compared to the α-helix (Figure 1.16). The energetic differences between these two structural motifs are very low and it has been observed that the
amino acid sequence, the dielectric constant of the solvent and length of the peptides can lead to the co-existence of α and 3₁₀ helices in solution.⁴⁰

Figure 1.12: a) Comparative representation of 3₁₀-helix and α-helix looking down the axis, b) representation of the hydrogen bonding pattern for the two secondary structures.

Table 1.1: Principal structural differences between α-helix and 3₁₀ helix.

<table>
<thead>
<tr>
<th></th>
<th>φ</th>
<th>Ψ</th>
<th>Amino acids per turn</th>
<th>Residue per ring formed by hydrogen bonds</th>
<th>Hydrogen bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helix</td>
<td>-63°</td>
<td>-42°</td>
<td>3.6</td>
<td>13</td>
<td>1 &lt;-&gt; 4</td>
</tr>
<tr>
<td>3₁₀ helix</td>
<td>-57°</td>
<td>-42°</td>
<td>3.2</td>
<td>10</td>
<td>1 &lt;-&gt; 3</td>
</tr>
</tbody>
</table>

Hydrogen bonds with motif 1 <-> 3 are also typical for the type III β-turn, as classified by Venkatachalam et al.² Multiple sequences of type III β-turn will lead to in a 3₁₀ helix.
1.9.4 Polyproline II helix (PPII)

This secondary structure is found in nature with or without the presence of proline and it seems an important structural element for some unfolded proteins. This structural motif is often found in loops connecting two secondary structures in a protein.\(^\text{41}\) PPII helix adopts a left-handed helix with dihedral angles of \(\varphi = -78^\circ\) and \(\Psi = +146^\circ\), which in a Ramachandran plot occupies an area adjacent to the \(\beta\)-region (see Figure 1.14).

1.9.5 Other helices

The structural motifs discussed previously are just few of the vast group of helices found in nature. To name few more, \(\pi\)-helix and collagen helices are also worth mentioning, with the \(\pi\)-helix being the thickest helix formed by a single strand \((n=5)\). The collagen helix is formed by these left-hand helices containing the repeating pattern Gly-Pro-hydroxyproline on each of the peptide chains.

1.9.6 \(\beta\)-sheet structures and \(\beta\)-turns

1.9.6.1 \(\beta\)-sheet

The \(\beta\)-sheet is along with \(\alpha\)-helix one of the most common structural motifs. It is formed by peptides in extended conformations (\(\beta\) strands) that are connected to each other via hydrogen bonds and organized in a planar sheet. Typical dihedral angles for each polypeptide chain are \(\varphi=-135^\circ\) and \(\Psi = 135^\circ\). Based on the orientation of the \(\beta\) strands \(\beta\)-sheets are classified as parallel or anti-parallel or mixed (Figure 1.17) Antiparallel \(\beta\)-sheet is the most stable because
the geometry of the hydrogen bonds allows an optimized N-H- \(-\)-O=C alignment compared to parallel \(\beta\)-sheet.

Figure 1.13: Schematic representation of a) antiparallel and b) parallel \(\beta\)-sheet. The arrow represent the backbone on the peptides, the dot line represent the hydrogen bonds.

1.9.6.2 \(\beta\)-turn

This secondary structure connects different structural motifs together such as two \(\beta\)-strands, or of a \(\beta\)-strand and \(\alpha\)-helix, or two \(\alpha\)-helices. It is characterised by a hydrogen bond between the C=O and NH groups of amino acids 3 residues apart. Such motifs have been classified by Venkatachalam into three different types: I, II and III, which are characterized by right-handed folding, and their enantiomers, I’, II’, III’. Different types are differentiated by dihedral angles values of residues \(i+1\) and \(i+2\). Table 1.2 shows reported dihedral angles for \(\beta\)-turn I, II, III.\(^{42}\)
Table 1.2: Dihedral angles $\phi$ and $\Psi$ for $(i+1)$ and $(i+3)$ in type I, II and III $\beta$-turn.

<table>
<thead>
<tr>
<th>$\beta$-turn type</th>
<th>$\phi$ $(i+1)$</th>
<th>$\Psi$ $(i+1)$</th>
<th>$\phi(i+2)$</th>
<th>$\Psi$ $(i+2)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>-60°</td>
<td>-30°</td>
<td>-90°</td>
<td>0°</td>
</tr>
<tr>
<td>Type II</td>
<td>-60°</td>
<td>+120°</td>
<td>+80°</td>
<td>0°</td>
</tr>
<tr>
<td>Type III</td>
<td>-60°</td>
<td>-30°</td>
<td>-60°</td>
<td>-30°</td>
</tr>
</tbody>
</table>

Figure 1.14: a) Generic structure of $\beta$-turn, showing hydrogen bond.

1.9.7 Peptide secondary structure

The word “peptide” derives from the Greek word πεπτός meaning digested. It was first introduced by Fisher in relation to the peptone, the fragment of protein produced by the gastric acids (pepsin). Nowadays, there is a branch of chemistry dedicated to the synthesis, isolation and characterisation of peptides. Since the publication of the first peptide synthesis by Fisher in 1902 peptide chemistry has grown exponentially. Peptides are found in almost every living organism and can act as hormones (insulin, vasopressin), neurotransmitters (substance P and opioid peptides) and antimicrobial agents (defensin, peptaibols).
1.9.8 Biological importance of peptide conformation

Owing to their short sequence these molecules are conformationally flexible and very much influenced by to their environment. It is known that only a few of the several conformations that a peptide can explore are of biological significance, and it is thought that nature preserves the conformational flexibility as a mechanism of survival. Thanks to the topological influence on the folding of these molecules it is possible to overcome, within certain limitations, the changes that may occur at the corresponding receptor.

However; the conformational flexibility has been used by nature as a mechanism of survival, as topological influence can induce one preferred conformation over another. The changes that occur at receptors can be overcome, within certain limits, by the conformational changes in the corresponding peptide. It is also know that the same peptide can interact with different isoforms of the same receptor in the same animal. An example is the [Leu]encephalin can an opioid peptide capable of interacting with the several different opioid receptors present in the human body.

1.9.9 Conformational studies of peptides

Understanding the link between biological activity and peptide conformation is important for peptidomimetic drug design, however it has proved to be challenging as the environment greatly affects the folding propensity of short sequences. Moreover, conformational studies are limited by the intrinsic characteristics of the technique used for the investigation. For instance, spectroscopic techniques, such as X-ray crystallography, study the conformation of the crystalline state which does not necessarily represent the conformation adopted in solution or
at the biological receptors. Crystallography, however, was the first approach used for conformational studies of peptides.\textsuperscript{43} The first solution studies of small peptides started in 1954 with IR studies of protected dipeptides in solution.\textsuperscript{44} Raman spectroscopy investigation started about a decade later\textsuperscript{45,46} when the spectra of poly-L-alanine fibres was published. Nonetheless, for decades the principal spectroscopic methods used for the investigation of peptides in solution were circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopies. Theoretical methods have also been of great use in the examination of peptide conformations, in this context, it is worth noting the pioneering work by Ramachandran and co-workers. Raman Optical Activity (ROA) investigation of peptides had a slow start and only recently has gained more attention from the scientific world for the investigation of molecular conformations in solution.\textsuperscript{13}

\subsection*{1.10 Antimicrobial peptides}

Antimicrobial peptides (AMPs) form part of a defensive mechanism against pathogens that are produced by both prokaryotes and eukaryotes. The first peptide of this class to be isolated and recognized as active against pathogens was gramicidin in 1939, extracted from \textit{Bacillus brevis}.\textsuperscript{47} Complex organisms, such as humans, present an adaptive immune system which protects them from the microbial infection. On the contrary, plants and insects do not have the ability to adapt their immune system. The lack of adaptation is compensated by the production of AMPs. Although the first isolated plant antimicrobial peptide goes as far back as 1896, the focus on this class of antimicrobial peptides does not start till the 1960s, when the first resistant strain of bacteria started to appear. Since then, numerous studies on a variety of species have resulted in the isolation and synthesis of numerous peptides. This class of biological peptides
is evolutionary conserved and can be active against a wide range of organisms, such as, fungi, protozoa, virus and bacteria. Despite the vast numbers of AMPs found in nature, there some communal characteristics that are shared by this class. Usually, naturally occurring AMPs are short molecules, less than 100 residues long; the majority of them present a positive net charge and fold into an amphipathic structure exposing both hydrophobic and hydrophilic domains. They are principally classified based on their secondary structure into four categories: loop, extended peptides, linear peptides (which form amphipathic and hydrophobic α-helix) and the disulfide-bonded β-sheet. The mechanism of action of antimicrobial is not completely understood. Different theories have been proposed, such as, pore formation, blebbing, vesciculariziation, budding, interfering with the metabolism cytoplasmic components and so on. Different behaviour is expected for different peptides acting on one bacterial strain and for different type of bacteria with a given peptide.

Overall, since the majority of the AMPs are cationic and display antimicrobial activity by perturbing the membrane, a few models of action are considered most plausible: barrel stave, micellar aggregate, toroidal and carpe-like model. These models differs in the way changes are induced in the membranes of the pathogen, nevertheless, the initial step is common to all models, with the peptides orienting on the membrane surface until a critical concentration is reached and the peptides organize following one of the methods proposed.

In the Barrel-Stave mechanism the peptides align on the membrane to expose the hydrophobic side-chains to the lipids, while the polar side-chains align inward and interacting with the core of the lipid bilayer and the hydrophilic components point into the core of a water permeable pore (Figure 1.19). The size of the pores is sufficient to allow leakage of cytoplasm components, which can kill the cell. In the carpet model the peptides align on the surface, remaining in contact with the phospholipid head group. The molecules subsequently align
covering the surface of the bilayer, like a carpet, and dissolve the area forming cracks in the bilayer; resulting in cytoplasm leakages, Figure 1.19. In the toroidal model, the peptides insert in the membrane but remain associated with the head group of the lipids. The peptides than bend forwards, forming pores (Figure 1.19). The aggregate mechanism proposes that, after the peptides are inserted into the membrane, they form irregular aggregates of lipids and peptides, leading the death of the cell, Figure 1.19.

![Diagram of AMP mechanism of action](image)

Figure 1.15: Representation of the mechanism of AMP mechanism of action. a) barrel stave model, b) carpet-like mechanism, c) toroidal pore model, and d) disorder toroidal model.  

### 1.11 Phospholipid membranes

In order to understand the mechanism of action of the antimicrobial peptides, a combination of *in vivo* (bacteria growth studies) and *in vitro* studies using model membranes are required. Biological cell membranes are complex systems composed primarily of phospholipids with the addition of proteins, carbohydrates and cholesterol. However, it is often very difficult to reproduce data from biological cell membranes and more simple models are used instead. Phospholipid vesicles are some of the most commonly used as model membranes for
protein/membrane studies. These are formed by phospholipid bilayers that have folded to form a spherical supramolecular structure with an enclosed aqueous volume.

Phospholipids are organic molecules composed of phosphate esters of diglycerides. A vast number are found in nature, but an ubiquitous class of phospholipid is the phosphatidylcholines, in which the head group is a choline with a quaternary ammonium cation. As an example, Figure 1.20 shows the structures of 1,2-dioleoyl-\(sn\)-glycero-3-phosphocholine (DOPC) and 1,2-dimyristoyl-\(sn\)-glycero-3-phosphocholine (DMPC) lipids, which were used in this thesis to perform peptide/membrane interaction studies. A key characteristic of phospholipids is their amphiphilic nature. The fatty acids attached to the phosphate “headgroup” are hydrophobic and are often referred to as “tails”. Phospholipids in solution self-assemble into structures such as micelles. Vesicles can form spontaneously. When the phospholipid are exposed to water, the hydrophilic head group is exposed to the water and the tails are locked in the hydrophobic core in order to reduce exposure to water.

Simple dispersion in water results in vesicles with a variety of sizes and number of bilayers in membrane. In order to obtain uniform unilamellar (one bilayer) vesicles a number of methods can be applied, such as extrusion or sonication. Based on the size they are classified as: with a diameter less than 100 nm they are called small unilamellar vesicles (SUVs), between 100 and 1000 nm large, unilamellar vesicles (LUVs); greater than 1 \(\mu\)m, giant unilamellar vesicles (GUVs). Vesicles formed by more than one bilayer are referred to as multilamellar vesicles (MLVs). Over time the vesicles in suspension will fuse to form larger aggregates of multilamellar structures, however in most cases the time scales for this process are known and it is possible to choose the appropriate phospholipids for experiments to be performed.
Figure 1.6: Structures of a) 1,2-dimyristoyl-\textit{sn}-glycero-3-phosphocholine, b) 1,2-dioleoyl-\textit{sn}-glycero-3-phosphocholine.

Phospholipid in a bilayer can exist in different phases. Single component bilayers are typically found in either ordered solid phase (L$_{\beta}$), also referred as gel phase, or a disordered liquid phase (L$_{\alpha}$).\textsuperscript{50} In their gel phase the lipid bilayer is highly ordered and lateral diffusion of the single lipids trough the membrane is slow. For any given lipid there is a temperature ‘$T_m$’ (known as transition temperature or ‘melt’ temperature) above which the bilayer changes from the gel phase into the liquid phase in which lipids can diffuse more quickly laterally.

1.11.1 \textbf{Vesicle Preparation by extrusion or sonication}

The standard procedure for the preparation of vesicles starts with the lipids dissolved in organic solvent. The desired amount of lipids is transferred in a round-bottomed flask and dried first under reduced pressure and subsequently with a flow of dry N$_2$(g). The thin lipid film obtained is then rehydrated by adding water or buffer and mixing with a vortex mixer until a lipid suspension is obtained. The vesicles obtained with this method are polydisperse and the majority of them will be multilamellar vesicles (MLVs) as shown in Figure 1.21.
Figure 1.7: Scheme of hydration of a dry lipid film re-suspended into a mixture of MLVs. Extrusion or sonication yield uniform vesicles.

The vesicles can be converted into uniform SUVs by using sonication or extrusion. Sonication converts the MLVs into ~ 50 nm SUVs by disrupting the vesicles and the bilayers reorganize into uniform size vesicles. In the extruder apparatus, shown in Figure 1.22, the suspension of polydisperse MLVs is forced through a membrane/filter at the T_m, the membrane can be chosen of the appropriate size for the desired diameter of the SUVs.

Figure 1.8: Schematic representation of vesicles extrusion apparatus. The polycarbonate filter disk (b) is placed between two rubber O-rings (c). Gas tight syringes (a) are fixed onto the supports, and the vesicles suspension (d) is extruded through the polycarbonate membrane.
1.12 Peptaiboles

Peptaiboles are a class of AMPs produced by the fungi in soil-borne and plant-pathogen of the genera *Acremonium*[^51], *Paecilomyces*[^52], *Emericellopis*[^53] and *Trichoderma*[^7]. The latter fungal class is the strain that produces the most peptaibols among of all of these species and is the most studied class. Indeed, the first peptibol to be isolated was alamethicin, produced by *Trichoderma viride*[^54] in 1967. Peptaibols are generally linear peptides of lengths between 5 to 21 amino acids, endowed with high hydrophobicity and a high content of non-proteinogenic amino acids, synthetized via enzymatic process. The two main features that distinguish these peptides from other AMPs is the absence of a net charge on the peptide and the relatively high content of quaternary amino acids.

The most abundant residue in peptaibols is α-aminoisobutyric acid (Aib), to which they owe their name: **pept** = peptides; **aib** = α-aminoisobutyric acid; **ol** = because the C-terminus is normally functionalised with a hydroxyl group. The N-terminus is generally acetylated; therefore, both C and N termini are capped to mask the charges at the termini. Although Aib is the most common residue, other quaternary amino acids are often incorporated in the primary sequence, such as isovaleric acid and secondary amino acids like proline and hydroxyproline. As already mentioned, peptaibols are synthetized via protein synthetase complexes that are also responsible for including the non-standard amino acids. Often the synthases do not show strict selectivity between the different amino acids leading to the synthesis of numerous isoforms within the same organisms[^55]. To date, more than 300 peptaibols have been isolated and the common feature identified among these peptides is their propensity to fold into helical secondary structures. The primary structure of peptides dictates the main folding pattern and it is known that quaternary amino acids; in particular the Aib residues, are helix promoters because of the restricted number of dihedral angles accessible. For instance, the Aib residue,
compared to the proteinogenic alanine, has an extra methyl group on the \( C_{\alpha} \) and as a consequence it can occupy only restricted areas of the Ramachandran plot (Figure 1.23), i.e. a restriction in the angles \( \varphi \) and \( \psi \) to those that promote helical folding. Aromatic residues are also common, probably acting as membrane anchors and stabilizers of the membrane orientation.\(^{56}\) It has also been also noted that the secondary structure of the natural occurring peptibols is not greatly influenced by degrees of solvation because the peptides have in large internal hydrogen bonding networks. On the contrary, the dielectric constant of the solvent significantly affects the spectroscopic properties of these peptides.\(^{57, 58, 40, 59}\)

Although the mechanism of peptaibol activity is yet to be fully understood, it has been proposed that they mainly act by modifying the permeability of the membrane \( \textit{via} \) either transmembrane pore formation or acting as an ion carrier. Thanks to the effect of the permeabilizing cell of the membranes, peptibols can exhibit cytotoxicity and hemolytic effects. They can also form voltage dependent channels, demonstrated for alamethicin.\(^{57}\) As previously stated, this peptide is among the most studied peptibols to date. Its mechanism of action has been studied using a large set of spectroscopic techniques and it has been demonstrated that it forms ion channels \( \textit{via} \) a barrel-stave mechanism.\(^{15}\)

Interestingly, the length of the peptaibol does not seem to have a negative impact on the antimicrobial activity. For instance the lipopeptides KB I and KB II, with 11 residues, have the same antimicrobial efficacy as the peptide KA V, which is 19 residues long.\(^{60}\) For this reason predictions of the general mechanism are quite difficult. Not only the antimicrobial activity of peptibols but also the peculiar characteristics of the Aib residue have gained the attention of the scientific community, with great intereste in the influence of this particular residue on the folding of short synthetic peptides.
Figure 1.9: Ramanchandran plots: a) α-aminoisobutyric acid. b) glycine. c) alanine. The pink areas represent the allowed value for φ and ψ torsional angles for these residues in a polypeptide. The abundance of quaternary amino acids seems to promote a helical folding for this class of antimicrobial peptides, in particular α-helices and 3_10 helices.

1.13 Foldamers: synthetic mimics of peptide folding.

Foldamers are small synthetic molecules that mimic the folding properties of natural proteins and peptides. It is possible to design foldamers with specific secondary structures by choosing the appropriate building blocks. They are divided into two main categories: “aliphatic foldamers” and “aromatic foldamers”. Figure 1.24 reports some examples of aliphatic and aromatic backbones.
Aib rich foldamers can be designed to mimic closely the folding and biological activity of natural occurring peptaibols and represent a useful tool for investigation of their peculiar characteristics. In case of Aib based foldamers, the steric hindrance caused by the geminal methyl groups favour helical folding due to the Thorpe-Ingold effect and the $3_{10}$ helix conformation seems to be the most favourable one.

It has been noted that if the primary sequence includes proteinogenic amino acids as well as quaternary amino acids, the balance between $3_{10}$-helix and $\alpha$ helix depends upon the nature of the solvent as well as the length of the peptide.  

Later in the thesis the experimental analysis of Aib-rich foldamers in solution and within membranes will be discussed.
These studies will focus on the use of Raman and ROA and IR to investigate the folding and self-assembly of Aib-rich foldamers in solution and in the membranes. However, before these studies were carried out on hard to obtain Aib-foldamers, the use of these techniques to study the self-assembly of peptides comprising proteinogenic peptides was initiated.

In particular, Raman, ROA and IR were used for the investigation of the self-assembly amyloid-forming peptides (GNNQQNY, YTIAALLSPYS-FTIAALLSPES) and the formation of β-sheet hydrogels.
1.14 **Project outline**

The evolution of multi-resistant microbial strains such as MRSA and *Clostridium difficile* presents a serious threat to the provision of basic healthcare. The development of new and effective antibiotics is therefore important. Antimicrobial peptides (AMPs) are a defensive mechanism against microbes produced by most organisms. Peptaibols are a class of AMPs produced by fungi via non-ribosomal synthesis and are characterized by a high content of non-proteinogenic residues, mostly α-aminoisobutyric acid (Aib; pept-aib-ol), which give them a high propensity to fold into helical structures, a C-terminal 1,2-amino alcohol (pept-aib-ol) and usually acetylated or acylated at the N-terminus. The antimicrobial activity of AMPs is mediated by modification of the membrane permeability, however, the general mechanism has been theorized, a detailed mechanism of action is yet to be identified.

This thesis fits into this picture, with the dual purpose of improving the understanding mechanism of the action of peptaibols and foldamers as well as pioneering the application of relatively novel spectroscopic techniques for studying peptides/membranes interactions.

In particular, Aib-foldamers have been studied in solution and within membranes in order to understand the conformational changes that these peptides undergo as well as to study the orientation and interaction within membranes.

Solution studies were also focused on the identification of spectral feature for 3$_{10}$-helix conformation in Raman and Raman Optical Activity spectroscopies. Most of the peptides presented in this thesis have been previously studied with other spectroscopic techniques, such as X-ray and ECD. Raman spectroscopy has long established itself as a powerful technique for the investigation of proteins and peptides secondary structures. Principal secondary structure motifs have been abundantly studied; however, the assignment for more rare secondary structures is still a matter of debate, particularly for the 3$_{10}$-helical conformation. This helical
conformation differs from the $\alpha$-helix by the number of residues per turn and intramolecular H-bonds network, with the orientation of the H-bonds between C=O- - - H- NH at a slightly higher angle to the helical axis compared to $\alpha$-helix. This geometry, along with the less favourable Vander-Walls interactions, destabilise the $\beta_{10}$-helix conformation. However, it has been reported that quaternary amino acids can support the $\beta_{10}$-helix conformation. This crucial information has led to the synthesis and investigation of a number of Aib-oligomers with various spectroscopy techniques in the attempt to identify spectroscopical differences between $\alpha$-helix and $\beta_{10}$-helix. By comparing the structural features of Raman and ROA with the structural information already established we aim to address main feature characteristic for $\beta_{10}$-helix conformation. The information obtained in solution will then be compared with the conformation for the peptides within phospholipid bilayers.

