Membrane-active peptides

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Declaration

I declare that this thesis has been composed by myself, and that the work described herein is my own except where stated.
This thesis is dedicated to my parents and sister who have all given me much love and support especially over the last three years; and also to my black lab, Sasha, whose neverending commitment for the search of food always astonishes me!
Acknowledgements

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First and foremost, may I thank Dr. Jeremy P. Bradshaw for his wise council and patience over the last three years.

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Most of the work described in this thesis has actually been carried out at other institutions. Without the assistance of each of the ‘local contacts’, the research would not be possible. Therefore, I thank Dr. Sharon Kelly at the CD facility at Stirling University, Dr. Thomas Hauss and Dr. Silvia Dante at V1 (HMI), Dr Edith Bellet-Amalric at D16 (ILL), Mrs. Sue Slawson at station 2.1 (SRS) and Dr. John Katsaris at C5 (Chalk River).

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The work of the technical staff can sometimes go unsung. I would like to acknowledge Dr. Celia Goodhew for letting me break her pH meter!, Mr. Derek Penman for his assistance with my many claim forms, Mr. Graham
Moodie who made the isolator and the administrative skills of Mrs. Frances Fettes at the Department of Pre-clinical Veterinary Sciences.

Thanks to the Wellcome Trust for their sponsorship.

Last, but by no means least, may I give a big shout to the Dunbar boys for their legendary nights out that have certainly relieved some of the pressure on my shoulders.
Abstract

Neurokinin A, (NKA), belongs to the tachykinin family, a group of small amphipathic peptides that bind to specific membrane-embedded G-protein-coupled receptors. The cell membrane acts as a solvent to accumulate peptide and an inducer of peptide secondary structure. The 3-dimensional shape that the peptide assumes when associated to the cell membrane will be an important parameter with regards receptor selectivity and affinity. Receptor affinity appears to depend on the different secondary structures of each tachykinin, which share the same hydrophobic C-terminal sequence, FXGLM. Binding of tachykinins to phospholipid bilayers may take place both on the aqueous membrane surface and in the hydrophobic region. Therefore, neutron diffraction measurements were carried out on highly aligned phospholipid multi-bilayers in order to define the location of the N- and C-terminus of NKA. This study reports that the bilayer location of NKA is remarkably similar to that of substance P, thereby inferring that finer levels of structure must control receptor specificity.

Circular dichroism studies were carried out in order to define the conformation of NKA in structure-inducing solvents and in a range of disperse systems. NKA was found to be in a random coil conformation in many of the solvents but exhibited considerable β-sheet conformation in octan-1-ol. These results are related to the neutron diffraction data. A model of the NKA-bilayer
interaction is presented