Figure 1.25: One of the aim of the project is to investigate the secondary structure of Aib foldamers in solution.
1.14.1 Membrane studies

Studies using model membranes, specially by NMR, FTIR and CD, have been reported for the investigation of conformational changes of peptide from solution to membranes.67 68, 69 These studies have successfully investigated the conformational changes of peptides partitioning into membranes interfaces and their folding. Although the general principals of partitioning of membrane-active peptides are generally understood, the details of such interactions are often elusive. In particular, the literature is lacking in the investigation of the helicity preference of membrane-active peptibols. In this thesis the interaction of foldamers, designed to mimic natural occurring peptibols, with membrane has been deeply investigated by using a combination of spectroscopy techniques, specifically: ROA, IR, VCD, LD and NR. The information obtained by each of these techniques are complementary and can lead to a deep understanding of the folding, thermodynamic and kinetics orientation of interaction of peptibols with phospholipid membranes.

ROA, IR and VCD analysis highlight the structural and helicity preference for Aib-foldamers within the membranes as well as allowing a comparative study between solution and membrane environment.
Figure 1.26: Representation of the type of membrane/peptide interaction studies presents throughout this thesis. Interaction between foldamers and membranes will be studies on bilayers with NR, and in interaction with vesicle with LD, Raman, ROA and VCD.

Linear Dichroism allows the investigation of the orientation of peptide interactions with phospholipid vesicles. This spectroscopy technique has been successfully used for investigating the orientation of gramicidin \(^\text{70,12}\). However, LD studies, being a relatively new technique, are quite challenging and many factors can influence the outcome of the experiments. Moreover, the results outlined in this thesis refer mainly to the initial orientation of the peptides with respect to the vesicles. Kinetic orientation of peptides and the final equilibrium, i.e. the thermodynamically preferred orientation can be different. The concept provided by the experiment performed with gramicidin showed the changes that the orientation of the peptides overtime.

Finally, the interaction of the Aib-foldamers with bilayers was tested with Neutron diffraction. This spectroscopic technique has been successfully used to study the orientation of peptide and proteins with respect to supported lipid bilayers. It has been reported the investigation of
the Trichogin GA IV (GAIV) and Aib-foldamers designed based this natural occurring peptibols using NR. This spectroscopic techniques has the potential of detecting, the orientation and partitioning of the molecules within the membrane as well as reports the effects of the latter on the bilayer structure. 71

The orientation and the conformation of the peptides within the membrane hopefully will give the information necessary to understand the preferred mechanism of action for the foldamers under investigation. Ultimately, the information obtained for these small peptides could give a better understanding of the mechanism of action of natural occurring peptibols.
2. Amyloid Fibers: vibrational spectroscopy analysis

2. Amyloid fibers: model peptides for amyloidogenic pathways

Alterations of protein secondary structure may lead to conditions known as “conformational diseases” or “amyloid diseases”, characterized by the accumulation of insoluble aggregates in tissues. Although the proteins involved are different for different diseases, the fibrilous mechanisms are similar. When proteostasis (the mechanisms that regulate protein synthesis, and folding/misfolding in cells) fails to remove small unfolded proteins or polypeptides with exposed hydrophobic parts, these will interact with the exposed hydrophobic residues of other proteins. This interaction leads to aggregates, and when the aggregation process is irreversible the fibers start to accumulate either inside or outside the cells, causing the death of cells. A number of neurodegenerative diseases, such as Parkinson’s, Alzheimer’s or prion-mediated diseases are characterized by the formation of these fibrillar protein aggregates named “amyloid fibers”. Although the fibers are ultimately responsible for the loss of function, it is now established that the small intrinsically unfolded peptides are responsible for fibril formation and are therefore cytotoxic. Amyloid deposits have a β-sheet structure that is bonded together to form nanometre-sized cross-β fibrils. Individual β-strands are perpendicular to the fiber axis (Figure 2.1).
Understanding the aggregation pathway to amyloid is crucial because the aggregation is irreversible and the most effective therapeutic strategy would be to prevent the accumulation of the amyloid deposits. Small self-assembled peptides are often used as a model to study the interactions involved in amylogenesis because, in many cases, only a small part of the protein is responsible for the aggregation process and short sequences extracted by the native protein can faithfully reproduce the amyloidogenic pathways. Of these short sequences, models of amyloidogenic peptides have been studied during this thesis using Raman and ROA spectroscopy (GNNQQNY) and IR spectroscopy (YTIAALLSPYS).

Figure 2.11: Schematic representation of fibrillation.
2.1.1 **GNNQQNY: results and discussion**

The heptapeptide GNNQQNY, found at the 7 to 13 position of the protein Sup35 (extracted from the yeast *Saccharomyces cerevisiae*), has been widely studied to investigate both the amyloid formation and prion-like transmission. Although GNNQQNY is only seven amino acids long it reproduces the amyloidogenic process of the full native protein Sup35 and has a tendency to self-assemble into hydrogels. This peptide was initially tested within our research group as a possible candidate for the formation of fibril gels for cell culture; however, due to difficulties encountered in the synthesis it was ruled out for this purpose. Although this peptide gel was not used for cell culture, the fibres in the gel were studied with vibrational spectroscopies. In particular, Raman and ROA experiments gave promising result for the investigation of amyloidogenic processes using these techniques.

Figure 2.2 shows the Raman and ROA spectra for GNNQQNY in solution with water. The solution was prepared by heating the sample at 60°C, to obtain a clear solution left to self-aggregate into a gel at room temperature. Figure 2.3 shows the spectrum of the peptide in its prefibrillar state (black line) and in its fibrillar state (orange line).
Figure 2.2: a) Raman spectra and b) ROA spectra of the solution at room temperature. The solution was prepared 1h before the experiment by dissolving the sample at 60°C GNNQQQNY in HPLC grade water, (33 mg/mL). The sample was left at room temperature to cool down an hour before starting collecting Raman. The experiment was performed at room temperature using a laser power of 1.20 W, data accumulation time 2.5 s over 24 h. The water has been not subtracted by the spectra.

Figure 2.3: Raman spectrum of the GNNQQQNY gel (orange line) performed after 9 days from the solution was prepared at the concentration of 33 mg/mL. GNNQQQNY in solution (black line). In both spectra the water was not subtracted and the same baseline was applied using MatLab. Experimental conditions were laser power of 1.20 W, data accumulation time 2.5 s for 24 h.
The main amyloid features are often found in the amide III region of the Raman spectrum. The Raman spectrum in solution presents a broad peak at 1248 cm\(^{-1}\) with a shoulder at 1269 cm\(^{-1}\) and another broad peak at 1340 cm\(^{-1}\) with a shoulder at 1327 cm\(^{-1}\). The hydrogel presents a broad peak at 1240 cm\(^{-1}\) with two shoulders at 1256 cm\(^{-1}\) and a peak at 1290 cm\(^{-1}\); at higher wavenumbers there is a peak at 1330 cm\(^{-1}\) with two smaller peaks at 1338 cm\(^{-1}\) and 1353 cm\(^{-1}\), while both prefibrillar and fibrillar forms show peaks at 1212 cm\(^{-1}\) typical of in amyloid fibres and is characteristic for β-sheet strands. The peaks at 1290 cm\(^{-1}\) and 1248 cm\(^{-1}\) are assigned to α-helices and random coil respectively. The amide I region for the heptapeptide in solution presents a marker band at 1672 cm\(^{-1}\) typical of β-type structures. The amide I region of the hydrogel is dramatically different with an increase of the intensity of the signal at 1672 cm\(^{-1}\) and the appearance of a signal at 1654 cm\(^{-1}\), typical of α-helix.\(^{77,78}\) Comparison between the β-shift Raman intensity at 1212 cm\(^{-1}\) and α-helix intensity at 1290 cm\(^{-1}\) is indicative of conversion between β-sheet and α-helix. The aromatic side chain of tyrosine is responsible for the Fermi double at ~831-851 cm\(^{-1}\); these two peaks known as Fermi resonance between the ring-breathing vibration and the overtone of an out-of-plane ring-bending. The relative intensity of these peaks is sensitive to the hydrogen bonding state or ionization state of the hydroxyl group of the tyrosine aromatic ring.\(^{79}\) In particular if the intensity ratio \(I_2/I_1\) (higher frequency/lower frequency) is greater than 1, the tyrosine is exposed to the solvent and act both as donor and acceptor of hydrogen bonding.\(^{79}\) Conversely, if the ratio is lower than 1 the tyrosine is buried, interlocked in hydrogen bonding with other part of the fibre but not with the solvent. The calculated ratio for GNNQQNY in solution is 1.7, whereas in the fibril form is 1.5; the decrease in the ratio suggests that part of the solvent-exposed tyrosine in the prefibrillar state became less accessible to the solvent in the fibril form. The tyrosine is also contributing to the band at 1620 cm\(^{-1}\) assigned to the ring vibration.\(^{80}\)
ROA spectra of amyloid-fibril are quite difficult to record because of the birifringence of the sample, therefore only the ROA spectrum of the prefibrillar solution was recoded (Figure 2.2). The ROA spectrum shows a peak at 1251 cm\(^{-1}\) and 1685 cm\(^{-1}\) assigned to β-turn and a small positive at 1646 cm\(^{-1}\) assigned to α-helix, the positive intense peak at 1316 cm\(^{-1}\) is assigned to PPII helix conformation.

Taking into account the information extracted by the Raman and ROA spectral assignments, it can be hypothesized that the prefibrillar state of the GNNQQNY is formed by the coexistence of different secondary structures with random coil and β-sheet being the main components with minor contribution from α-helix. By considering the ROA spectral analysis it seems that the PPII helix is the main contribution to the random coil folding of the peptide. In the fibril state, the α-helix component became more appreciable and the β-sheet secondary structure is much more ordered. X-ray and NMR studies have demonstrated that GNNQQNY is found in three distinct conformations within a fibril.\(^81\) X-ray and NMR studies have demonstrated that GNNQQNY is found in three distinct conformations within a fibril.\(^81\,82\) In particular, magic angle spinning-NMR (MAS-NMR) studies report the analysis of the torsion angles of the three different conformers, for one of the conformers dihedral angles correspond to both α-helix and extended β-sheet. Moreover, it was also observed that there was a high degree of order in the structure.\(^82\) Taking into account the Raman and ROA spectral assignments, the prefibrillar state of the GNNQQNY seems to be formed by the coexistence of different secondary structures with random coil and β-sheet being the main components with a minor contribution of α-helix. The ROA analysis seems to suggest that the PPII helix is the main contribution to the random coil folding of the peptide. In the fibril state the α-helix component became more appreciable and the β-sheet secondary structure is much more ordered. The results seem to be in line with what was observed in the solid state with MAS-NMR and X-Ray crystallography, and suggests that the PPII helix might play a key role in the fibrillation process. The role of
PPPII helix in the amyloidogenic process has already been proposed from the Raman and ROA investigation of the prefibrillar state of lysozyme. The ROA analysis demonstrate the in the intermediated prefibrillar state of lysozyme there are large ammount of PPII helix.

2.1.2 GNNQQNY: conclusion

Raman and ROA investigation of the prefibrillar GNNQQNY provided detailed information on the structural composition of the peptides. In particular, Raman investigation is in line with the typical marker bands reported for amyloid fibrillation and shows evidence of the coexistence in solution of multiple secondary structures, in particular, random coil and β-type structure. Intrestingly, ROA spectroscopy was able to identify the random coil secondary structure as PPII helix conformation. These data seem to suggest that the heptapeptided in its prefibrillar state contains large ammount of PPII strucutre. Unfortunately it was not possible to obtain an ROA spectra of the fibrillar state, however, Raman investigation successfulely diagnoses changes in the secondary structure occuring between the two different aggregation states.
Additional work on amyloid peptides was conducted in collaboration with Dr Jakub Ujma with the investigation of the peptides YTIAALLSPYS and FTIAALLSPFS via cold ion spectroscopy to obtain highly resolved, IR and UV spectra. The IR spectra recorded in the gas phase where then correlated with IR spectra in solution.

The protein transthyretin (TTR) is known to undergo amyloidosis process, with consequent accumulation of amyloids fiber in the peripheral nervous system and or autonomic nervous system resulting in conditions such as peripheral neuropathy, characterized by the loss on sensitivity to stimuli such as pain touch, heat diseases and kidney diseases to name some. YTIAALLSPYS peptide is a part of a TTR protein that is implicated in amyloidogenic process. A great deal of work has been done to study the kinetics and structure of the mature fibrils. Notably, fluorescence methods were used to investigate kinetics of the assembly process. Mature fibrils peptide TTR$_{105-115}$ have been previously studied via solid state NMR (SSNMR) revealing that the peptide strands are organized parallel to each other and in-register with the adjacent β-sheets in an antiparallel orientation. However, very little is known about the structure of the soluble prefibrillar aggregates. The prefibrillar state features a multiplicity of oligomeric states with short living nature that are quite difficult to isolate and study with classic solution or solid state techniques. Gas phase ion spectroscopy methods can address this problem by allowing mass separation prior spectroscopy characterization. In result, non-covalently bound, transient assemblies can be analyzed.

In the original experiment the wild type sequence (YTIAALLSPYS) was analyzed in gas phase, however the UV spectra of both monomer and clusters of WT were very broad and undefined because of the interference of the electronic transition of tyrosines. To overcome this problem the experiment was re-designed with the use of a mutated peptide YΔ F TTR$_{105-}$.
in which the tyrosines was replaces by phenylalanines. This substitution has been found to affect very mildly the IR and mass spectra, suggesting that aggregation mechanism was unaltered. However, the absence of the hydroxyl group on the aromatic rings allowed the collection of double resonance IR/UV spectra of the clusters in gas-phase.

The FTIR spectra in solution in the aggregating condition (Figure 2.4) shows typical amyloid features, in particular in the amide I shows two peaks shifted at 1626 cm\(^{-1}\) and 1695 cm\(^{-1}\) typically observed in amyloids. The FTIR analysis on the same sample was performed after seven days showing a significant change of the feature in the amide I region. The band at 1695 cm\(^{-1}\) was significantly reduced and the band at 1626 cm\(^{-1}\) became much broader with the formation of a broader shoulder between the spectral features at 1695 cm\(^{-1}\) and 1626 cm\(^{-1}\). The FTIR analysis was also performed on the peptide in solid state showed in Figure 2.4. Interestingly, the solid state FTIR spectrum presents spectral features from both the fresh and incubated solution with a peak at 1645 cm\(^{-1}\) and smaller peak at 1695 cm\(^{-1}\) with a shoulder in between the two main peaks.

The peptides were also studied in DMSO, also reported in Figure 2.5. The amide I region presents a very different shape compared to the experiment in water with a peak at 1664 cm\(^{-1}\) and 1688 cm\(^{-1}\) suggesting the coexistence of β-turn type structures and disordered structures.
Figure 2.4: FTIR spectra of FTIAALLSPFS. A. Aggregating condition (8.5 mM), freshly prepared solution (solid line) and after 7 days (solid line). B. Freeze dried solid as purchased. C. Sample in a solution of DMSO (8.5 mM).

These results were compared with the experiments performed by Dr Jakub Ujma in the gas phase, the technique used is beyond the scope of this thesis but information can be found in the literature.\textsuperscript{87-89} In particular, the spectral features in the amide I region in solution and in gas phase were compared in order to support the gas phase assignment as well as investigate the different shift for same IR band from solution to gas phase.
Figure 2.5: IR spectra of the monomer and trimer clusters of FTIAALLSPFS. The spectra were recorded by Dr Jakub Ujma using the “IR Gain” scheme. Filled area in the spectra of the [M+2H]^{2+} and [3M+2H]^{2+} reports the standard deviation calculated from several repeats. It has been reported in order to show the reproducibility of the β-sheet/α-helix signature bands.

It has been reported that in the gas phase some secondary structure signatures may be shifted to lower wavenumbers because of the absence of interaction with the solvent and possible interactions with protons. The gas phase of the FTIAALLSPFS have shown sharp Amide I showed 1656, 1669 and 1692 cm\(^{-1}\) which have been assigned to β-sheet. The IR spectra in the solid state and under aggregating conditions strongly support the presence of β-sheet rich aggregates. In particular, the spectrum measured in DMSO solution shows band at 1663 cm\(^{-1}\) assigned to disordered structures and β-stands at 1688 cm\(^{-1}\) which are very similar to the gas-phase amide I region of [3M+2H]_{2+}. The comparison between solid state and solution state seems to suggests that the prefibrillar state (with β-sheet/β-turn content, 1688-1692 cm\(^{-1}\)) can be observed even under non-aggregating conditions. Moreover, the DMSO environment seems to resemble the gas phase environment. This is in line with what observed in case of Gramicidin S previously. It was reported that in DMSO solution the amide I presented a peak at 1661 cm\(^{-1}\) and a peak at 1680 cm\(^{-1}\). In contrast, the gas phase amide I
region showed a peak at 1610 cm\(^{-1}\) and 1680 cm\(^{-1}\). The results also lead to suggest the band around 1690 cm\(^{-1}\) may be seen as a reproducible feature common to both DMSO and gas-phase IR spectra (DMSO) indicative of antiparallel \(\beta\)-sheet/\(\beta\)-turn arrangement.

2.2.1 Conclusion

The IR experiments on amyloid peptide FTIAALLSPFS supported the interpretation of the IR spectra of cold ions. This technique is increasingly gaining more attention from the scientific world thanks to the ability of isolating single species with different charges. Future work will be focused on the evaluating the possibility of creating non-specific aggregates from non-aggregating solutions. This process will be used to “catalyse” the aggregation in order to perform efficient screening of potentially toxic amyloid intermediates.
2.3 Two component amyloid EEFKWKFKEE with KKFEWFEKK

Self-aggregation of proteins is not always negative, indeed amyloid fibrils play a key role in a number of biological processes, they form the protective envelope on fish and insect eggs or they are components of the spider silk. As already discussed, the amyloids are formed by cross-β-sheet structures and they are endowed with robustness and tensile strength. Moreover, by changing the environments or the primary sequence it is possible to regulate the physicochemical properties of the material. The characteristics just described make amyloid fibre highly compatible with cell culture.

A two component self-assembled amyloid hydrogel system formed by two complementary building block; EEFKWKFKEE (p1) and KKFEWFEKK (p2) were investigated using Raman and ROA analyses. The peptides were synthetized based on the pattern “hphphp” that has been previously reported to support β-sheet folding. The peptides arrange into antiparallel β-sheet that self-assembles into fibrils with desirable properties for cell culture. The gel is transparent, self-healing and compatible with numerous buffers. Moreover, by introducing at the N-terminus of p1 the motif RGD it was possible to grow 3T3 mouse fibroblast on the hydrogel made of 5 mol % p1-RGD into p1 + p2 hydrogel.
Figure 2.6: a) to c) Scheme showing the arrangement of p1 (EEFKWKFKKE, pI = 4.7) and p2 (KKFEWFEKKE, pI = 9.7) into β-sheet structures. b) and c) representation of the pH-dependent self-assembled hydrogel-forming binary amyloid fibres from the unstructured subunits. (d) TEM image of p1 + p2 hydrogel samples (scale bar 100 nm).

The peptides are formed as TFA salts and once dissolved water the pH of the resulting solution was approximately of 4. During the course of the investigation it was observed that both peptides are soluble in water at low or high pH whilst insoluble at neutral pH. Despite the peptides have been purified twice using high pressure liquid chromatography (HPLC) the peptides presented intrinsic fluorescence and it was necessary the use of a quencher, in this case, it was used sodium iodide. The interference by the fluorescence did not affect greatly the Raman experiments; however, in absence of the quencher it was not possible to record the ROA spectrum of the peptides.
2.3.1 **Studies on KKFEWFEKK (p2)**

The Raman amide I region of p2 (Figure 2.7) is composed of a broad peak suggesting the absence of a preferred conformation, as previously reported for poly-L-lysine and poly-L-glutamic peptides.\textsuperscript{99}

ROA analysis (Figure 2.7) gives a better insight of the conformation of the peptide. The amide II region is formed by a sharp couplet centred at 1448 cm\(^{-1}\) negative at lower wavenumbers. The amide III region shows positive bands at 1316 cm\(^{-1}\) typical of PPII helix conformation and a couplet negative at 1268 cm\(^{-1}\) assigned to β-turn type structures and a small positive band 1352 cm\(^{-1}\) given by α-helix. The sharp nature of these ROA bands and also that of the ROA couplet at 1488 cm\(^{-1}\) indicates that these conformations are highly ordered, with limited conformational mobility. Previous studies have shown that mobile and dynamic structures lead to broad and lower intensity ROA bands while more highly ordered structures give rise to more intense and sharper ROA futures\textsuperscript{99, 100}. The results suggest that the solution of p2 is characterized by an unfolded conformation with limited mobility.
Table 2.3: Band assignments for Raman spectra and ROA of p2 (NaI). 99, 101, 102

<table>
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<th>Wavenumbers (cm⁻¹)</th>
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<td>1675</td>
<td>Poly-proline 2 helix</td>
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Figure 2.7: a) Raman spectrum b) ROA spectrum of p2 (NaI). The ROA spectrum shows sharp peaks in the Amide I and Amide III regions indicative of restricted order in the conformation. The peptide has an unfolded conformation with restricted mobility.

2.3.2 Studies on EEFKWKFKEE (p1)

It was found that p1 self-assemble into a weak hydrogel at low pH with low elasticity when stressed. It has been experimentally observed that basic pH prevents the self-assembled the peptide. Therefore, Raman and ROA analysis was performed at pH 11 (Figure 2.8). Band assignments are listed in Table 2.4. By comparing the spectra and the band assignment, it is apparent that the Raman of peptides p1 and p2 are very similar. In particular, the amide I bands are, in both cases broad, suggesting the absence of preferred secondary structure.

The ROA spectra show significant differences in the intensity and shaped of the main features. The amide III region presents a negative band at 1268 cm\(^{-1}\) assigned to β-turn, and a positive band at 1327 cm\(^{-1}\) assigned to PPII helix. The amide II region shows a low intensity couplet positive a lower wavenumbers at 1342/1345 cm\(^{-1}\). To better understand the structural
differences between the two different peptides as solutions in water. Figure 2.7 shows the ROA of both hydrogel building blocks, \textbf{p2} presents sharper and higher intensity bands in the amide III compared to \textbf{p1} which suggests a more ordered structure for \textbf{p2} compared to \textbf{p1}. This conclusion is supported by the difference observed in the ROA tryptophan bands for the two peptides. Raman spectra of both peptides present a very similar tryptophan band at 1553 cm\textsuperscript{-1}. However, spectral features typical for this amino acids are found in the ROA spectrum of \textbf{p2} but not for the \textbf{p1}. As the final ROA spectrum is the results of the difference in intensities between $I_R$ and $I_L$, opposite conformations cancel each other out. Taking this into consideration, it is apparent that \textbf{p2} has more restricted conformation mobility around the tryptophan compared to \textbf{p1}. However, it is important to take in account the possibility that the different pH influenced the experimental ROA. Harada \textit{et al.} have previously shown that the difference in the shift of the tryptophan in the Raman spectrum is related to the angles between the indolic ring and the $C_\beta$.\textsuperscript{103} In this case, calculation of the band shift suggests and orientation of the aromatic ring of roughly 100\degree angle with an error of +/- 10\degree. Barrone used the correlation between Raman and ROA bands of the tryptophan to determine the angles and the orientation of the indole ring.\textsuperscript{103,104} Positive ROA bands are indicative of a positive orientation of the ring, the opposite in case the band is negative. In the ROA spectra of the \textbf{p2}, even if with low intensity, it is possible to recognise a positive band at 1553 cm\textsuperscript{-1}, this is not the case for \textbf{p1}. However due to the nature of the peptides; it was not possible to test the single peptides at the same pH.
Figure 2.8: a) Raman spectrum b) ROA spectrum of p1 (NaI). The ROA spectrum broad peaks, which is indicative of conformational mobility of the conformation. 105

Table 2.4: Band assignments for Raman spectra of p1 in presence of the quenching fluorescence (NaI). 105

<table>
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<th>Wavenumbers (cm$^{-1}$)</th>
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</tbody>
</table>
Figure 2.9: Raman spectra of a) p1 (blue line) at pH 11 and b) p2 (black line) at pH 4 in water. However at different pH, the Raman spectrum results quite similar apart a slight difference in the intensity and shape of the Amide I region.
The ROA spectrum of p1 shows bands with lower intensity in the Amide I compared to the p2. The lower intensity, along with the absence of the Trp band at 1553 cm\(^{-1}\) for p1 is indicative of high mobility of the conformation for this peptide compared to p2.

2.3.3 Two component gel of EEFKWKFKKEE (p1) and KKFEWEFEKK (p2) at pH 7

Raman and ROA analyses were also performed on the two component gel a concentration of 4 mmol. Unfortunately, because of the birefringence induced by the fibers the ROA spectra was dominated by distortion and it had been decided not to present the spectra. However the Raman spectrum was successfully recorded and it is presented in Figure 2.11.

The amide I region presents an intense peak at 1644 cm\(^{-1}\) with a shoulder at 1670 cm\(^{-1}\) originating from β-sheet, in the amide III there is peak at 1246 cm\(^{-1}\) also arising from β sheet and a relatively high intensity peak at 1338 cm\(^{-1}\) alpha helical \(\psi/\phi\) angles with a shoulder at 1363 cm\(^{-1}\) originating from Trp. The hydrogel amide III region does not shows dramatic changes compared to the solutions spectra (Figure 2.11). By contrast the amide I region of the hydrogel shows an intense water contribution, which is to be expected in a hydrogel.
2.3.4 Conclusion

The results of the Raman and ROA analysis of the two components hydrogel strongly support the importance of the application of these spectroscopy techniques for amyloid fibrillation process. Raman analysis of \( p_1 \) at pH 11 and \( p_2 \) at pH 4 suggested that single the components are unstructured when in solution. However, ROA analysis suggests that \( p_1 \) adopts \( \beta \)-turn and PPII helix conformation, and \( p_2 \) \( \beta \)-turn and PPII helix with a small contribution of \( \alpha \)-helix conformation. Moreover, the tryptophan bands gave useful insight about the indolic ring \( C_\beta \) bond angle calculated to be about 100°. These results strongly correlate with the ROA analysis of the prefibrillar state of GNNQQNY and of lysozyme. \(^83\)
2.4 Conclusion and Future work

Amyloid fibres are long recognized as responsible for ‘amyloid diseases’, however it is now establish that small unfolded peptides trigger the fibrillation. Raman, X-ray crystallography and NMR, are routinely applied for the investigation of prefibrillar and fibrillary states. However, these techniques have some limitation due to their intrinsic nature. The analysis of amyloid prefibrillar state for peptides GNNQQNY, p1 and p2 seems to suggest that the PPII helix conformation is a communal feature in the solution state. It has been reported that ROA is able to distinguish between two types of ‘disorder’ classified based on the dynamic of the conformers. A ‘dynamic disorder’ characterized by a distribution of dihedral angles, which lead to a cluster of conformers and a ‘static disorder’ characteristic of loops turns. The PPII helix conformation has been proposed to be a common feature for the static disorders. The information collected with the ROA studies presented in this thesis do support this findings. Moreover, the IR investigation and cold ion gas phases analysis represent a novel approach to the study of the fibrillation process. Future work will be focused on the optimization of the experimental condition in order to obtained ROA spectra of the fibrillary state.
3. **Chapter 3: VA and VOA analysis of Aib-foldamers in solution**

3 Introduction to the chapter

This chapter comprises an investigation of a series of Aib-rich oligomers in solution in order to identify Raman and ROA marker bands for $3_{10}$-helix. Aib-foldamers are short hydrophobic molecules, for this reason they need to be studied in organic solvents. The preferred conformation was analysed in a solution of chloroform, a solvent with a low dielectric constant (4.81) and dipole moment 1.0D. This solvent is often used to mimic the centre of the membrane which presents a low polarity due to the fatty acids. However, this solvent is highly volatile and there is the potential for intermolecular aggregation at the concentration required for the experiments; for this reason the peptides were also tested in DMSO-$d_6$. This organic solvent has a low volatility, a dielectric constant of 46.7, with a dipole moment of 3.96D, and minimizes the intermolecular aggregation by acting as a hydrogen bonding acceptor. The DMSO-$d_6$ was chosen over the non-deuterated solvent, because the solvent bands of the deuterated solvent do not overlap with the amide I, II or III regions.

Although, much evidence has been reported in the literature that this solvent destabilizes secondary structures of proteins and peptides, NMR studies on peptide CbzAib$_3$LeuAib$_2$OMe in this solvent demonstrate that the $3_{10}$-helix conformation is retained. The experiments were performed using Raman spectroscopy, IR, ROA and where applicable VCD, in Figure 3.1 are listed the peptides studied.
Figure 3.1: Chemical structure of the achiral peptides (N$_3$Aib$_n$O$^t$Bu) and chiral peptides (CbzXAib$_n$O$^t$Bu).

### 3.1 Length studies: N$_3$Aib$_n$O$^t$Bu

Raman spectroscopy has been successfully used for the investigation of protein and peptide secondary structures. The most common structural motifs such as α-helix and β-sheet have been widely studied with this spectroscopic technique. However, less common secondary structures, among which is the 3$_{10}$-helix, have not been fully assigned yet. The length study outlined in this chapter aims to identify Raman marker bands for 3$_{10}$-helix, as well as to explore the stability of this conformation when the sequence of the peptides is progressively increased. The formation of a sizable hydrogen bonding network is necessary for stabilising the secondary structure, therefore a minimum number of residues is often required to ensure the stability of the folding pathway. However, it has been reported that Aib-oligomers present an incipient 3$_{10}$-helix starting from three residues, and it is also well established that the octamer is fully folded into a stable 3$_{10}$-helix conformation.$^{106, 107}$ The characterisation of secondary structures in vibrational spectroscopies is usually performed by analysing several vibrational modes, in particular, the amide I, amide II and amide III regions of a Raman spectrum. These vibrational
modes are the most informative in the cases of protein and peptide secondary structures, as they originate from the polypeptide chains, and independently from the side groups. However, for Aib-rich peptides, the amide III is difficult to interpret. For example, Figure 3.2 shows the stacked graph of the region between 900-1400 cm\(^{-1}\) of the Raman spectra of peptides 1 to 6, and in Figure 3.3 shows the amide I region. By comparing the two stacked graphs, it is clear that the changes in the amide III region are less informative than the changes in the amide I region, leaving the latter the more sensitive to the length variation and to conformational preferences for these peptides. For this reason, throughout this thesis, secondary structure characterisation has been performed by focusing on the amide I vibration modes.

A crystallographic investigation of peptides 1 to 6 has reported evidence of 3\(_{10}\)-helix hydrogen bonding pattern in the solid state, therefore it is believed that this peptide adopts the same conformation in organic solvents. Naturally occurring peptibols present both N- and C- termini functionalized, for this reason; the peptides here presented have been modified at the C- and N- termini. The nature of the moieties can affect the stability of the secondary structure. In particular, the t-Bu ester induces formation of the Schellman-like motif at the C-terminus of the peptides.\(^{108}\) NMR and solid state studies report that the longest chain tested, 6 (N\(_3\)Aib\(_8\)O\(_t\)Bu) adopts a stable 3\(_{10}\)-helix conformation; conversely, peptide 1 (N\(_2\)Aib\(_2\)O\(_t\)Bu) is too short to form a helical structure.\(^{106, 107}\)
Figure 3.2: Stacked graph of Raman spectra of N$_3$Aib$_n$O$^t$Bu from 900 cm$^{-1}$ to 1400 cm$^{-1}$. Conditions: peptides concentration 121 mg/mL in CHCl$_3$. Data collection time varies as appropriate from 1 to 4 h depending upon the fluorescence of the sample. Laser power for all the experiments: 1.2W. Peptides of different length are shown in different colours; each analyte is labelled on the side. The two vertical black lines indicate bands from the organic solvent.
Figure 3.3: Stacked graph of the Raman amide I region of peptides $\text{N}_3\text{Aib}_n\text{O}^\text{Bu}$ with the deconvoluted bands (fine green line). Conditions: peptide concentrations 121 mg/mL in CHCl$_3$. Data collection time varies as appropriate from 1 to 4 h depending upon the fluorescence of the sample. Laser power for all the experiments: 1.2W. Peptides of different length are shown in different colours; each analyte is labelled on the side. Experimental Raman spectra for each peptide are shown in different colours. The dotted vertical black line indicates the wavenumber shift at 1668 cm$^{-1}$, assigned to $\beta$-helix conformation.

The amide I region for these peptides is shown in Figure 3.3. It is apparent that the shapes of the bands vary with the increase of the length of the sequence. The presence of multiple bands suggests the co-existence of multiple conformations; second derivative and deconvolution analyses have been used for qualitative and quantitative analysis of the complex bands. Mathematical resolution-enhancement of the different components reveals the most accurate wavenumber shift, as well as the relative intensity of the bands, which directly relates to the relative stability of the conformations in solution. The deconvoluted bands are shown in Figure 3.3 as a fine green line. In Figure 3.4 deconvolution and second derivative analyses for each...
peptide are shown. Table 3.1 summarizes all deconvolution analysis for the peptides studied. The deconvolution has been performed using Origin Pro, the same baseline with 8 anchor points has been applied to all Raman spectra for the peptides and the hidden peaks were identified second derivative. The fitting of the hidden peaks was achieved using the Gaussian setting. The dimer N₃Aib₂O'Bu (Figure 3.3 black line) presents in the amide I a peak at 1682 cm⁻¹, even after deconvolution appears to be generated by one conformation. This band has been assigned to an unstructured conformational state. The N₃Aib₈O'Bu (orange line) shows an amide I band at 1668 cm⁻¹. Deconvolution and the second derivative confirmed the contribution of only one conformation to the amide I band. NMR and X-ray studies on the octamer have reported that this peptide adopts a fully 3₁₀-helix conformation, therefore assigned to the band at 1668 cm⁻¹.

Having established marker bands for 3₁₀-helix and unstructured conformations characteristic of the longest and shortest Aib-foldamers, it was possible to perform the analysis of intermediate chain lengths. The spectral features for these peptides are evidently composed of multiple bands, indicating the co-existence of multiple conformational states.

The tetramer, N₃Aib₄O'Bu (green line), is long enough to show a partial 3₁₀-helix folding; however, it is not fully folded due to the lack of sizable hydrogen bonding. The amide I band profile reveals a shoulder at 1667 cm⁻¹ on the main band at 1688 cm⁻¹, with a relative percentage area of 40% and 60% respectively. Based on the assignment of the dimer and octamer amide I bands, it is clear that the peptide is mainly in an unstructured conformation. The N₃Aib₅O'Bu amide I region (brown line) shows a broader band, which after deconvolution is found to be formed by the overlapping of three components: a peak at 1667 cm⁻¹ (27%), the main band at 1683 cm⁻¹ (59%), and a minor contribution from a band at 1692 cm⁻¹ (14%). The smaller component is likely to have arisen from the ‘fray’ of the terminal amino acids not interlocked.
in the hydrogen bonding network. Although the peptide is partially folded, as was the case for the shorter tetramer, it is mainly in its unstructured conformation. The N₃Aib₆O'Bu amide I band (pink line) is formed by a main peak at 1677 cm⁻¹ and a shoulder at 1662 cm⁻¹; deconvolution reveals relative intensity areas of 73% and 27% respectively. This peptide is still not long enough to show a significant 3₁₀-helix nature. The N₃Aib₇O'Bu (blue line) presents similar wavenumbers at 1666 cm⁻¹ and 1676 cm⁻¹, but the relative intensity areas of these two bands are significantly different to those calculated for the hexamer, with the main band at 1666 cm⁻¹ contributing 64% to the amide I vibrational mode, and 1676 cm⁻¹ contributing only 36%. This peptide is only one residue longer compared to the hexamer; however the stability of the 3₁₀-helix conformation is significantly increased.
Figure 3.4: Amide I region band deconvolution (top of each pair) and second derivative (bottom of each pair) for peptides N₃AibₓOᵦBu. Deconvolution was performed using Origin Pro 9. In black is reported the experimental data and in green the calculated components of secondary structure and in red the cumulative calculated peak (top spectra). Underneath each experimental Raman is reported the second derivative.
Table 3.1: Amide I frequencies (cm\(^{-1}\)) and percentage areas of the Raman spectra.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>amide I bands</th>
<th>% Area</th>
<th>3(_{10})-helix</th>
<th>unstructured</th>
<th>3(_{10})-helix</th>
<th>unstructured</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 N(_3)Aib(_2)OtBu</td>
<td>n/a 1681</td>
<td>n/a 100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 N(_3)Aib(_4)OtBu</td>
<td>1667 1688</td>
<td>40 60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 N(_3)Aib(_5)OtBu</td>
<td>1666 1682/1692</td>
<td>27 59/14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 N(_3)Aib(_6)OtBu</td>
<td>1661 1679</td>
<td>27 73</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 N(_3)Aib(_7)OtBu</td>
<td>1666 1680</td>
<td>64 36</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 N(_3)Aib(_8)OtBu</td>
<td>1668 n/a</td>
<td>100 n/a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In order to confirm the assignment of the Raman bands, peptides 1 to 6 were also analysed via IR spectroscopy. It has been reported that peptides forming stable 3\(_{10}\)-helices show a peak in the amide I spectrum at 1666-1662 cm\(^{-1}\).\(^{109, 110}\) Using second derivatives and deconvolution was identified a small contribution to the band at 1681-1678 cm\(^{-1}\) and also a contribution at 1646-1644 cm\(^{-1}\) when the structure adopts a \(\beta\)-ribbon conformation.\(^{109, 110}\)

The profiles of the IR spectra (Figure 3.5) closely resemble the profiles of the Amide I region of the Raman spectra. As was the case for Raman analysis, the amide I region of the IR spectra changes with the increase of the peptide length. The main bands and of the amide I band are reported in Table 3.2. In Figure 3.6 the second derivative and the deconvolution profile for each analyte are shown. The IR analysis for these series of monodisperse Aib-oligomers are in good agreement with the literature assignment for the 3\(_{10}\)-helix conformation\(^{109, 110}\), and the results strongly support the Raman assignment already outlined.
Table 3.2: FTIR amide I frequencies (cm\(^{-1}\)) and percentage areas for FTIR spectra.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>amide I bands</th>
<th>% Area</th>
<th>% Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(3_{10}) helix</td>
<td>unstructured</td>
<td>(3_{10}) helix</td>
</tr>
<tr>
<td>1 N(_3)Aib(_2)OtBu</td>
<td>1667 1617/1642</td>
<td>54 11/34</td>
<td></td>
</tr>
<tr>
<td>2 N(_3)Aib(_4)OtBu</td>
<td>1659 1674/1687</td>
<td>18 72/9</td>
<td></td>
</tr>
<tr>
<td>3 N(_3)Aib(_5)OtBu</td>
<td>1666 1634/1681</td>
<td>83 1/16</td>
<td></td>
</tr>
<tr>
<td>4 N(_3)Aib(_6)OtBu</td>
<td>1664 1646/1651/1678</td>
<td>12 31/6/52</td>
<td></td>
</tr>
<tr>
<td>5 N(_3)Aib(_7)OtBu</td>
<td>1668 1654</td>
<td>48 52</td>
<td></td>
</tr>
<tr>
<td>6 N(_3)Aib(_8)OtBu</td>
<td>1669 1651/1683</td>
<td>85 14/1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.5: Stacked graph of the FTIR amide I region of peptides $N_3\text{Aib}_nO^t\text{Bu}$ (colour coded) with the deconvolution of the bands (fine grey line). Conditions: peptides concentration 121 mg/mL in CHCl$_3$. Experimental Raman for each peptide is shown in different colours; each analyte is labelled on the side. In pale grey is reported the calculated hidden peak. The dotted vertical black line indicates wavenumber shift of 1668 cm$^{-1}$, assigned to $3_{10}$-helix conformation.
Figure 3.6: FTIR Amide I region deconvolution and second derivative for peptides $N_3\text{Aib}_nO'\text{Bu}$. Deconvolution was performed using Origin Pro 9. In black is reported the experimental data and in green the calculated components of secondary structure and in red the cumulative calculated peak (top spectra). Underneath each experimental Raman the second derivative is reported.

3.1.1 Conclusion

Overall, the investigation of the Amide I region of the Raman spectra for peptides 1 to 6 showed two main bands falling between 1662-1668 cm$^{-1}$ and 1679-1692 cm$^{-1}$. By correlating the experimental data with NMR and X-ray crystallographic analyses in the literature, the bands
between 1662 -1668 cm$^{-1}$ are assigned to $3_{10}$-helix conformation. The bands found at higher wavenumbers have been preliminarily assigned to an unstructured conformation. However, it has to be noted that, as discussed in Chapter 2 (section 2.2.3 and 2.5), in the case of Aib-foldamers there is a very restricted number of dihedral angles that can be explored because of the steric hindrance of the two methyl groups on the Ca. Therefore, even in their ‘unstructured conformation’, Aib-foldamers are believed to adopt characteristic spatial organization. Solid state analysis of N$_3$Aib$_n$O'Bu studied in this thesis, reports that these peptides adopt hydrogen bonding patterns of the $3_{10}$-helix in their crystal form. Conversely, in solution these peptides behave differently, and despite showing a partial $3_{10}$-helix nature, it is necessary to have a minimum length of 8 Aib residues to form a full $3_{10}$-helix conformation. It has been mentioned that repetition of three or more type III or III' β-turn dihedral angles leads to $3_{10}$-helices (refer to section 2.2.3), therefore it could be argued that the unstructured conformation has a type III β-turn nature. However, experimental data are insufficient at present to assign the bands in the region between 1672 and 1692 cm$^{-1}$ to turn-like structures.
3.2 DMSO-\textsubscript{d$_6$} influence on $\text{3}_{10}$Helix conformation

The preferred conformation of peptide 1 to 6d was also tested in DMSO-\textsubscript{d$_6$}. DMSO-\textsubscript{d$_6$} solution analysis was performed on the same library of achiral Aib-foldamers analysed in CHCl$_3$. However, due to the low solubility in this solvent only some of the peptides have been successfully analysed. In particular it was possible to record the spectra of the monomer, the dimer, the tetramer and the hexamer. Figure 3.7 presents the amide I region and Figure 3.8 shows the deconvolution and the second derivative of the amide I region.

As previously stated, $\text{N}_3\text{Aib}_2\text{O}^\text{tBu}$ is too short to have a secondary structure and the amide I band is broader, and shifted to a lower wavenumber compared to CHCl$_3$ possibly because of the formation of hydrogen bonding with the solvent.

The amide I region of $\text{N}_3\text{Aib}_4\text{O}^\text{tBu}$ (green line) presents two distinct peaks at 1662 cm$^{-1}$ and 1684 cm$^{-1}$; deconvolution analysis showed intensity areas of 40% and 60% respectively. The amide I region for the tetramer in DMSO-\textsubscript{d$_6$} is quite different in shape from that of the same peptide in chloroform. The band corresponding to the 3$_{10}$-helix conformation (1666 cm$^{-1}$), which is just a shoulder of the major band in chloroform, appears as a distinctive band in DMSO-\textsubscript{d$_6$} (Figure 3.7).

The $\text{N}_3\text{Aib}_6\text{O}^\text{tBu}$ (pink line) amide I band is significantly different from the one in chloroform, with the peak at 1668 cm$^{-1}$ being the major peak, whereas in CHCl$_3$ it was a small shoulder of the main peak at 1688 cm$^{-1}$, as observed in Figure 3.7. Deconvolution values for the same peptide in different solvents are remarkably different, with calculated intensity area of 90% and 10% (1668/1688 cm$^{-1}$) in DMSO-\textsubscript{d$_6$} and 30% and 70% (1668/1688 cm$^{-1}$) in CHCl$_3$. 

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Figure 3.7: a) Raman amide I region deconvolution and second derivative for peptides N₃Aib₆O'Bu. Conditions: peptides concentration 121 mg/mL in DMSO-d₆. Experimental Raman for each peptide is shown in different colours; each analyte is labelled on the side. In pale grey is reported the calculated contribution to the secondary. The dotted vertical black line indicates wavenumber shift of 1668 cm⁻¹, assigned to 3₁₀-helix conformation. a) Stacked graph represents a comparison between the amide I region for the same N₃Aib₆O'Bu in DMSO-d₆ and CHCl₃ at the same experimental conditions.
Table 3.3: Comparison of amide I deconvoluted bands (cm\(^{-1}\)) of the Raman spectrum of \(N_3\text{Aib}_n\text{O}^\text{Bu}\) in DMSO-\(d_6\) and CHCl\(_3\) solutions.

<table>
<thead>
<tr>
<th>Foldamer</th>
<th>Amide 1, band 1</th>
<th>Amide 1, band 2</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n=2)</td>
<td>n/a</td>
<td>1681 (100 %)</td>
<td>CHCl(_3)</td>
</tr>
<tr>
<td>1 (n=2)</td>
<td>n/a</td>
<td>1674 (91%) 1684 (9%)</td>
<td>DMSO-(d_6)</td>
</tr>
<tr>
<td>2 (n=4)</td>
<td>1667 (40%)</td>
<td>1688 (60%)</td>
<td>CHCl(_3)</td>
</tr>
<tr>
<td>2 (n=4)</td>
<td>1662 (38%)</td>
<td>1685 (62%)</td>
<td>DMSO-(d_6)</td>
</tr>
<tr>
<td>3 (n=5)</td>
<td>1666 (27%)</td>
<td>1682/1692 (59%/14%)</td>
<td>CHCl(_3)</td>
</tr>
<tr>
<td>4 (n=6)</td>
<td>1661 (27%)</td>
<td>1679 (73%)</td>
<td>CHCl(_3)</td>
</tr>
<tr>
<td>4 (n=6)</td>
<td>1668 (90%)</td>
<td>1688 (10%)</td>
<td>DMSO-(d_6)</td>
</tr>
<tr>
<td>5 (n=7)</td>
<td>1666 (64%)</td>
<td>1680 (36%)</td>
<td>CHCl(_3)</td>
</tr>
<tr>
<td>6 (n=8)</td>
<td>1688 (100%)</td>
<td>n/a</td>
<td>CHCl(_3)</td>
</tr>
</tbody>
</table>
Figure 3.8: Raman amide I region deconvolution and second derivative for peptides N\textsubscript{3}Aib\textsubscript{0}O\textsubscript{t}Bu in DMSO-\textit{d}\textsubscript{6}. Deconvolution was performed using Origin Pro9. In black is reported the experimental data and in green the calculated components of secondary structure and in red the cumulative calculated peak (top spectra). Underneath each experimental Raman the second derivative is reported.

The stacked graph of the IR amide I region of peptides 1, 2, 4 and 5 is reported in Figure 3.9. As was the case for Raman spectra, the position of the IR amide I bands are influenced by the number of residues. The deconvolution and second derivative of peptides 1, 2, 4 and 5 (Figure 3.9) shows two main contributors to the secondary structure. The dimer (black line) presents a major peak at 1670 cm\textsuperscript{-1} with a shoulder at 1657 cm\textsuperscript{-1}. The tetramer (green line) presents two bands that are equally intense, one at 1658 cm\textsuperscript{-1} and one at 1680 cm\textsuperscript{-1}. The hexamer amide I band, unlike for the shorter peptides, results from three components: the main absorption band, which occurs at 1661 cm\textsuperscript{-1}, and two smaller components at 1664 cm\textsuperscript{-1} and 1681 cm\textsuperscript{-1}. The IR amide I bands are very similar in terms of wavenumber shift to the IR amide I bands in CHCl\textsubscript{3}. The IR amide I bands in DMSO-\textit{d}\textsubscript{6}, similarly to CHCl\textsubscript{3} support the Raman assignment already outlined.
Figure 3.9: a) Comparison between the FTIR amide I region for a series of N_3Aib_3O^tBu in DMSO-d_6 and CHCl_3. b) FTIR amide I region, deconvolution and second derivative for peptides N_3Aib_3O^tBu. Conditions: peptides concentration 121 mg/mL in DMSO-d_6. Experimental IR for each peptide is shown in different colours; each analyte is labelled on the side. In pale grey is reported the calculated contribution to the secondary. The dotted vertical black line indicates wavenumber shift of 1668 cm^{-1}, assigned to 3_{10}-helix conformation.

Table 3.4: FTIR amide I deconvolution values for peptides N_3Aib_3O^tBu.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>amide I bands</th>
<th>% Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3_{10} helix</td>
<td>unstructured</td>
</tr>
<tr>
<td>1 N_3Aib_2OtBu</td>
<td>1657</td>
<td>1671</td>
</tr>
<tr>
<td>2 N_3Aib_4OtBu</td>
<td>1658</td>
<td>1980</td>
</tr>
<tr>
<td>4 N_3Aib_6OtBu</td>
<td>1661/1664</td>
<td>1981</td>
</tr>
</tbody>
</table>
3.2.1 Conclusion

Overall, the investigation of Aib-rich foldamers in deuterated DMSO demonstrates that, unlike peptides whose primary sequence is mainly composed of coded amino acids, these peptides retain a stable helical structure. Raman analysis in both CHCl$_3$ and DMSO-$d_6$ have successfully allowed for the identification of a range in the amide I region where 3$_{10}$-helix marker bands are placed between 1662 and 1668 cm$^{-1}$. 
3.3 **Vibrational optical activity analysis**

Peptides 1 to 6 have a primary structure formed by achiral amino acids, which leads to a symmetric Ramachandran plot with right-(P) and left-(M) handed helices being equally energetic, with a barrier to inversion estimated at $\Delta G^{\ddagger} = 4.6 \text{ kJ mol}^{-1}$ per Aib residue. The conformational equilibrium between M\(\rightleftharpoons\)P in the case of Aib oligomers is hence finely balanced, dynamically oscillating between the two different states (Figure 3.10).

![Figure 3.10: Representation of the interconversion between left-handed (M) and right-handed (P) 3_{10}-helix. Covalent bonds are represented as solid line, hydrogen bonds are reported in red dotted line.](image)

It has been reported that it is possible to perturb this finely tuned equilibrium by adding a single chiral residue in the sequence of Aib-oligomers. The chiral residue perturbs the M\(\rightleftharpoons\)P conformational equilibrium, inducing a screw-sense preference. The location and the nature of the chiral residues have been investigated by both Toniolo’s and Clayden’s research groups.\(^{112}\) In particular, it has been observed that quaternary L-amino acids bearing two different
substituents at the $\alpha$-carbon (such as, L-$\alpha$-Methylvaline), support a right-handed helix when placed at the N-terminus of Aib oligomers. The opposite screw-sense preference has been found in the case of tertiary residues, such as L-Phe. Therefore, Cbz(L)PheAib$_4$OtBu is mainly left-handed in solution, although with little difference between the population of the two screw-sense conformers.$^{113}$

3.3.1 ROA spectroscopy of chiral foldamers

ROA spectroscopy is a powerful biophysical technique that allows the discrimination of enantiomers and conformers.$^{46, 114, 115}$ Since the 1990s, numerous biological molecules have been investigated by ROA, both experimentally and computationally.$^{83, 116-119}$ Despite the high number of proteins and peptides investigated, identification of the $3_{10}$-helical conformation using ROA is elusive mainly because the interpretation of the spectral features are quite challenging. This is because, despite the amide III region being the most informative part of a standard peptide’s ROA spectrum, these vibrations are missing in the case of quaternary amino acids like Aib, leaving the amide I region the most useful part of the ROA spectrum for investigating the conformational preference of Aib-rich foldamers.$^{120}$ Enantiomers of CbzPheAib$_4$OtBu and Cbz($\alpha$Me)ValAib$_4$OtBu (Figure 3.11) were analysed by Raman spectroscopy, ROA, IR and VCD in organic solvents. In both cases, the peptides present a C-terminus esterification with a t-Bu ester and at the N-terminus capping with a Cbz-group, which is believed to induce aggregation due to $\pi$-$\pi$ stacking. These studies allow for the correlation between spectral response and conformation, aiming to both identify chiral marker bands for $3_{10}$-helix in ROA spectra, as well as to investigate the sensitivity of this technique for distinguishing between $\alpha$-helix and $3_{10}$-helix.
3.3.1.1 CbzPheAib4OtBu enantiomers: Experimental and calculated ROA

Initial studies on peptides 7 and 8 in solution with CHCl₃ as organic solvent proved to be difficult because of the volatility of the solvent and the degradation of the concentrated solutions induced by the strong laser irradiation and the long data accumulation times required (between 24-28 h). For this reason, the ROA spectra for these two peptides have been recorded in DMSO-d₆.

The experimental Raman spectra of both D and L enantiomers of CbzPheAib₄OtBu are presented in Figure 3.12. The two different enantiomers generate essentially the same Raman spectra, because Raman spectroscopy is not sensitive to the stereochemistry of the analyte. Raman spectral features give rise to the bands in the corresponding ROA spectra, which provides an empirical approach to analysing the ROA spectra of these peptides. The Raman spectra of peptides 7 and 8 show a small peak at 1610 cm⁻¹. These peaks are both assigned to...
the aromatic vibrations arising from both the Cbz- group and the phenylalanine. The methyl side-chain vibrations are found at 1454 cm$^{-1}$, the amide I band, originating from C=O stretching vibrations, presents a broad peak with a maximum at 1680 cm$^{-1}$ accompanied by a small shoulder at 1668 cm$^{-1}$ for the right-handed enantiomer, and at 1678 cm$^{-1}$ with a shoulder at 1664 cm$^{-1}$ for the left-handed enantiomer. The deconvolution of the amide I profile reveals, for both enantiomers, a percentage fit of 60% and 40% respectively. Deconvolution of the amide I region identifies two bands contributing to the secondary structure, to assign them the band assignment discussed for peptides 1 to 6 can be taken into consideration. In particular, for the Raman investigation of the achiral peptides (1 to 6), it was concluded that the $3_{10}$-helix marker bands fall between 1661 and 1668 cm$^{-1}$, and the unstructured conformation is found between 1680 and 1690 cm$^{-1}$. Tacking this into consideration, the band at 1668 cm$^{-1}$ can be assigned to the $3_{10}$-helix conformation, and the band at 1680 cm$^{-1}$ to an unstructured conformation. The comparatively high amount of disorder observed for this peptide is attributable to its length (only 5 residues) along with the lack of a sizeable intramolecular H-bonding network.

By contrast, the ROA spectra of peptides 7 and 8 are approximately mirror images (Figure 3.12). The slight differences between the two spectra may be caused by a combination of factors, such as small differences in the concentration of the samples, or different levels of purity. The greatest difference between the ROA spectra of the two enantiomers is found in the amide II region. The ROA spectrum of Cbz(D)PheAib$_4$O'Bu presents an intense band at 1457 cm$^{-1}$, while the ROA spectrum of the enantiomer presents a couplet that is positive at lower wavenumber and is centred at 1450 cm$^{-1}$. 
An investigation of peptide Ac-L-(αMe)Nva-L-ATANP-L(αMe)Nva2-L-ATANP-L(αMe)Nva2OMe in H₂O and D₂O solutions by Toniolo and colleagues is the only reported experimental ROA data of peptides containing high amounts of quaternary amino acids. The amide I band in their work presented a small positive band at 1668 cm⁻¹ in both solvents and a band at 1465 cm⁻¹ assigned to CH₃ deformations in H₂O, shifted to 1439 cm⁻¹ in D₂O. In order to gain a detailed understanding of the ROA spectral features, a DFT computational analysis was performed on peptides 7 and 8 (Figure 3.13 and Figure 3.14). Calculations were performed by Professor Petr Bour (Prague) using QM/MM calculations at the BPW91/6-31G**/PCM level with the peptide conformation constrained to either standard 3₁₀-helix or PPII helix angles.
The calculation for Cbz(L)PheAibO'Bu (7) in a right-handed (P) 3_{10}-helical conformation can be used to assign the main spectral features. In the calculated ROA spectrum, the amide I couplet has a negative feature at ~1660 cm\(^{-1}\) and a very weak positive feature at a higher frequency (1720 cm\(^{-1}\)). However, the PPII helix model of the same peptide gave a positive feature at ~1680 cm\(^{-1}\). In contrast, the 3_{10}-helix model predicted a couplet in the amide I region that is negative a lower wavenumbers at ~1666 cm\(^{-1}\). Therefore, it appears that the computational modelling support the 3_{10}-helix for peptide 8. The opposite case was found for the enantiomer 8 which seems to agree better with the PPII helix calculation.

Figure 3.13: Experiment + BPW91/6-31G**/PCM calculation/comparison. Comparison between a) experimental (left) and calculated (right) Raman spectra.
Figure 3.14: Calculated Raman (bottom) and ROA (top) spectra for $3_{10}$-helix (blue) and PPII helix (red) conformations of peptide Cbz(L)PheAib$_n$OtBu.

### 3.1.1 Cbz($\alpha$Me)ValAib$_n$O$_t$Bu: experimental Raman and ROA

The Raman and ROA spectra of peptides 9 and 10 are shown in Figure 3.15. As previously stated, X-ray crystallography, NMR and ECD spectroscopies have been used to investigate the preferential helicity of a peptide that was N-capped with the non-natural residue (L)$\alpha$MeVal. By taking into consideration the different screw sense preference observed between tertiary and quaternary amino acids, the ROA spectrum should reflect the different screw-sense
preference of peptides containing unnatural residues. Peptides 9 and 10 were less susceptible to aggregation in CHCl₃, and their stability enabled their ROA spectra to be measured in CHCl₃.

The Raman spectra of peptides 9 and 10 in CHCl₃ are very similar, as can be observed in Figure 3.15. The amide I band is formed for both enantiomers as a broad peak, and the second derivative followed by band deconvolution reveals a band at 1680 cm⁻¹ and another band at 1664 cm⁻¹, with relative areas of 55:45%. ROA analysis of the two enantiomers in CHCl₃ gave a mirror image response, despite great distortions from the solvent. Compared to the ROA spectra of peptides 7 and 8 in DMSO-d₆, the signal/noise ratio is not as good. Despite the peptides being more stable in CHCl₃, the high concentration (121 mg/mL for these experiments) and the long time required for ROA analysis (between 24-48 h) are incompatible with the tendency of Aib-peptide to aggregate in CHCl₃, which causes thermal decomposition of the analyte. However, a few distinguishing bands can be identified in the amide II and amide I region. In particular, the right-handed enantiomer (Cbz(L)αMeValAib₄O'Bu) presents a couplet, negative/positive at 1434/1454 cm⁻¹, in the amide II region, arising from the side chain vibrations, and a negative/positive peak at 1660/1680 cm⁻¹. The amide I region of peptide 9 shows a positive peak at 1660 cm⁻¹ and a negative peak at 1680 cm⁻¹, the opposite case is for the enantiomer (10).
Figure 3.15: a) Raman spectra and b) ROA spectra of peptide 9 (black line) and 10 (red line) measures in CHCl₃ (dotted line) at a concentration of 121 mg/mL, laser power 1.2 W. Time for the accumulation of the spectra of about 24 h for peptide 9 and 6 h for peptide 10 (peptide 10 was thermally decomposed by the laser very quickly, maybe because of impurity). The solvent has not been subtracted from the spectra.

3.3.1.2 Conclusion

The ROA analysis of chiral peptides 7, 8, 9 and 10 successffully distinguishes between enantiomers with opposite screw-sense preferences. The experiments were performed in two different solvent systems in order to evaluate their influences on chiral amide I bands for Aib-rich peptides. A calculation analysis also support the helicity assignment. Before proposing a definitive chiral marker band for 3₁₀-helix more data will be acquired.
3.3.2 VCD analysis of chiral foldamers

The chiral peptides 7 to 10 discussed above were also studied with IR and VCD spectroscopies. These two vibrational spectroscopic techniques discussed in Chapter 1 are complementary and a combination of the two can emphasize different structural information.\textsuperscript{121, 122} VCD measures the differential absorption between left- and right-circularly polarized IR radiations from chiral molecules, while ROA measures the difference in their scattering of left- and right-circularly polarized light. The former has proven to be particularly sensitive for the discrimination between 3\textsubscript{10}-helix and \(\alpha\)-helix conformation by interpreting the amide I and amide II band shapes and frequencies of several peptides studied in both organic solvents and water.\textsuperscript{109, 120, 123} This discrimination between helical types is performed mainly by looking at the difference in intensity between amide I/amide II intensities.\textsuperscript{8-12} Although both helical structures present similar features with a negative couplet in the amide A region, a positive couplet in the amide I, and a negative band in the amide II region, the relative intensities are significantly different between the two helical conformations.\textsuperscript{109, 124} In the case of 3\textsubscript{10}-helix, the amide I band has a weaker or similar intensity to the amide II band, while the amide I band for \(\alpha\)-helix is more intense compared to the amide II band. Moreover, the amide II features for 3\textsubscript{10}-helix have been reported to be sharp and at a similar wavenumber shift compared to the IR absorption. In contrast, the amide II region for \(\alpha\)-helix is broad and shifted to a lower wavenumber compared to the corresponding absorbance band.

3.3.2.1 VCD analysis of CbzPheOH in solution

In order to unambiguously assign the VCD amide I band, the VCD spectra of the single amino acids, Cbz(L)PheOH and Cbz(D)PheOH, were also recorded, as is shown in Figure 3.16 during the first accumulation performed over a period of 30 min indicated as time 0 h (black line), the
measurement at the 10 h (red line) and the average overtime (blue line). The IR spectra for both amino acids present a peak at 1715 cm⁻¹ that is assigned to CO stretching of the t-Bu moiety. The VCD spectrum of Cbz(L)PheOH presents a positive peak at 1732 cm⁻¹ and a negative band at 1711 cm⁻¹; the enantiomer Cbz(D)PheOH presents the reverse band signs as expected. This VCD investigation of the single amino acids has confirmed that the amide I region does not present a band from the amino acid Phe and, therefore, any band observed in this region can be confidently assigned to the secondary structure of the peptide, in either aqueous or organic solvents.

Figure 3.16: Comparison of the IR (bottom spectra) and VCD (middle) spectra of Cbz(L)PheOH and Cbz(D)PheOH amino acids both at a concentration of approximately 40 mg/mL in DMSO-d₆. The measurements have been colour coded, in black is reported the measurements after 30 min. In red is reported the measurement over a period of 1 h recorded after 6 h from the beginning of the experiment. In blue is reported the average of the spectra over a total of 6 h. On top of the graph is reported the measured level of noise for the experiment.
3.3.2.2 VCD analysis of CbzPheAib4O^tBu

Figure 3.17 shows the IR spectra and VCD spectra of D- and L- forms of the Cbz-PheAib4O^tBu peptide in DMSO-\textit{d}_6 at a concentration of 50 mg/mL. The IR spectra for the two enantiomers are very similar.

The amide I region of the IR spectra for peptide 7 shows a main peak at 1674 cm\textsuperscript{-1} with a shoulder at 1621 cm\textsuperscript{-1}. Similarly the enantiomer presents a main peak at 1670 cm\textsuperscript{-1} with a shoulder at 1621 cm\textsuperscript{-1}. The VCD spectra of the left-handed peptide 7 are presented at 0h (black line), the measurement at the 18h after the experiments started (red line) and an average over time (blue line). The amide I region shows a positive band at 1682 cm\textsuperscript{-1} and a negative band at 1662 cm\textsuperscript{-1}, with Cbz(D)PheAib4O^tBu 8 showing the reverse. It is apparent that over time there is a decrease in the intensity of the amide I band in both the IR and VCD spectra. It has been suggested that these changes are caused by the aggregation of the peptide in solution, as was observed in the case of the Lys and Leu polymer (LKKL)\textsubscript{6}.\textsuperscript{125}

Interestingly, Cbz(D)PheAib4O^tBu presents a different behaviour in solution, and the intensity of the IR band for this enantiomer does not change over time, suggesting the aggregation state of the peptide remains constant during the experiment. In contrast, the VCD amide I bands invert sign over time, going from positive/negative at lower/higher wavenumber to negative/positive after 18 h. The experiment was repeated “recycling” the sample prepared for the ROA experiment (Figure 3.18). The VCD signal in this case did not flip over time; however, the intensity distribution between positive and negative lobes changed during the measurements, with the band at 1662 cm\textsuperscript{-1} reducing in intensity during the course of the experiment. Therefore, it is apparent that the VCD signal did not flip, but intensity distribution between positive and negative lobes changed during the measurements.
Figure 3.17: IR (bottom spectra) and VCD (middle) spectra of peptide 7 and 8 in DMSO-$d_6$; 50 mg/mL; 50 μm path-length; BaF$_2$ cell; 29°C. The measurements have been colour coded, in black is reported the measurements after 30 min. In red is reported the measurement over a period of 1 h recorded after 18 h from the beginning of the experiment. In blue is reported the average of the spectra over a total of 18 h. On top of the graph is reported the measured level of noise for the experiment.
Figure 3.18: IR (bottom spectra) and VCD (middle) spectra of Cbz(D)PheAib4OtBu peptide in DMSO-$d_6$; 50 mg/mL; 50 μm path length; BaF₂ cell; 29°C. Repeat from the same ROA sample (after ~9h the negative lobe was dominating). The measurements have been colour coded, in black and is reported the first two scan of 30 min. in red is reported the measurement from 30 to 60 min from the start of the experiment. In blue is reported the average of the spectra over a total of 6 h. On top of the graph is reported the measured level of noise for the experiment.

3.1.2 VCD analysis of CbzαMeValAib₄OtBu in solution

The preferred conformation of peptide 9 and 10 were also investigated with VCD and IR. The experiments were performed in solution of DMSO-$d_6$ in order to evaluate by VCD analysis the effect that DMSO-$d_6$ has on the secondary structure of Aib-foldamers. The VCD and IR spectra
are presented in Figure 3.19. It is apparent that the two enantiomers do not have a mirror image and nor do they have a comparable IR spectrum. There was insufficient sample of peptide 10 (Cbz(D)αMeValAib₄OtBu) to repeat these experiments. As soon as more compound will be synthetized the experiment will be repeated. The VCD and IR spectra of peptide 9 will also be repeated, however the IR amide I region shown a main peak at 1663 cm⁻¹ that can be assigned to 3₁₀-helix, with a shoulder at 1680 cm⁻¹ that can be assigned to unstructured conformation.¹⁰⁹

Figure 3.19: IR (bottom spectra) and VCD (middle) spectra of peptide 9 (black line) and 10 (red line). Samples were dissolved DMSO-<i>d</i>₆; 40mg/mL; 50μm pathlength; BaF₂ cell; 29°C. On top of the graph is reported the measured level of noise for the experiment.
3.4 Conclusion and Future work

The experiments outlined in this chapter allowed for $3_{10}$-helix investigation using vibration spectroscopy. The experiments were performed first on a set of achiral foldamers of different lengths in order to identify the range in the amide I region where signature bands for this secondary structure can be found. The experiments successfully identify this region being between 1661 and 1668 cm$^{-1}$. The analysis of peptide 7, 8, 9, and 10 reports for the first time the investigation of Aib-foldamers of different screw-sense preference using VOA spectroscopies. ROA and VCD successfully allowed for the detection of enantiomers with opposite screw-sense preference. Moreover, it was possible to perform calculation analysis that allowed for the assignment of the sign of the VOA spectra. The peptides analysed in this thesis were not long enough to form a stable $3_{10}$-helix, therefore it was not possible to unambiguously assign chiral marker bands for opposite screw-sense for the shorter foldamers. The synthesis of longer foldamers is quite complex, nevertheless, future work will be focused on the screening of a library of chiral Aib-foldamers with increased length to fully assign ROA marker bands for $3_{10}$-helix.
4. Membrane/Foldamers interactions studies

4. Introduction

The experiments outlined in this chapter aim to explore the application of a set of spectroscopy techniques to the investigation of the preferred conformation and orientation of Aib-rich foldamers within membranes. As discussed in Chapter 2, the antimicrobial activity of AMPs depends, among other factors, on the secondary structure adopted at the cell surface as well as their orientation/partitioning in the bilayer.\textsuperscript{64, 126} For example, the antimicrobial activity of alamethicin has been shown to follow a barrel-stave mechanism.\textsuperscript{127-129} In this model the peptides sit on the surface of the bilayer, in equilibrium with each other, until an appropriate concentration is reached inside the membrane and the peptides insert in bundles into the membrane, like the staves in a barrel. \textsuperscript{127-129} Therefore, understanding the peptides conformation and their orientation in the bilayer is important to understand the model of action of the antimicrobial peptides, like peptaibols, as well as of synthetic foldamers. The experiments performed during this thesis aim to address some of these matters by using IR, VCD, NR, ROA, ECD and LD. VCD experiments were carried out in Prague with the help of Dr Valery Andrushchenko, NR at the ISIS-SURF facility in collaboration with Dr Mario Campana and ECD and LD at the University of Warwick in collaboration with Prof. Alison Rodger.
4.1 Vibrational Circular Dichroism and Raman Optical Activity of 3\textsubscript{10}-helical foldamers

IR spectroscopy has been successfully used to study the secondary structure of proteins and peptides within the membrane.\textsuperscript{67} However, this spectroscopy technique is not sensitive to chirality and cannot give information about the screw-sense preference of the chirally controlled Aib Foldamers. VCD, on the other hand, could give detailed information about the handedness adopted by the peptide in the membrane; however, there are very few examples in the literature about the use of this technique to investigate protein/peptide interactions with membranes. In a recent publication, VCD was successfully used to investigate the preferred conformation of antimicrobial peptides melectin and antapin interacting with the membrane.\textsuperscript{130}

Foldamers can adopt different screw-sense preferences in solution, however, once interacting with the membrane, the conformation and screw-sense preference may change. VCD can potentially address this question and the use of this technique for studying Aib-foldamers/membrane interactions is described here. In particular, the preferred conformations of peptides 7, 8 and 11 in interacting with lipid bilayer were studied with VCD and IR. As was the case for the solution studies, the spectra interpretation will be focused on the amide I and amide II region of the VCD spectra.
Figure 4.1: Schematic representation of the VCD experiments. The peptide in solution can adopt different screw-sense preferences based on the N-terminus capping. Once interacting with the vesicles the conformation and screw sense preference may vary. VCD spectroscopy has been used to detect the preferred screw-sense within the membrane.

The peptides were tested in interaction with small unilamellar vesicles prepared with DOPC lipid (Δ9-cis, 1,2-dioleoyl-sn-glycero-3-phosphocholine, 220mg/mL) with a 4% molar ratio, with the final concentration of the peptide of about between 30 and 50 mg/mL. The experimental procedure is reported in detail in Chapter 5.

4.1.1 VCD and IR of small unilamellar vesicles of DOPC

The VCD and IR spectra of the suspension of liposomes in deuterated PBS buffer are reported in Figure 4.2 along with the noise level measured for the experiment. This measurement is
reported in order to highlight interference by experimental artefact. The spectra presented in the figure refer to the VCD measured with in the first blocks of scan (30 min, black line); the average of 1 hour measurement taken 7 h after the beginning of the experiment (red line) and the average of the total experimental (blue line). The amide I region of 7h average, shows an intense broad peak at 1725 cm$^{-1}$ assigned to the $\nu$ (C=O) vibration. This band has a corresponding band in the VCD spectrum with a negative peak at 1742 cm$^{-1}$.

Figure 4.2: IR (bottom spectra) and VCD (middle) spectra of blank DOPC SUV in PBS/D$_2$O; 220mg/mL; 50µm path length; BaF$_2$ cell; 29°C. The top graph is the measured noise of the experiment; the middle graph is the VCD spectra of the DOPC SUV. The graph shows the measurement after 30 min from the start of the experiment (0h, black hour), the measurement at 7.5 h from the beginning of the experiment (red line) and the average of the all measurements (blue line). The last graph is the IR spectra of the SUVs, in black is reported the measurements at 0h and in red is reported the measurement at 7.5h.
The DOPC SUV spectrum shows that there is a window between 1500 and 1700 cm\(^{-1}\) free from lipid vibrations, therefore the VCD could potentially be applied for the investigation of the Aib-foldamers within the membrane, as this allows the amide C=O stretches (amide I) to be observed.

### 4.1.2 Cbz-PheAib\(_4\)O\(_{iBu}\) enantiomers within the membrane

The VCD and IR spectra of peptides 7 and 8, in DMSO–d\(_6\) solution and bound to membrane, are presented in Figure 4.3. The vesicles were prepared using PBD/D\(_2\)O buffer at pH=7.4. The enantiomers are presented overlaid on the same graph to highlight differences in sign of the VCD spectra. The IR amide I regions for the two peptides in DMSO solution are very similar; differences in the intensity may be caused by the slight differences in the concentration of the sample. As discussed in Chapter 3 (section 3.3.2), in DMSO solution, the IR spectra of both peptides are dominated by a band at 1670 cm\(^{-1}\) as observed in Figure 4.3. The amide I region of the peptide in SUVs still presents a peak at 1670 cm\(^{-1}\), accompanied by two peaks at 20 cm\(^{-1}\) higher and 20 lower than the band at 1670 cm\(^{-1}\). The amide I profile suggests the presence of multiple conformations within the membrane.

On the contrary, VCD spectra of the two enantiomers in DMSO solution did showed an inversion and a mirror image response in both amide I and amide II regions. By contrast, the VCD was successfully used for the investigation of Aib-foldamers within membranes. The amide I region of the Cbz(D)PheAib\(_4\)O\(_{iBu}\) bound to membrane (red line) shows a series of overlapping bands. In particular, a negative feature at 1726 cm\(^{-1}\), a positive band at 1713 cm\(^{-1}\), a negative band at 1700 cm\(^{-1}\) and a positive peak at 1686 and 1688 cm\(^{-1}\) with a sharp couplet negative/positive at 1650/1639 cm\(^{-1}\). The VCD amide I region of the
Cbz(L)PheAib₄O'Bu bound to membrane (black line) shows a different profile with a positive feature at 1726 cm⁻¹, a negative band at 1713 cm⁻¹ a positive peak at 1695 and negative at 1681 and 1668 cm⁻¹ with a negative band at 1656 and 1644 cm⁻¹. Interestingly, the VCD bands corresponding to the lipid vibration absorption are also inverted between the two spectra. This is evidence of interaction between the peptides and the C=O of the fatty acids. The interpretation of this changed are difficult at this stage.

By taking into account the IR and VCD spectra in Figure 4.3, it can be suggested that the peptides adopt multiple dihedral angles values within the membrane. It is apparent, in particular for peptide 7, that the amide I region presents a “W” shaped pattern, reported to be indicative of a mixture of helix and coil structures or a mix of α-helix and β-sheet conformations in solution.³¹,³² The VCD spectra for the antimicrobial peptides melectin and anrapin in DOPC and DOPC/DMPC (2:1) liposomes presents a similar “W” shaped pattern within membranes, and it has been suggested in this case, to be indicative of the coexistence of α-helix and PPII helix conformations.³² Interestingly, the two enantiomers do not give a mirror image in the amide I region. The differences could be the result of different factors. It could be diagnostic of a different screw-sense equilibrium within the membrane for the enantiomers. It has already been discussed in Chapter 3 (section 3.3), that chiral foldamers adopt a screw-sense preference based on the chirality and type of N-terminal residue. However, once in interaction with lipids this equilibrium between left-handed and right-handed screw-senses might be influenced by the environment, as DOPC lipids themselves are chiral.

4.1.2.1 Conclusion

The interpretation of the data at this stage is quite challenging and more experiments need to be performed in order to better understand and interpret the data. Despite the many unanswered questions, the experiments performed are promising and demonstrate the successful application
of VCD as a probe for screw-sense preferences and the preferred conformation in membranes as well as in solution.

Figure 4.3: a) IR and VCD of 7 (black line) and b) 8 (red line) in DMSO, c) PBS/D$_2$O, bottom graph. Final concentration of the peptide was 40mg/mL; 50µm path length; BaF$_2$ cell; 29°C.
4.1.3 CbzAib\textsuperscript{-}FibTEG

Peptide 11 is achiral therefore VCD analysis in DMSO has not been presented in Chapter 3. However the comparison between the spectra for solution and the membrane bound peptide shows interesting results (Figure 4.4).

The IR amide I region for peptide 11 in solution (black line) presents a peak at 1662 cm\textsuperscript{-1} that undergoes a slight blue shift to 1659 cm\textsuperscript{-1} (red line) for the peptide within the membrane. As already discussed in Chapter 3, the IR band at 1662 cm\textsuperscript{-1} is diagnostic for 3\textsubscript{10}-helix.

![Figure 4.4: a) Measured noise, b) VCD and c) IR of 11 in DMSO-d\textsubscript{6} (black line, concentration 40 mg/mL) and in DOPC SUVs (red line) at a concentration of 36 mg/mL.](image)

By contrast, it is apparent in Figure 4.4, that the VCD spectra in solution and within the membrane are significantly different. This peptide is achiral and the VCD spectrum is
dominated by noise, however, the same peptide embedded to the SUVs produces a stronger VCD spectrum. The amide I region is formed by a positive peak at 1683 cm\(^{-1}\), an intense negative peak at 1665 cm\(^{-1}\) and a small negative at 1646 cm\(^{-1}\). The sign of the amide I band is similar to the sign for peptide 7 (Figure 4.3), which adopts a left-handed helix in both solution and SUVs. The VCD analysis of 11 seems to be in agreement with the spectra of 7 and 8, however, unlike these peptides, the amide I region does not show a defined “W” shaped pattern as the signals at 1665 cm\(^{-1}\) and 1646 cm\(^{-1}\) do not contribute equally as was the case for the shorter enantiomers.

4.1.3.1 Conclusion

Peptide 11 is longer than CbzPheAib\(_4\)O\(_\text{Bu}\) (8) discussed in the previous section, and NMR data within our research group have demonstrated that this peptide adopts a full 3\(_{10}\)-helix in solution.\(^{113}\) By taking into consideration the information extracted from Figure 4.4, it can be argued that the peptide is mostly in its helical conformation both in the membrane and in solution; as in both cases the IR amide I region presents a similar band indicative of 3\(_{10}\) helix conformation. The equilibrium between \(M\) and \(P\) in the membrane should be equal because there is no chiral control at the N-terminus. However, it appears that the interaction with the chiral DOPC lipids may shift the equilibrium towards the left-handed screw-sense helix. Alternatively, it could be argued that the chirality of the lipid is influencing the VCD of the peptide. The novelty of this data does not allow for a definitive interpretation and more spectra need to be collected in order to give a clear interpretation of the screw-sense preference adopted by the peptides interacting with membranes. Nevertheless, the experiment proves the relevance of VCD spectroscopy for studying the foldamer/membrane interaction compared to more common spectroscopy techniques such NMR, X-ray or ECD.
4.2 Cbz PheAib₄OtBu enantiomers in DOPC SUVs, Prague

The experiments on peptides 7 and 8 were recorded on ChiralRaman 2x in the laboratory of by Prof. Petr Bour (Czech Republic) because of technical problems with one of the components of the instrumentation in Manchester that needed to be replaced. The Prague instrument has the same SCP-ROA strategy and is also equipped with a 532 nm laser; therefore the data obtained with the two instruments will be comparable. The vesicles were prepared using PBS/D₂O buffer. The deuteration is expected to have an effect on the position of the bands in the ROA spectra because of the H-D exchange. The lipid/peptide molar ratio was kept the same as the VCD experiments with final concentration of the peptide of 40 mg/mL, and lipids concentration of about 130 mg/mL.
The amide I region of the DOPC SUVs presents a small positive band around 1655 cm\(^{-1}\). The amide II region presents a peak at 1445 cm\(^{-1}\).\(^{133}\)

In Figure 4.7 are shown the ROA spectra of DOPC SUVs in PBS/D\(_2\)O buffer, with and without the two enantiomers 7 and 8. The three main ROA bands observed for DOPC at 1264, 1445 and 1655 cm\(^{-1}\) show strong responses to addition of the foldamers. The band at 1655 cm\(^{-1}\), which originates from the \(cis\) C=C stretch, is positive for DOPC by itself but becomes strongly negative in presence of either foldamer. This indicates that both foldamers are interacting with
the double bond of the fatty acids tails. This observation suggests that both peptides are successfully incorporated into the bilayer. The band at 1264 cm\(^{-1}\), which also originates from \textit{cis} C=C vibrations, is also perturbed by the interaction with the foldamers, although this response is more complex as the left-handed foldamers shows loss of signal at that wavenumber while the right-handed foldamer shown sign inversion. The band at 1445 cm\(^{-1}\), arising from CH\(_2\) bend, shows a strong sign inversion for peptide 8 (right-handed foldamer) whereas for peptide 7 (left-handed foldamer) a weak positive signal is observed.

4.2.1 Conclusions

The interpretation of the ROA spectra of peptides 7 and 8 interacting with the membrane is not easy. Nevertheless, the data showed that ROA can give useful insight about the interaction of peptides with the lipids. We hypothesize that this is monitoring a difference of interaction between each foldamer and the chiral centre of the lipid. The ROA data suggest that the peptides are inserting in the bilayer as the C=C vibration undergoes to the same variation in the presence of either foldamer. Moreover, the data indicate that the right-handed foldamer (with the Cbz(D)Phe at the N-terminus) interacts preferentially with the lipid molecules as there are more significant changes in the ROA bands of the lipid.
4.3 **Linear Dichroism (LD)**

Linear dichroism (LD) measurements were carried out at the University of Warwick in collaboration with Professor Alison Rodger with the help of Dr Claire Broughton. As discussed in Chapter 1, LD gives information regarding the orientation of molecules by measuring the difference in absorption between a parallel and perpendicular polarized light (section 1.1). It has been observed that by measuring the LD of small peptides in interaction with vesicles, it is possible to understand the orientation of peptides bound to vesicles. The experiments outlined in this section aim to understand the orientation of a small library of Aib-foldamers. The peptides studied with this technique were selected based on HPTS and NMR studies previously published within my research group.\textsuperscript{134,135}

The LD signs for the most common secondary structures are summarized in Figure 4.8. Although the $3_{10}$-helix conformation have never been reported before, the electronic transition are similar to the one of the $\alpha$-helix, therefore they can be used as a reference.\textsuperscript{1} Great effort was put into optimizing the experimental procedure and numerous conditions and peptides were tested. The main difficulty encountered during the experiments was the reproducibility of the experiment, as liposomes behave in the cuvette flow in an unpredictable way, inducing aberrations of the spectra. Only a selection of the data will be presented here, chosen based on the reproducibility or relevance of the spectra. The peptide and the peptide/lipid ratio studied are listed in Table 4.1.
Figure 4.6: Schematic representation of the predicted LD sign of a) α-helix and b) β-sheet, when aligning on the surface or in insertion between the lipids.
Table 4.1: Lists of the peptides tested in the LD experiments. In the table are reported the sequence and the number coding of the peptides.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Peptide/Lipid ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>N$_3$Aib$_5$O$^t$Bu (2)</td>
<td>1/5</td>
</tr>
<tr>
<td>N$_3$Aib$_5$O$^t$Bu(2)</td>
<td>1/10</td>
</tr>
<tr>
<td>N$_3$Aib$_8$O$^t$Bu(4)</td>
<td>1/5</td>
</tr>
<tr>
<td>N$_3$Aib$_8$O$^t$Bu(4)</td>
<td>1/10</td>
</tr>
<tr>
<td>N$_3$Aib$_6$O$^t$Bu(6)</td>
<td>1/5</td>
</tr>
<tr>
<td>N$_3$Aib$_6$O$^t$Bu(6)</td>
<td>1/10</td>
</tr>
<tr>
<td>Cbz(L)PheValAib$_4$O$^t$Bu(7)</td>
<td>1/5</td>
</tr>
<tr>
<td>Cbz(L)PheValAib$_4$O$^t$Bu(7)</td>
<td>1/10</td>
</tr>
<tr>
<td>Cbz(D)PheValAib$_4$O$^t$Bu(8)</td>
<td>1/5</td>
</tr>
<tr>
<td>Cbz(D)PheValAib$_4$O$^t$Bu(8)</td>
<td>1/10</td>
</tr>
<tr>
<td>Cbz(L)$\alpha$MeValAib$_4$O$^t$Bu(9)</td>
<td>1/10</td>
</tr>
<tr>
<td>Cbz(L)$\alpha$MeValAib$_4$O$^t$Bu(9)</td>
<td>1/5</td>
</tr>
<tr>
<td>Cbz(D)$\alpha$MeValAib$_4$O$^t$Bu(10)</td>
<td>1/5</td>
</tr>
<tr>
<td>Cbz(D)$\alpha$MeValAib$_4$O$^t$Bu(10)</td>
<td>1/10</td>
</tr>
<tr>
<td>Cbz(L)$\alpha$MeValAib$_5$FibTEG(12)</td>
<td>1/10</td>
</tr>
<tr>
<td>Cbz(L)$\alpha$MeValAib$_5$FibTEG(12)</td>
<td>1/10</td>
</tr>
<tr>
<td>CbzAib$_9$FibTEG(11)</td>
<td>1/5</td>
</tr>
<tr>
<td>CbzAib$_9$FibTEG(11)</td>
<td>1/10</td>
</tr>
</tbody>
</table>

4.3.1 **Linear dichroism method development**

The experimental conditions were optimized to obtain the best quality information possible from the LD spectra. Different concentrations of the vesicles were tested in order to find the right balance between lipid concentration and the scattering of light. Based on previous data acquired in Prof. Alison Rodger’s research group, the optimized size of the vesicles was fixed.
to 100 nm; however, because of the size of the particles, comparable with the wavelength of light, they may scatter light, thereby affecting the measurements.\textsuperscript{1,6} It has been observed during the experiments that the higher the concentration of the lipids the greater the scattering of light. The lipid employed for the experiments was DOPC and the concentrations tested were: 1 mg/mL, 2 mg/mL, 5 mg/mL and 10 mg/mL. The lowest concentration tested gave the best results in terms of scattering; however, the final concentration of the peptide was too dilute and the LD signal was quite weak. The highest concentration had too much scattering and the experiments were unsuccessful. The concentration of lipid at 5 mg/mL was the best compromise between scattering of light and quality of the LD signal and it was used for all the experiments hereafter outlined.

As already mentioned, the orientation of the peptide within the membrane might change when a threshold is reached; for this reason two different peptide ratios were tested, at a 10\% molar ratio and a 5\% molar ratio. It was not advisable to use concentrations above or below these, because if the ratio is too low the peptides might not record a LD spectrum, and if too high they might disrupt the membrane.\textsuperscript{136-138} The optimization of the experiments was quite laborious and time consuming and more conditions and concentrations will be tested in the future.

The experiments were performed in all cases by adding the right amount of peptide dissolved in acetonitrile to the suspension of liposomes at the concentration of 5 mg/mL. Acetonitrile was selected because there are evidence that by adding small amount of CH\textsubscript{3}CN (4:6 ration and below) DOPC vesicles are not affected\textsuperscript{139}, and the cut off of this solvent is 190 nm, sufficiently low to allow the collection of the LD spectra. The peptides were added to the vesicles suspension and mixed by vortexing and then the LD spectrum was measured immediately after. It was observed that the mixing in this way was necessary in order to obtain an LD spectrum. However, the time necessary to collect one complete LD spectrum is of 25 min. The
experiments were performed by measuring first the LD spectrum of the oriented sample by rotating the cuvette, then for the non-oriented sample (not spinning). More details about the exact experimental procedure are outlined in Chapter 6.

4.3.2 LD Studies of N₃AibₙOtBu peptides

The LD experiments of peptide 2, 4 and 6 at a concentration of 5% molar ratio are reported in Figure 4.10, and at 10% molar ratio in Figure 4.9. The LD spectrum for foldamers in the vesicle suspension can be analysed for most samples down to 205 nm. Any absorption signal below this wavenumber is not reliable due to the light being scattered by the vesicles.

![LD spectra](image)

Figure 4.7: Experimental LD spectra measured a 10% molar ration of peptides N₃Aib₉OtBu (2), N₃Aib₆OtBu (4) and N₃Aib₈OtBu (6) in vesicles in PBS buffer (lipid concentration= 5mg/mL, pH= 7.4).
Figure 4.10: Experimental LD spectra measured a 5% molar ratio of peptides N₃Aib₆O'Bu (3) and N₃Aib₈O'Bu (4) in vesicles in PBS buffer (lipid concentration = 5mg/mL, pH= 7.4).

N₃Aib₈O'Bu at a 10% molar ratio presents a negative sign at 275 nm. The lower concentration presents a very weak band around the same wavelength, despite the experiment having been repeated several times with different liposome preparations it was not possible to improve the quality of the spectra. The signal at 245 nm is a distortion induced by the vesicles. The N₃Aib₆O'Bu LD spectrum for the peptide at 10% molar ratio also shows a negative band at 275 nm, however, because of the insufficient amount of the sample it was not possible to perform the experiment with lipid/peptide at 5% molar ratio. N₃Aib₈O'Bu at 10% molar ratio presents a very weak signal at about 215 nm, and at a 5% molar ratio presents a positive signal at about 206 nm, and a weak negative signal at about 274 nm.

To understand these data it has to be consider the functional groups in the azido group (-N₃) is a linear moiety as the three nitrogen atoms are involved in two double bonds with the angles
between \(\text{N=N=N}\) approximatively \(180^\circ\) and the \(\text{R-N-(NN)}\) angle is about \(120^\circ\) (Figure 4.11). Since the angle between the \(\text{R-N-(NN)}\) is fixed this group can only rotate around the helical axis, therefore by determining the orientation of the azido group we can evaluate the orientation of the peptide embedded in the liposomes.

In the literature it has been reported that alkyl azides in organic solvents present two electronic transitions at 215 nm and 285 nm, the latter being perpendicular to the plane of the molecule. Professor Petr Bour (Prague) performed a calculation on model \(\text{CH}_3\text{N}_3\) optimized at the B3LYP/6-311++G**/PCM level to determine the direction of the electronic transition dipole moment for the transitions at 215 nm and 285 nm. The latter was calculated with \(\mu_{285} = 0.1\) D with the direction perpendicular to the molecular plane. By using the information extracted from the literature and this calculation, the negative transition at about 275 nm observed in Figure 4.9 can be assigned to the azido electronic transition. The LD is calculated by subtracting the absorbance between the perpendicular and parallel polarized light, as reported in Equations 7 and 8:

\[
\text{LD} = A_\parallel - A_\perp = A_\parallel > 0 \quad (7)
\]

\[
\text{LD} = A_\parallel - A_\perp = A_\perp < 0 \quad (8)
\]

The negative sign of the signal at 275 nm suggests that the perpendicularly polarized light is absorbed more than the parallel light. Based on the calculated direction of electronic transition vector and the restrain angle between R-N-(NN) the negative LD signed observed at 275 nm, indicates that the peptides are inserting approximately in a perpendicular orientation to the plane of the bilayer.
Figure 4.8: a) Calculated electronic transition and geometry for the CH₃N₃ molecule, and b) is a two-dimensional representation to show the rotation allowed around the R-N-(NN) bond. Beneath (c) is a representation of the possible orientation of the peptide responsible for the negative signal at 275 nm.

4.3.3 LD Studies of CbzPheAib₄OtBu enantiomers

The LD spectra were recorded for peptide 7 (Cbz(L)PheAib₄O⁴Bu) 8 (Cbz(D)PheAib₄O⁴Bu) and the racemic mixture at both a 5% peptide:lipid molar ratio (Figure 4.12) and at a 10% molar ratio (Figure 4.13). The vesicles scatter light therefore the spectra have an aberration at 200 nm and below. However, despite the interference due to scattering, the UV region for all peptides shows a strong negative band around 222 nm and a series of weak positive signals between 250 and 280 nm. The LD signal is dominated by the aromatic electronic transition; the intensity of the band at 222 nm suggests the presence of exciton coupling between the aromatic rings on the Phe and the Cbz at the N-terminus. The exciton coupling is observed when two chromophores are asymmetrically oriented and close enough to have coupling of the excited states. This phenomenon has been used in ECD to experimentally determine the absolute
stereochemistry of molecules in solution as the exciton coupling gives a characteristic Cotton effect in the spectra.\textsuperscript{141-143}

Figure 4.9: Experimental LD spectra measured a 5\% molar ratio of peptides Cbz(L)Aib\textsubscript{4}O\textsubscript{2}Bu (7), CbzAib\textsubscript{4}O\textsubscript{2}Bu (8) and a racemic mixture of 7 and 8 in vesicles in PBS buffer (lipid concentration = 5 mg/mL, pH= 7.4).

Figure 4.10: Experimental LD spectra measured a 5\% molar ration of peptides and a racemic mixture of 7 and 8 in vesicles in PBS buffer (lipid concentration = 5 mg/mL, pH= 7.4).
The LD signal at 222 nm coming from the aromatic rings masks the n → π* transition of the amides, therefore the spectra gives no information regarding the backbone orientation. However, key information regarding the relative orientation of the Cbz and Phe groups can be extracted from the spectra. The aromatic electronic transitions are summarized in Figure 4.14. It is observed that there are two dipole moment vectors, one along the plane of the aromatic and one perpendicular to it (Figure 4.14). The transition at 222 nm is most likely arising from the aromatic transition that is usually found at 210 nm that, in this case, is shifted to higher wavelength because of the exciton coupling effect. It is apparent from the LD data that the sign at 222 nm is negative suggesting that the aromatic is aligning flat on the surface of the bilayer. This orientation results in a greater absorbance of the perpendicular light compared to the parallel light resolving in a negative sign according to equations 7 and 8.

Figure 4.11: Representation of the possible orientation of the peptide responsible for the negative signal at 222 nm theorized based on the aromatic dipole moment vector (red arrows). The transition at 260 nm is perpendicular to the plane of the aromatic; while the transition at 210 nm occurs on the plane of the aromatic ring. The negative signal at 222 nm is originated by the transition at 210 nm. However, it is shifted to higher wavelength because of the influence of the lipid environment. The negative sign of the signal suggests that named transition is oriented parallel to the surface of the bilayer.
4.3.4 ECD studies of CbzPheAib₄O'Bu enantiomers

The spectrometer used to record the LD spectra simultaneously records the ECD spectra for the same sample. The quality of the data has been previously investigated in Prof Alison Rodger’s research group, and it was demonstrated that the instrument can successfully measure both spectra in the same experimental session. The ECD spectra of Cbz(L)PheAib₄O'Bu in acetonitrile solution (black line) and in interaction with the lipid bilayer at ratio 5:1 (red line) were recorded (Figure 4.16). The ECD spectrum of a vesicle suspension can be analysed down to 205 nm, but any absorption signal below this wavenumber is not reliable due to the light being scattered by the vesicles. However, a transition at 222 nm is clearly observed. The enantiomers were also studied in a solution of methanol, which showed each enantiomer gave mirror image signals for the $\pi \rightarrow \pi^*$ and the $n \rightarrow \pi^*$ absorption bands (Figure 4.15). By comparing the information obtained in Figure 4.15 with the spectra in Figure 4.16, we can observe that the transition at 222 nm of the peptide in solution has the opposite sign in the bilayer.

Figure 4.12: ECD spectrum of both enantiomers of CbzPheAib₄O'Bu in methanol at concentration of 1mg/mL.
ECD spectra were recorded for the enantiomers and racemate of CbzPheAib₄O'Bu interacting with the vesicle membranes at all loadings (5% and 10%) (Figure 4.17). Cbz(L)PheAib₄O'Bu at concentration of 10% (black dotted line) molar ratio showed a negative peak at about 230 nm, with a $\Delta\varepsilon$ value of -4. The LD sign at 5% molar ratio (solid black line) is significantly different with a negative sign at 222 nm, and $\Delta\varepsilon$ value of -10. Cbz(D)PheAib₄O'Bu at concentration of 10% molar ratio (red dotted line) showed a positive peak at about 230 nm, with $\Delta\varepsilon$ of 7, whereas the LD sign at 5% molar (solid red line) presents a positive peak at 225 nm, and $\Delta\varepsilon$ of 11. The racemic mixtures do not have a strong ECD signal.
The ECD spectra, which were recorded at the same time as the LD spectra, were collected straight after adding the peptide dissolved in acetonitrile to the liposome suspension in buffer and vortexing for about 30 s. Therefore the question remains if the ECD signals and LD are from a mixture of peptides in solution and peptides embedded to the membrane.

4.3.4.1 Discussion

The ECD signal from solution to the membrane undergoes a dramatic change in the sign of the signal that suggests that there is a substantial change in the screw-sense preference of the peptides. However, the ECD signal is very sensitive to the mutual spatial separation and orientation or the aromatic rings and it has been found that often the orientation influence the final ECD signal greater than the actual structural rearrangement like it was the case for
Gramicidin. In particular, as already discussed in Chapter 1 (see section 1.1), during its insertion the antimicrobial peptide refolds from non-channel to channel-active form. The preliminary ECD investigation lead to the conclusion that major structural changes were occurring as the sign of the ECD signal between the forms was dramatically different. However, LD spectra of the two forms shared the same sign, therefore have a similar orientation proving that the ECD signal was influenced by a rearrangement of the relative orientation of the Trp residues rather than changes in orientation or conformations between the two forms. Taking this into consideration, it can be argued that the inversion of the ECD sign observed for CbzPheAib\textsubscript{9}O\textsubscript{Bu} enantiomers from solution to membrane is the result of the changes in the relative orientation of the aromatic groups rather than structural changes. By taking into consideration the VCD and ROA analysis discussed in section 4.1 and 4.2, VOA investigation did not show dramatic changes in the screw-sense preference of the CbzPheAib\textsubscript{9}O\textsubscript{Bu} enantiomers bound to DOPC SUVs as the ECD data suggests that the signal is indeed influenced by the relative orientation of the Cbz and Phe aromatic rings.

4.3.5 **LD Studies of CbzAib\textsubscript{9}FibTEG (11)**

The LD spectrum for peptide 11 at a 10% molar ratio in 5 mg/mL vesicles, both freshly prepared and measured after 14 h is reported in Figure 4.19. In the far UV there is a positive peak at 218 nm in both the fresh sample (solid line) and the old sample (dotted line). The old sample presents two negative peaks at 262 and 268 nm.

Based on the information described in Figure 4.14, the positive sign of the electronic transition at 218 nm suggests that the aromatic group is inserting perpendicular to the plane of the bilayer. This sharp nature of the signal at 218 nm suggests the presence of exciton coupling between
the Cbz protecting group at the N-termini, as was the case for peptides 7, 8, 9 and 10. This exciton coupling indicates that the peptide may aggregate once inserted in the vesicles. The experiments collected at two different time points do not show a significant change in the spectra; therefore the peptide may adopt the final orientation in a short window of time, within the 25 min necessary to record a LD spectrum.

Figure 4.15: Experimental LD spectra measured a 10% molar ratio of peptides CbzValAib3FibTEG (11) in vesicles (concentration 5mg/mL, pH=7.4), freshly prepared (solid line) and after 14 h (dotted line).

4.3.6 LD Studies of CbzαMeValAib3O\textsubscript{t}Bu enantiomers

The LD spectra of peptide 9 (Cbz(L)αMeValAib3O\textsubscript{t}Bu) and 10 (Cbz(D)αMeVal Aib3O\textsubscript{t}Bu) at a 5% molar ratio and 10% molar ratio for peptide 9 are shown in Figure 4.18. The spectra of 9 and 10 overlap completely at 5%, suggesting that both enantiomers adopt the same orientation and they have a similar effect on the behaviour of the SUVs.

The LD of peptide 9 (black line) and 10 (red dotted line) shows aberration caused by the scattering of the vesicles. The aberration has been found comparable throughout the experiment performed on peptides 9 and 10. At this stage, it is difficult to give an explanation for this
effect. The LD spectra present (for both enantiomers at 5% molar ration) a sharp negative signal at 218 nm, a small positive at 208 nm and a series a small positive between 250 and 280 nm. The shape of the band -/+ at 218/208 nm and the positive vibrionic signal between 250 and 289 nm the presence of exciton coupling can be elucidated. Peptide 9 and 10 have no aromatic residue in the sequence, therefore the signal observed is mainly influenced by the Cbz protecting group. In order to give exciton coupling a chromophore needs to be asymmetrically oriented and proximal to another chromophore. Therefore the presence of exciton coupling suggests that the foldamers are aggregating in the bilayer of the liposomes. Since the aromatic group is mainly responsible for the intense signal at 218 and the small positive between 250 and 280 nm, as was the case for peptide 7 and 8, we can extrapolate information regarding the orientation of the Cbz group. The information extracted from Figure 4.14 suggests that the Cbz groups orient parallel to the surface of the liposomes. The electronic transition at 208 nm could contain a contribution from the $\pi \rightarrow \pi^*$ transitions, however that transition alone is not sufficient to confirm the final orientation of the peptide.
Figure 4.16: Experimental LD spectra measured a 5% molar ratio of peptides Cbz(L)αMeValAib₄O'Bu (9), Cbz(D)αMeValAib₄O'Bu (10) and Cbz(L)αMeValAib₄O'Bu at 10% molar ratio in vesicles (concentration 5mg/mL, pH=7.4).

Cbz(L)αMeValAib₄O'Bu at 10% molar ratio has a very similar LD spectrum, with a intense negative signal at 218 nm, a positive at 208 nm, a small positive at 225 nm and a series of small positives between 250 and 280 nm. The aromatics transition, as it was the case for the peptides 7 and 8 (Figure 4.14) seems to suggest that the aromatic rings are aligning parallel to the surface of the lipids. The amides transition at 208 nm ($\pi\rightarrow\pi'$) and 225 nm ($n\rightarrow\pi'$) are positive indicating that the peptide is aligning on the surface of the vesicles (Figure 4.8). However, the very low intensity signals and presence of the exciton coupling might affect the signal coming from the peptides transition. The time spent optimising the experimental conditions did not allow for more experiments to be performed. In future kinetic experiments may be performed to understand if the LD data informs only on the initial orientation of the peptide.
4.3.7 LD Studies of CbzαMeValAib5FibTEG (12)

The LD spectrum for peptide 12 at a 10% molar ratio prepared fresh and measured at 2 different time points are reported in Figure 4.20. In the far UV region there is a negative peak at 225 nm at all the concentrations studied. The aromatic vibration transitions are represented in this case by small negative signals between 250 and 270. There are some differences in the shape of the vibrionic transition but overall the spectra are consistent from samples measured at different times.

The negative sign of the vibrionic transition has been assigned to aromatic groups inserting perpendicular to the plane of the bilayer. There was no exciton coupling, which suggests two possible explanations, the peptides are not aggregating or the chromophores are oriented symmetrically. The experiments collected at different time points do not show a significant change; therefore the peptide orients and adopts the final orientation in a short window of time (within the 25 min necessary to collect the LD spectrum).

![Figure 4.17: Experimental LD spectra measured a 5% molar ratio of peptides CbzαMeValAib5FibTEG (12), freshly prepared (solid black line) and at 10 % molar ratio (dotted line) and after 14 h (blue line).](image-url)
4.4.8 Conclusions on LD studies of Aib foldamers interacting with bilayers

The LD experiments discussed here give key information regarding the orientation of the peptides within the membrane. The set of experiments performed on the achiral foldamers suggest that the peptides are inserting in the membrane rather than aligning on the surface. Reproducibility for these experiments was difficult to obtain and it was observed that the freshness of the sample greatly affected the outcome of the experiment. Overall, the LD signal coming from the N3Aib6O'Bu was weaker than the peptides containing aromatic residues (Cbz-protecting group, Phe) but interpretation of the band at ~275 nm for the longer foldamers (n=6,8) suggests the foldamers are inserting with the helical axis perpendicular to the bilayer plane. The peptides with a Phe or Cbz incorporated in the sequence gave more intense LD signals and much more reproducible data, thus suggesting that the aromatics support the peptaibols interaction with the liposomes as suggested for the naturally occurring peptaibols. Moreover, it is suggested that the peptides aggregate in the membrane, giving characteristic exciton coupling for each peptide. The sign of the exciton coupling revealed that the aromatic groups align parallel to the surface of the vesicles for the tetrameric foldamer 7, 8, 9 and 10. Conversely, the long peptide 11 modified at the C-terminus with a TEG protecting group inserts into the membrane and the aromatic group is perpendicular to the vesicle surface. The same orientation was found for the shorter 12 whose aromatic signal was very weak. The exciton coupling is observed if the chromophores are asymmetrically oriented; the lack of excitonic signal seems to suggest that, for this peptide, the relative orientation of the Cbz group is symmetrical.

The optimized experimental condition and the reproducibility of the data here presented demonstrate the potential of the LD as a tool for Aib-oligomers interacting with membranes.
4.4 Neutron Reflectometry (NR) experiments on foldamers 9, 11, 12

The interaction of peptides 9, 11, 12 with lipid bilayers and their effects on the membrane structure was investigated by neutron reflectometry on planar supported DMPC bilayers. Every sample was measured under three different water contrasts: D$_2$O, water contrast matched to silicon surface (CMSi) that supports the bilayer and H$_2$O. The DMPC bilayers were either fully hydrogenous or partially (tail) deuterated form in order to constrain the fitting routine and achieve additional sensitivity to the adsorbed lipid bilayer. All contrasts used in this experiment are listed in Table 4.2.

Table 4.2: The set of contrasts shown was used to determine the conformation of the lipid bilayer in absence and in presence of the Aib foldamers.

<table>
<thead>
<tr>
<th>Water Lipid</th>
<th>D$_2$O</th>
<th>CMSi</th>
<th>H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>d</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

4.4.1 Data analysis

The data analysis was performed using the standard optical matrix method where the interface is modelled by dividing it in a number of layers, each of them characterised by a certain thickness ($d$), scattering length density ($Nb$) and roughness ($\sigma$). The reflectivity profile is then calculated from the model and compared to the experimental data. The procedure is iterated to obtain a good fit to the data. Since the scattering length density of every component (or part
of them) is known and reported in Table 4.3, it is more convenient to break up the layer \( Nb \) using the following equation:

\[
Nb_{layer} = \sum_i Nb_i \Phi_i
\]

(31)

\( Nb_i \) and \( \Phi_i \) are the scattering length density and volume fraction of each component, respectively. The variation in \( Nb_{layer} \) with contrast change is then attributed to the presence of water (bilayer defects / hydration). The water volume fraction can be easily modelled using the hydration parameter (\( \Phi_{water} \)). As no water is expected to be found in the inner tail group region, the hydration parameter for this region was used to determine the total lipid coverage. This calculation can be easily performed for the bare lipid bilayer. In the presence of the peptaibol this calculation can be complicated by the possible presence of water associated with the Aib backbone. The calculated values are thus potentially an underestimation of the true lipid coverage; these values are reported nonetheless for comparison. The Nb values for all components are given in the table below.

Table 4.3. Nb values for all compounds used in the experiment. The change in peptaibol Nb because of H/D substitution was accounted for, this however does not affect the Nb of lipid head groups.

<table>
<thead>
<tr>
<th>Component</th>
<th>Nb (( \times 10^{-6} \text{ Å}^2 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicon</td>
<td>2.07</td>
</tr>
<tr>
<td>Silicon oxide</td>
<td>3.47</td>
</tr>
<tr>
<td>Head group</td>
<td>1.18</td>
</tr>
<tr>
<td>Tail group hydrogenous</td>
<td>-0.35</td>
</tr>
<tr>
<td>Tail group deuterated</td>
<td>6.2</td>
</tr>
<tr>
<td>Peptaibol</td>
<td>1.18</td>
</tr>
<tr>
<td>( \text{D}_2\text{O} )</td>
<td>6.35</td>
</tr>
<tr>
<td>CMSi water</td>
<td>2.07</td>
</tr>
<tr>
<td>( \text{H}_2\text{O} )</td>
<td>-0.56</td>
</tr>
</tbody>
</table>
Figure 4.18: Schematic representation of the scattering length density ($N_b$) profiles for DMPC bilayers deposited on a silicon surface. The cartoon above clearly sketches the different features observed in the $N_b$ profiles.

### 4.4.2 Data fitting and membrane thickness

In order to obtain a unique fit to the data, for every sample all 6 profiles were fitted simultaneously. In the fitting routine almost all parameters were maintained constant: the only parameter which was allowed to vary was the hydration parameter to account for defects in the lipid deposition. Even then, the head group and tail group hydration were coupled at all times.

In a typical neutron reflectivity experiment refer to the scattering length density profile $N_b$ (see section 1.6). The $N_b$ profiles for all contrasts for the DMPC bilayer are shown in Figure schematically in Figure 4.20, together with a schematisation of the experimental chamber.
interface to highlight the different regions of the Nb profile. Generally, the Nb is reported in the y axis, while the x axis represents the distance perpendicular to the interface expressed in Angstrom. The zero position on the x-axis is arbitrary; for this set of experiments the zero was placed at the oxide/head group boundary. The standard procedure for the data analysis is to use the minimum number of layers to describe the interfacial region. This result is in line with the hydrophobic nature of the peptides. Overall, the data fitting confirmed 100 % partitioning in the lipid bilayer for all the foldamers.

Finally, all data were fitted to a four layer model (Figure 4.21), representing four distinct regions of the interface: silicon oxide, inner head group region, tail group region and outer head group region.

The silicon oxide layer, at the silicon surface, is the first feature that appears in the Nb profile corresponding to the first peak on the left hand side of the plots. The oxide layer was found to be 14±2 Å, consistent with a typical native oxide layer, containing 20±5 % water. An interlayer roughness of 4 Å was used at the silicon/silicon oxide and oxide/head group boundaries. The second peak, only clearly visible when deuterated DMPC was used, represents the inner head-group region. The layer is significantly hydrated and the Nb changes quite dramatically with different contrasts. The third region represents the tail group and, in case of 100% lipid coverage of the silicon surface, it should be completely free from water molecules. (Table 4.4)
Table 4.4. Lipid coverage for the systems studied

<table>
<thead>
<tr>
<th>Peptide</th>
<th>h-DMPC</th>
<th>d-DMPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare lipid</td>
<td>90%</td>
<td>82%</td>
</tr>
<tr>
<td>Cbz-(L)αMeVal-Aib₄-FibTEG (12)</td>
<td>95.00%</td>
<td>92.00%</td>
</tr>
<tr>
<td>Cbz-Aib₆-FibTEG (11)</td>
<td>87%</td>
<td>88%</td>
</tr>
<tr>
<td>Cbz-αMeVal-Aib₄-OtBu (9)</td>
<td>97%</td>
<td>95%</td>
</tr>
</tbody>
</table>

Figure 4.19: Neutron reflectivity profile of \( h \)-DMPC and \( d \)-DMPC with peptides 8 (red line), 11 (blue line) and 12 (pink line).
Finally, the last layer represents the outer head group region. Since the bilayer is deposited from an SUV suspension, it is expected to be symmetrically distributed, although some differences may arise after the bilayer is deposited onto the silicon surface because of steric hindrance. Nevertheless, the symmetry parameter was considered constant throughout the data fitting procedure, as was the hydration parameter of the head groups of the inner and outer head group regions. The layer thickness for the two head groups regions was also kept constant where applicable. The only exception was the data fitting process for Cbz(L)αMeValAib₄OtBu for which some differences between the inner and outer head group regions were found, with the outer head group region being 2 Å thicker than the inner one. The fitting parameters for all reflectivity profiles are listed in Table 4.5.

Table 4.5: Fitting parameters for all reflectivity profiles reported in this study. The volume fraction of water is only reported for the h-lipids, the values scale linearly for the d-lipids.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Layer d/Å</th>
<th>σ/Å</th>
<th>Φᵳ/%</th>
<th>Φᵳ/Å</th>
<th>Φᵳ/Å</th>
<th>Φᵳ/Å</th>
<th>Φᵳ/Å</th>
<th>Φᵳ/Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG1</td>
<td>8 4 25 7 7 38 7 7 22 7 3.5 25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tail</td>
<td>33 4 10 35 2 12 33 10 5 32 4.5 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HG2</td>
<td>8 4 25 7 7 38 7 7 35 9 4.5 25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The bare lipid bilayer (DMPC only, no foldamers) presents a thick ordered region for the tail groups (33 Å, fully extended thickness is ~ 37.5 Å). The tail groups are also almost fully extended at 8 Å (the expected value is ~ 10.5 Å). Both the head group thickness and interlayer roughness are in close agreement with what reported for similar DPPC bilayers. The structure presents itself as relatively well-ordered with an overall thickness of 49 Å.
4.4.3 CbzAib₉FibTEG (11) and Cbz(αMeVal)Aib₄FibTEG (12)

The $N_b$ for peptaibol and the head group regions are very similar for the experiments with different contrasts. Therefore, the set of contrasts used are not sensitive to the volume fraction or conformation of peptaibol in the head group region. The tail group region for the h-lipid also offers little sensitivity to the peptide because of the small difference in $N_b$. However, the $N_b$ between the d-lipid tail groups was much more informative giving us information about the approximately concentration of the peptides within the bilayer. For all peptaibols studied, the $N_b$ of the d-tail group region was found to decrease compared to the bare lipid bilayer, consistent with approximately 5 to 10 % peptaibol by volume.

The addition of CbzAib₉FibTEG (11) to the bilayer causes relatively few changes to the structure, although the three sections of the bilayer are more defined and the interlayer roughness is reduced compared to the bare lipid. This suggests that there may be some degree of ordering within the head group regions even though the effect is rather small. The peptide is sufficiently long to span the bilayer, sitting comfortably with both ends in the inner and outer head group regions.

The overall thickness of the membrane with Cbz(αMeVal)Aib₄FibTEG (12) and without is comparable with a length of roughly 48 Å. However, Cbz(L)(αMeVal)Aib₄FibTEG greatly affected the structure of the bilayer. In particular, the peptides affect the degree of disorder within the lipid bilayer. It has been reported that large values of roughness are related to intermixing between adjacent regions of the bilayer, and are therefore indicative of the loss of spatial constraint by the head groups of the bilayer. These will then indicate a significant intermixing between the tail group and head group regions of the lipids.
Considering the length of 12, this peptide compared to the 11 (CbzAib₉FibTEG) is 5 amino acids shorter, which corresponds roughly to 6 Å less when the peptides adopt a helical conformation. A model that can explain the increase of the roughening within the lipid bilayer is similar to what observed for peptide GAIV. If the peptide is too short to span the bilayer it may pull the head groups together comparable to the effect on the membrane by peptide GAIV. Moreover, similarly to CbzAib₉FibTEG, the tail group region for the d-lipid decreases from $6.20 \times 10^{-6} \text{Å}^{-2}$ to $5.78 \times 10^{-6} \text{Å}^{-2}$, again consistent with approximately 7.5% peptaibol by volume (Figure 4.22).

4.4.4 NR from DMPC bilayers containing Cbz(L)(αMeVal)Aib₄OtBu

The Cbz(L)αMeValAib₄OtBu (9) is much shorter compared to both the CbzAib₉FibTEG (11) and the Cbz(L)(αMeVal)Aib₄FibTEG (12) and, even in its fully extended conformation, it cannot span the bilayer. Analysis of the tail group region for the d-lipid, was found to decrease from $6.20 \times 10^{-6} \text{Å}^{-2}$ to $5.66 \times 10^{-6} \text{Å}^{-2}$ consistent with approximately 10% peptaibols by volume suggesting a significant partitioning of the peptides in the hydrophobic region of the membrane despite the peptide being shorter than the width of the lipid bilayer. Two possible scenarios can be pictured to explain this effect. Either the peptides assembled into large aggregates that formed channels across the lipid membrane, or alternatively the foldamers behave like lipids or surfactants, segregating the hydrophobic domains in the inner region of the bilayer and exposing their hydrophilic head. Although at this stage it is difficult to propose a definite explanation for the observed effect, these experiments prove that Cbz(αMeVal)Aib₄OtBu penetrates the lipid bilayer despite being relatively short. However, unlike the Cbz(L)(αMeVal)Aib₄FibTEG, the roughness of the lipid for peptide 12 is comparable to one of the lipid bilayers (Figure 4.22).
Figure 4.20: Illustration of the observed experimental effect on the thickness of the lipid bilayer for peptides 9, 12 and 11. Peptide 12 after insertion in the membrane, pulls together the polar heads of the opposite leaflets. Both peptides 9 and 12 have no effect on the thickness of the bilayer despite peptide 9 being shorter than peptide 11.

Overall, Neutron scattering experiments allowed for the clarification of the effect of a sample of Aib-foldamers on the lipid bilayer and are in agreement with the LD data discussed in section 4.4. NR data lead to conclude that peptides 9 and 11 have little effect on the roughness and thickness of the membrane. In contrast the peptide 12 reduces the thickness of the membrane. Peptide 9 is too short to span the membrane; however, has no evidence of reducing the thickness of the membrane and a proposed explanation of such effect is ascribed to aggregation of the peptides to form aggregates that can span the membrane. Linear dichroism analysis indeed reports evidence of exciton coupling between the aromatic groups at the N-terminus. This observation seems to support the aggregating model. As for peptide 11, both LD and NR are of difficult interpretation. Ideally calculation would allow for a definitive interpretation of the effect observed by this peptide on the bilayer.

4.5 Antimicrobial susceptibility tests

All the membrane/peptide interaction studies have demonstrated that the Aib-foldamers presented in this thesis interact with the membrane; therefore it was decided to test the antimicrobial activity with in vitro tests. The peptides tested for antimicrobial activity are listed
in Table 4.6. The design and synthesis of 15 to 18 has been performed by Dr Jennifer Jones and Dr Catherine Adams. The length of the peptides has been chosen according to the data collected in our research group in order for the peptides to adopt a full 3$_{10}$-helix conformation in solution and at the same time, to be long enough to span the length of the membrane. It has been observed, via planar bilayer conductance (PBC) assays and 8-hydroxypyrenetrisulfonate (HPTS) assays of ionophoric activity, that different Aib foldamer lengths have a different ability to transport ions through the membrane, with the LD experiments presented in this thesis. In particular PBC studies performed by Dr J. Jones and Dr C. Adams have proved that peptides with length of eight residue or more could span the membrane and lead to ion channel formation.

The C-termini modification to an alcohol has been chosen in order to better investigate the role of the termini modification of natural occurring peptibols. It has been reported in Chapter 2 that peptibols are commonly found with an Ac group at the N-terminus and at the C-terminus an amino alcohol is found such as leucinol (Lol), phenylalaninol (Phol), valinol (Vol). The biological importance of such modifications is not yet clear. The N-termini of 15-18 had an azido protecting group. Foldamers with an acetyl protecting group were also synthetized by Dr C. Adams, however little of these peptides were available.

![Chemical structure of the peptides tested for the antimicrobial susceptibility assay.](image)

Figure 4.21: Chemical structure of the peptides tested for the antimicrobial susceptibility assay.
4.5.1 **Agar disk-diffusion methods**

Preliminary studies were conducted on foldamer 7 using a culture of *E.Coli* in agar at the concentration of 100 μg/mL and 5 μg/mL using the protocol outlined in Chapter 5. Since foldamer 7 is not water soluble, the experiment was performed by adding the right amount of peptide in a solution of DMSO. DMSO at concentration higher than 0.1% vol/vol can be toxic for cells. The strain of *E. Coli* selected is modified to be resistant to the ampicillin that was also added during the preparation of the agar plate (in order to avoid cross contamination with bacteria in the surrounding environment). The antimicrobial susceptibility test was unsuccessful as the bacteria growth was undisturbed. The peptides are not soluble in agar and precipitated, therefore, the bacteria cells were not exposed to it and grew freely.

4.5.2 **Bacteria growth-inhibition tests**

Since the peptides precipitated in the agar, it was decided to test their antimicrobial activity using bacteria growth-inhibition assays instead. This method, detailed in Chapter 5, requires the addition of the foldamers to a solution of Lysine Decarboxylase Broth (LD Broth) inoculated with the bacteria. The solution is in constant agitation throughout the experiment, therefore, even if the peptide is insoluble in water the bacteria will be exposed to it and its antimicrobial activity can be assessed. In a growth-inhibition experiment, the health of the bacteria is measured indirectly by measuring the turbidity of the solution they grow in. The turbidity is measures by reading the absorbance or optical density on an aliquot (1 mL) of the inoculation mixture at regular interval at 600 nm wavelength. In an ideal case, the OD$_{600}$ measured for the mixture bacteria/antimicrobial peptide should be attenuated compared to the mixture of bacteria without compounds present. Antimicrobial tests usually use a positive or
negative control in order to compare the antimicrobial susceptibility of the compound being assessed. In this case gramicidin mixture from *Bacillus aneurinolyticus* was used (a mixture of gramicidins A, B, C, and D). This antibiotic mixture is potent and selective for Gram positive bacteria.  

The peptides were tested several times at a concentration of 100 µg/mL. (Figure 4.22) and OD$_{600}$ measured over time. It is apparent that the growth curve for the bacteria/peptides mixture and cells alone are very similar (Figure 4.23). These results demonstrate that the peptides are not active on Gram negative as for the Gramicidin mixture. Future work will be testing the antimicrobial activity for the same peptides on Gram positive.

![Figure 4.22: Inhibition growth curve OD$_{600}$/time. The measurements have been taken every hour over a period of 10 h. In the graph are presented peptides 13 to 16 against cell+DMSO as a control.](image-url)
Figure 4.23: Inhibition growth curve OD_{600}/time. The measurements have been taken every hour over a period of 10 h. In the graph are presented peptides 13 against cell+DMSO as a control, gramicidin and cells alone.
The experiments described in this chapter permit insight into the preferred conformation and orientation of Aib-rich peptides when bounded to membrane. The novelty of the experiments has resulted, in some cases, to a limited interpretation of the data. Nevertheless, the VCD and the ROA studies have allowed the preferred conformation of the foldamers in membranes to be studied. In particular, the analysis of the ‘W’ shape pattern of the VCD amide I band reveals that the peptides, once bounded to membrane, adopt a complexity of helical conformations. The influence of the membrane environment on the equilibrium between the M and P screw-sense preference in these Aib foldamers was particularly noteworthy. VCD suggested the chiral amino acid at the N-terminus seems to compete with the bilayer in determining the equilibrium between the right and left handed helix. However, in the absence of chiral control, it appears that one screw sense preference dominates over the other. ROA was not able to either confirm or deny these results, and more conditions need to be explored in order to optimize the quality of the ROA data.

The LD studies allowed for a better understanding of the orientation of the foldamers with respect to the bilayer. Some of the peptides, such as the 11, 12 and N₃Aib₆O’Bu revealed that the peptides preferentially insert perpendicular to the plane of the bilayer. Moreover, kinetic studies on peptides 11 and 12 suggest that the preferred orientation is adopted quite quickly, within the 25 min necessary to collect the first LD spectrum, because the spectral features don’t change over time. These results are in line with the neutron scattering experiments that demonstrate that all the peptides tested were able to distribute themselves throughout the membrane. Moreover, the neutron reflectionary experiments revealed the effect that the peptides have on the membranes, which is important in order to understand the potential mechanism of action of the peptides. In particular, it was observed that the shorter peptides
increased the roughness of the membrane and ‘pulled’ the two bilayer leaflet closer, while the longer peptides inserted in the membrane without cause many disruption to the structure.

Overall these experiments were promising and more work will be done, especially with the VCD and CD studies which have proved to be easy to perform and provide consistent data.

In particular, a series of Aib-foldamers, chiral and achiral, will be analysed in phospholipid bilayers with opposite chirality in order to understand the effect of the chirality of the lipids on the VCD signal. As for the ROA, more conditions will be tested in order to improve the sensitivity of the instruments.

Lastly, the antimicrobial studies on *E. Coli* were unsuccessful. It can be concluded that unlike Alamethicin, the foldamers tested are not active on Gram negative bacteria. Future work will be focused on foldamers against of Gram positive bacteria using gramicidin as a positive control.
5. Conclusion and Future work

5 Conclusion

This thesis has described the use of a large set of spectroscopy techniques for the investigation of the conformation, orientation and interaction of Aib-rich foldamers in solution as well as when bonded to a membrane.

Raman and ROA spectroscopic investigations of Aib-foldamers (see Chapter 3) have successfully investigated the preferred conformation of these hydrophobic peptides in organic solvents. Substantial progress has been achieved towards the application of ROA for the investigation of $\beta$-helix conformation, and a marker band wavenumber range has been identified in the region between 1662-1668 cm$^{-1}$. Moreover, by using screw-sense controlled peptides, it was possible to obtain a signature of the right-handed and left-handed helicity of $\beta$-helix peptides. VCD studies have been performed on the same pool of peptides, when applicable, and the information was in agreement with the Raman and ROA data. This work is in the process of being submitted to a journal.

Figure 5.1: Illustration of the different ROA signatures recorded for chiral Aib-foldamers with different chiral control head groups. In particular, the recorded ROA spectrum for the right handed Cbz(D)PheAib$_4$O'Bu (red line) and its left handed enantiomer (black line).
The information obtained in solution has been beneficial for the development and interpretation of the conformation and preferred helicity of Aib-foldamers bound to membranes using VCD and ROA spectroscopies.

Figure 5.2: Illustration of the different VCD spectra recorded for chiral Aib-foldamers with different chiral control head groups. In particular, are presented the recorded ROA spectrum for the right handed Cbz(D)PheAib₄O'Bu (red line) and the left handed enantiomer (black line).

These experiments are novel, and both the experimental procedure and the interpretations need to be improved. However, this thesis successfully has set the groundwork for future investigations of foldamers bound to membranes using VOA techniques. VCD, in particular, has been demonstrated to have many advantages compared to other spectroscopic techniques routinely used for the investigation of secondary structures within the membranes. The experiments, although requiring longer acquisition times compared to ECD measurements or NMR, are sensitive to the screw-sense preference rather than the chiral centre, as is the case for ECD. Particularly interesting is the experiment performed on the achiral peptide in solution and within the membrane. Although it has been theorized that peptides may adopt a preferred screw-sense preference within the membrane, this is rather difficult to investigate. VCD has proven to be particularly sensitive to the structural differences that occur between the solution and the membrane, with the identification of bands useful for the determination of the structural preferences as well as the screw-sense preferences. In particular, the spectral feature for peptide
Cbz(L)Phe-Aib\textsubscript{3}O'Bu demonstrate that the peptide posits the coexistence of multiple secondary structures and that the overall screw-sense preference in solution is retained when partitioned into the lipid bilayer. By contrast, the enantiomers do not give a mirror image, suggesting interference from the membrane on the screw-sense equilibrium for this peptide. Moreover, the experiment on peptide CbzAib\textsubscript{3}FibNHTEG showed an interesting result. The peptide is achiral, and in solution interconverts rapidly between right and left screw-sense, so the peptide presents no VCD spectrum. However, once the peptide is embedded into the membrane, a VCD spectrum was recorded. The VCD experiments are very recent and more data needs to be acquired in order to confidently assign the bands. However, they are promising and call for future studies.

ROA investigations of peptides within the membrane at this stage are less easy to interpret and reproduce compared to VCD, nevertheless, it was possible to obtain information about the partition of the lipid by monitoring the change in the ROA bands of the lipid. The ROA gave evidence for the peptide to interact with the fatty acids and therefore suggesting the partition in the hydrophobic part of the membrane. Remarkably this results were also obtained by LD and NR measurements.

This thesis has also described the development of an experimental procedure for the identification of the orientation of the peptides at the membrane interface. In particular, LD and neutron scattering experiments performed on a small library of Aib-rich foldamers (Chapter 4) have produced promising results. Peptide CbzAib\textsubscript{3}FibNHTEG has been studied with VCD, LD, and neutron scattering. The information obtained suggests that this peptide is long enough to insert into the membrane and once inserted one of the two screw-senses might be preferred. Moreover, the insertion within the membrane is rapid, as evidence of insertion was obtained by LD analysis after the first experiment (dead time of 25 min) adding an aliquot
of peptide to a suspension of liposomes. The interpretation of the LD spectra is still in progress as additional data and calculations need to be undertaken, specifically for the short peptides Cbz-protected at the N-terminus. The LD signal for the named peptides is dominated by the exciton coupling of the aromatic moiety, therefore the interpretation of the orientation is important and needs additional data before we fully understand the LD data.

Interestingly, NR experiments performed on a mixture of peptides and supported lipid bilayers suggest that all peptides analysed insert into the membrane, however the effect on the membrane varies depending on the length. The shorter peptide modified with a NHTEG at the C-terminus seem to reduce the thickness of the membrane by pulling the head group close, while the longer chain and the short peptide with the t-Bu modification spans the bilayer without affecting the membrane properties.

![Figure 5.3: Illustration of the observed experimental effect on the thickness of the lipid bilayer for peptides 9, 12 and 11. Peptide 12 after insertion in the membrane pulls together the polar head of the opposite leaflets. Both peptide 9 and 12 have no effect on the thickness of the bilayer despite peptide 9 being shorter than peptide 11.](image)

Ultimately, the antimicrobial activity of a small library of Aib-rich peptides was tested, C and N termini modified accordingly to mimic the naturally occurring peptidibols. The question to be addressed with these experiments was not only a study of the antimicrobial potential, but
also the role of the different C and N terminus capping. The synthesis of the peptaibols in nature is non-ribosomal and there is evidence of post-synthetic modification of the peptides. We sought to understand the role of such modification as it seems important in the evolution of the synthesis of these peptides by fungi. These experiments have demonstrated that the peptides have no activity on Gram negative bacteria, and this was also the case for the naturally occurring peptide Gramicidin.
5.1 **Future work**

Overall, the most significant contribution of this thesis to knowledge is the identification of Raman and ROA marker bands for the $3_{10}$-helix conformation and the insight into non-common spectroscopic techniques for the investigation of peptide/membrane interaction and Aib-foldamers. The solution experiments have successfully identified marker bands for $3_{10}$-helices using Raman and ROA spectra. Great effort was put into the optimization of the experimental conditions in solution, and future work will mainly focus on the screening of a library of Aib-rich flavamers in different solvents. In particular, the synthesis of water soluble monodisperse Aib peptides could be investigated. There are very few examples of water soluble $3_{10}$-helix peptides, and the sequences obtained contain bulky hydrophilic amino acids such as Ac(L)$(\alpha$Me)NvaL-ATANP(3HCl)[L-$(\alpha$Me)Nva]$2$L-ATANP(3HCl)[L-$(\alpha$Me)Nva]$2$–OMe. The synthesis of fully water soluble Aib monodisperse peptides should be considered in order to investigate the equilibrium between $\alpha$-helix and $3_{10}$-helix in a water environment.

The studies of the interaction with the membrane are still at an early stage, and great effort will be put into the development of robust and sensitive data. The key questions to answer are how the membrane affects the equilibrium between M and P screw-sense preference, and how the chirality of the membrane influences such an equilibrium. The lipid used throughout this thesis had all the same chirality with $\Delta 9$-Cis configuration, this could have had an impact on the screw-sense preference or upon the signal recorded. Therefore, further investigation of other lipids is necessary. Following the experiment outlined in this thesis, the most suitable candidate to probe the screw-sense preference could be VCD in combination with ECD. A pool of peptides with specific probes would be synthetized and lipids with different chirality would be tested to ultimately address the unanswered questions. As for the ROA studies, the data presented in Chapter 4 are a novel and powerful means for discovering the screw-sense
preference of a peptide within the membrane. However, its utility is currently hampered by the uncontrolled reproducibility of the experiments. More work should be focused on the optimisation of the experimental conditions and conformation studies using this VOA technique should be repeated for the peptides presented in this thesis as well as for the novel compound synthesized.

The understanding of the orientation of the peptides should be carried on with LD experiments and, hopefully, a combined effort between conformational and orientational studies will finally reveal a pattern for the mechanism of action of the peptaibols.

Future work should also focus on testing the antimicrobial activity on a range of Gram positive bacteria. By correlating the antimicrobial susceptibility with the structural information acquired for this library of foldamers the current research will hopefully move a step closer to the final goal, which is to understand the minimum residue and modification required for the peptide to exhibit the antimicrobial activity and, thereby, design new antimicrobial drugs.
6. Material and Methods

6 Instrumentation

Raman and ROA studies were recorded using a ChiralRaman spectrometer and ChiralRaman-2x (BioTools Inc., USA). A schematic representation and description of the instrument is found in Figure 1.6. Both instrumentations adopt a backscattered SCP strategy and the sample are analyzed using a customized quartz cell cuvette. The incident laser excitation wavelength is 532.5 nm. Experiments were performed in Manchester and Prague (Czech Republic). There spectrometer in Manchester employs a Millenium Pro (Spectraphysic, UK) laser while the Prague spectrometer uses a OPUS 532 diode pumped solid-state (DPSS) laser (Laser Quantum LTD). The VCD experiments were performed using a ChiralIR-2x (BioTools Inc., USA) 50 μm pathlength in a BaF₂ cell at 29°C. LD was performed in a Jasco spectropolarimeter: J-1500 (Jasco, Dunmow, UK) equipped with the LD cuvette flow cells (Dioptica Scientific Ltd, Rugby, UK).

All samples for IR spectroscopy analysis were analyzed as solutions using a Bruker Alpha FT-IR (Billerica, Massachusetts, United States) spectrometer equipped with a platinum ATR module from 4000 to 400 cm⁻¹.

All the peptides presented in this project were purified when possible using a HPLC Agilent (Santa Clara, California, United States) 1100 series equipped with a semi-preparative C-18 column Agilent eclipse XDB-C18, 5 μm, 9.4 mm x 250 mm. Results were plotted, analysed and interpreted using OriginPro 8.5 software.

Vesicles were extruded using a LiposoFast liposome (vesicle) extruder from AVESTIN Europe GmbH. Sonication was performed using a VCX130PB probe-type sonicator (Sonics & Materials Inc.). Vortex mixing was performed using a Vortex Genie 2 (600-2700 rpm).
6.1 Data processing

Raman and ROA spectra were processed using Matlab 2010 and in-house toolbox. Conventional Raman spectra were baseline corrected according the method proposed by Eilers et al. In addition the Raman spectra were of water subtracted. For the Raman spectra recorded in DMSO-\textit{d}_6, the solvent was subtracted in addition to baseline removal procedures and intensity normalisation for comparative purposes. The ROA spectra were baseline corrected using a median filter, and smoothed using a second level Savitzky-Golay filter. Spectral deconvolutions were carried out in OriginPro 9 using the inbuilt peak fitting and deconvolution tool. The second derivatives were Gaussian peaks iteratively fitted until convergence criteria were satisfied. LD data were processed using the JASCO spectra manager.

6.2 Materials

All Aib-rich peptides were synthetized by Dr Sarah Pike and Dr Matteo De Poli collaborating with Prof J. Clayden at the University of Manchester. Peptides \textbf{p1} was synthetized according to the method reported following in this chapter. \textbf{p2} was provided by Dr Patrick King. YTIAALLSPYS and FTIAALLSPES were provided by Dr Jakub Ujma.

6.2.1 Lipids

DOPC lipids (1,2-dioleoyl-sn-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids Inc., (AL, USA). DMPC and tail-deuterated DMPC (d-DPPC, 1,2-dipalmitoyl(d62)-sn-glycero-3-phosphocholine) were purchased also from Avanti polar lipids (Alabaster, AL, USA).
6.2.2 Vesicles materials

PBS, was obtained from Sigma-Aldrich Co. (UK). Drain disc (10MM PE) and Nuclepore PC 19mm for the extrusion were obtained by Whatman (GE Healthcare, USA).

6.2.3 p1 and p2 synthesis

Chapter 2 presented a Raman and ROA investigation of peptides p1 and p2. The combined effort in our lab resulted in the publication in Soft Matter in 2016. One contribution to the paper was the synthesis and purification via reverse-phase high performance liquid chromatography (RP-HPLC) of the p1 and p1-RGD peptides employed for the biological assay. The synthesis was carried out via standard solid phase synthesis using a Fmoc strategy.

The synthesis was carried out on 0.25 mmol scale using a CEM Liberty microwave. The low-loading Wang resins (ca. 0.25 mmol/g), pre-loaded with Fmoc-Glu(tBu) or Fmoc-Arg(Pbf)-OH. The coupling mixture used were 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N,N-Diisopropylethylamine (DIEA) 1-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). Subsequently the peptides were cleaved from the resin, along with their conjugates, using 95 % trifluoroacetic acid (TFA) for 2-4 h under agitation at room temperature, using 2.5 % purified H2O and 2.5 % triisopropylsilane as scavengers. The peptides were then dried under reduced pressure to remove excess of TFA and the crude material was precipitated from an excess of cold diethyl ether. The crude was dissolved in water and then lyophilized to obtain a low-density powder.

Purification was performed using reverse-phase HPLC (Agilent 1100 series), equipped with a semi-preparative C-18 column (Agilent Eclipse XDB-C18, 5 μm, 9.4 mm × 250 mm) at room temperature. The solvent system was CH3CN and purified water both mildly acidified with 0.1 % TFA. The purity was achieved using a gradient strategy, in which CH3CN increased linearly
from 5 % to 95 % over 1 h. Peptide and peptide conjugate identities were determined using mass spectrometry, with a Waters LCT-TOF connected to a Waters Alliance LC (Waters, Herts, UK). The final purity was determined by analytical HPLC on the same system using an analytical C18 column.

6.3 Sample preparation for vibrational spectroscopy experiments

6.3.1 p1 and p2 solution and hydrogel formation

Chemicals were purchase from Sigma-Aldrich (UK), AGTC (UK) or Novabiochem. Solvents used were HPLC grade and purchased from Fisher or Sigma Aldrich.

The solution sample of p2 (peptide provided by Dr Patrick King) was prepared by adding 10 mg (7 µmol) of p2, purified twice with HPLC, dissolved in 300 µL of HPLC grade water and mixed with 20 mg of NaI (0.1 mol) giving a final concentration of p2 of approximately 30 mg/mL (0.023 mol/L). The pH, as measured with a pH paper, was between 4 and 5. The solution sample of p1 (synthesis reported in section 5.7) 20 mg (14 µmol, purified twice with HPLC) was dissolved in 250 µL of HPLC grade water and NaOH (45 µL, 1M) and mixed with 20 mg of NaI (0.1 mol) for a final concentration of 80 mg/mL (0.25 mmol ), pH 11. The hydrogel was obtained by mixing approximately 1 mg of p1 (0.71 mmol) dissolved in 120 µL of water (0.006 mol/L) and 1.5 mg of p2 (1.07 mmol) in 40 µL of water (0.026 mol/L) a temperature of 90° C following by neutralization using a NaOH (1 M). The solution was heated using the heating block Grant QDB2. The gel obtained from the mixture of p1 and p2 was 4 mM, with volume of 170 µL (14 mg/mL).
6.3.2 **GNNQQNY**

The peptide GNNQQNY, provided by Dr Patrick King, was prepared by dissolving 12.5 mg (0.4 mmol) in 400 µL of micro-filtrated H$_2$O to a final concentration of 31.25 mg/mL (50 mmol/L), using an ultrasound bath to avoid the accumulation of insoluble crystals. The ultrasound agitation was applied for further 15 min after the solution was obtained. The sample was stored overnight at room temperature. The gel was prepared by dissolving 12.5 mg of GNNQQNY (0.4 mmol) in 400 µL micro-filtrated H$_2$O for a final concentration of 25 mg/mL with heating of the solution. The sample was left in the heating block for 1.5 h.

6.3.3 **Aib-foldamers**

The samples were prepared by weighting each powdered analyte and then dissolved in the appropriate solvents. All peptides were previously purified with HPLC in order to reduce the fluorescent impurities. The final analyte concentration was 121 mg/mL for Raman, ROA and IR experiments of the achiral peptides. The final concentration for the VCD experiments was kept between 30 and 40 mg/mL.

6.3.4 **Vesicles preparation**

The liposome samples for the VCD and ROA experiments performed in Prague were prepared by combining the right amount of lipid (DOPC) from the stock solutions (20 mg/mL in chloroform) with the right amount of peptide dissolved in spectroscopic grade chloroform (4 % molar ratio) in a 5 mL round-bottomed flask. These amounts will give a final concentration of about 120 mg/mL (total lipid concentration) when resuspended in 1 mL of aqueous solution
and a final concentration of the peptide of 30 mg/mL. The chloroform was removed under reduced pressure using a rotary evaporator until a thin film was formed on the walls of the flask. The film was then further dried on a high vacuum line for at least 2 h. Deuterated PBS buffer solution (1 mL) was then added to the flask and the lipid film was suspended in the solution with the assistance of vortex mixing. The turbid suspension was sonicated at room temperature using a sonicator bath on the same day of the experiment. The experiments were performed in a customized VCD (International crystal laboratory, ICL, 11 Erie St # 2, Garfield, NJ 07026, United States) and ROA cuvette (Starna Scientific, UK) to perform the experiments.

6.3.5 Neutron scattering experiment

Model membranes were deposited (using vesicles prepared as in the method outlined in section 5.3.4) on single silicon crystals (SiO$_2$) after Piranha-clean of the surface. All bilayer deposition procedure were performed under ambient conditions and without subphase buffering before the NR experiment. Specular NR measurements were carried out using the SURF time-of-flight reflectometers at the Rutherford Appleton Laboratory (Oxfordshire, UK), using neutron wavelengths from 0.5 to 6.8 Å. The reflected intensity was measured as a function of the momentum transfer, $Q_z$.

$$Q_z = 4\pi \sin \frac{\theta}{\lambda}$$  \hspace{1cm} (27)

with $\lambda$ representing the wavelength and $\lambda$ the incident angle. The collimated neutron beam was reflected from the silicon–liquid interface at three glancing angles of incidence, 0.358, 0.658 and 1.58. For the experiments a flow cells was used that was connected to a liquid chromatography pump (L7100 HPLC pump, Merck, Hitachi), which exchanged the solution isotopic contrast within chamber of the cell (total volume 5 mL). For each solution isotopic contrast change, a total of 22.5 mL of 20 mM pH/D 7.0 sodium phosphate buffer solution was
pumped through the cell (7.5 cell volumes) at a speed of 1.5 mL/min. Each solution contrast was run in duplicate with the repeat analysis taken at 16 h intervals.

6.3.6 LD experiments

The final mixture of lipids was obtained by combining the right amount of lipid from the stock solutions in spectroscopic grade chloroform in a 5 mL round-bottomed flask to obtain a concentration of 1, 2, 5 and 10 mg/mL (total lipid concentration) when resuspended in 1 mL of PBS buffer at pH=7.4. The chloroform was removed under reduced pressure using a rotary evaporator until a thin film was formed on the walls of the flask. The film was then further dried on a high vacuum line for at least 2 h. PBS buffer solution (1 mL) was then added to the flask and the lipid film was suspended in the solution with the assistance of vortex mixing. The turbid suspension was freeze/thawed 5 times and then transferred into one of the 1 mL gas-tight syringes of the LiposoFast extruder. The syringe attached to the rest of the extruder assemblage, fixed with a polycarbonate membrane with 100 nm diameter pores. The suspension was passed through the membrane at least 15 times. The peptide/vesicles samples were prepared by mixing to a molar ratio of 10% or 5% molar ratio (lipid:peptide). The concentration of peptide was about 0.1 mg/mL and 0.05 mg/mL (15 μM, 7.5 μM) for all the peptides with a final volume in the coquette of 80 μL. The experiments were performed in a home build coquette flow LD, rotation speed was 3000 rpm, and the baseline was measured at 0 rpm. Spectra were gathered from 180 to 350 nm, with a speed of 100 nm/min integration time of 1 s and each spectrum average of 8 measurements.
6.4 Antimicrobial activity assays

6.4.1 Agar diffusion test

Antimicrobial assays were performed with the help of Dr Edward A McKenzie. Bacteria tests were carried out on *Escherichia Coli* JM109 (New England BioLabs, Ipswich, Massachusetts, United States), transformed with pHis plasmid (ampicillin selection) and plated out on LB amp (100 µg/mL) agar overnight at 37°C. A colony was selected and used to inoculate three petri dishes containing 100 µg/mL of ampicillin (total volume 20 mL), 100 µg/mL of peptide 7 and 5 µg/mL of peptide 7 and left over night with shaking at 37°C. Germination and growth of the bacteria was tested the next day.

6.4.2 Bacteria growth-inhibition test

Bacteria tests were carried out on *E.Coli* JM109 (New England BioLabs, Ipswich, Massachusetts, United States), transformed with pHis plasmid (ampicillin selection) and plated out on LB amp (100 µg/mL) agar overnight at 37°C. A colony was selected and used to inoculate a 5ml LB culture containing 100 µg/mL ampicillin and left over night with shaking at 37°C. Next day, 200 µl of the saturated culture was then added to 100 mL of LB + ampicillin. This was than divided into 10 mL sample tube either alone or in the presence of compounds with a concentration of 5 µg/mL (20 µL and 100 µL additions) in DMSO. Samples were left shaking in the incubator for 2h. Subsequently the OD_{600} was measured every 1h or 2 h over a period of 9 h or 7 h respectively after inoculation. The growth inhibition curve (OD_{600} vs time) was plotted using Origin Pro 9.
7. Supplementary information

7 Supplementary spectra

7.1 Raman spectra in CHCl3 and DMSO-$d_6$

The majority of the Raman spectra presented in Chapter 3 were a selection of the amide I region, in this chapter it will presented the Raman spectra in the region between 800-1800 cm$^{-1}$ in chloroform and 1100-1800 cm$^{-1}$ in DMSO-$d_6$. Spectral region above and below these range were dominated by the solvents band. The spectra are presented without solvent subtraction.

![Raman spectrum](image)

Figure 7.1: Raman spectrum of $N_3$Aib$_2$O'Bu in DMSO-$d_6$ shown between 1100 and 1800 cm$^{-1}$. 
Figure 7.2: Raman spectrum of $\text{N}_3\text{Aib}_6\text{O}^\text{Bu}$ in DMSO-$d_6$ shown between 1100 and 1800 cm$^{-1}$.

Figure 7.3: Raman spectrum of $\text{N}_3\text{Aib}_4\text{O}^\text{Bu}$ in DMSO-$d_6$ shown between 1100 and 1800 cm$^{-1}$.
Figure 7.4: Raman spectrum of N₃Aib₂O'Bu in CHCl₃ shown between 800 and 1800 cm⁻¹.

Figure 7.5: Raman spectrum of N₃Aib₂O'Bu in CHCl₃ shown between 800 and 1800 cm⁻¹.
Figure 7.6: Raman spectrum of N₃Aib₄O'Bu in CHCl₃ shown between 800 and 1800 cm⁻¹.

Figure 7.7: Raman spectrum of N₃Aib₄O'Bu in CHCl₃ shown between 800 and 1800 cm⁻¹.
Figure 7.8: Raman spectrum of $N_3Aib_4O'Bu$ in CHCl$_3$ shown between 800 and 1800 cm$^{-1}$.

Figure 7.9: Raman spectrum of $N_3Aib_7O'Bu$ in CHCl$_3$ shown between 800 and 1800 cm$^{-1}$.
Figure 7.10: Raman spectrum of N₃Aib₈O'Bu in CHCl₃ shown between 800 and 1800 cm⁻¹.
7.2 Chapter 3: IR spectra in CHCl$_3$ and DMSO-$d_6$

The IR spectra presented in Chapter 3 showed only selection of the amide I region, in this chapter it will presented the IR spectra in the region between 2500-1100 cm$^{-1}$ in chloroform and DMSO-$d_6$. The spectra presented are obtained by subtracting to the raw spectra of the sample the recorder IR of the solvent.

![Figure 7.11: IR spectrum of N$_3$Aib$_2$O$^t$Bu in DMSO-$d_6$ shown between 2500 and 1100 cm$^{-1}$](image-url)
Figure 7.12: IR spectrum of N₃Aib₄O'Bu in DMSO-d₆ shown between 2500 and 1100 cm⁻¹.

Figure 7.13: IR spectrum of N₃Aib₆O'Bu in DMSO-d₆ shown between 2500 and 1100 cm⁻¹.
Figure 7.14: IR spectrum of $N_3$Aib$_2$O$^t$Bu in CHCl$_3$ shown between 2500 and 1100 cm$^{-1}$.

Figure 7.15: IR spectrum of $N_3$Aib$_4$O$^t$Bu in CHCl$_3$ shown between 2500 and 1100 cm$^{-1}$. 
Figure 7.16: IR spectrum of N$_3$Aib$_5$O'Bu in CHCl$_3$ shown between 2500 and 1100 cm$^{-1}$.

Figure 7.17: IR spectrum of N$_3$Aib$_6$O'Bu in CHCl$_3$ shown between 2500 and 1100 cm$^{-1}$.
Figure 7.18: IR spectrum of $\text{N}_3\text{Aib}_7\text{O}^\text{tBu}$ in CHCl$_3$ shown between 2500 and 1100 cm$^{-1}$.

Figure 7.19: IR spectrum of $\text{N}_3\text{Aib}_8\text{O}^\text{tBu}$ in CHCl$_3$ shown between 2500 and 1100 cm$^{-1}$.
7.2.1 Growth inhibition test

As discussed in Chapter 4, the antimicrobial activity for peptides 15 to 19 has been tested using the growth inhibition test. The peptides have proven not to be active on Gram- E. Coli. Following are presented the growth rate inhibition curve performed. All peptides have been tested at a concentration of 100 μg/mL.

![Growth inhibition curve](image)

Figure 7.20: Growth inhibition curve of peptides 15 to 18 compared to cells plus DMSO. The OD$_{600}$ was measured every hour over a period of 9h.
Figure 7.21: Growth inhibition curve of peptides 15 to 17 compared to cells plus DMSO (control A, B and C) and cell only (Cell1 and 2). The OD$_{600}$ was measured every 2 h over a period of 7h.
### 7.3 Neutron Scattering

In Table 7.1 are reported all the fitting parameters, SF is the scale factor, Rough is the roughness, e is the unit in Angstrom, d is the layer thickness. Hydr is the hydration parameter that is 0 when there is no water and 100 when the sample chamber contains only water.

Nb is \( \ldots \) (see equation 1.29).

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7.4 Histidine

From the work in this thesis, in collaboration with PhD student Salvatore Cardamone, it was published the paper ‘The Computational Prediction of Raman and ROA Spectra of Charged Histidine Tautomers in Aqueous Solution’. The computational modelling of Histidine is problematic because of the possibility to adopt multiple formal charge states and tautomers. Mr Salvatore Cardamone aimed to recover the experimental Raman Optical Activity (ROA) spectra of histidine. The paper successfully introduces a novel conformer selection scheme that reproduces the experimental spectra. The agreement between the experiments and theory is amenable for band assignments. My contribution to the paper was collecting the experimental Raman and ROA for the (L) histidine at pH 7.8 and pH 4 (Figure 7.22).

![Figure 7.22: Raman (top) spectra and ROA (bottom) spectra of (L)His at pH 7.8 (black line) and pH 4 (red line). The spectra were collected at 1.0 W for a total of 6 h for each analyte.](image-url)
8 References

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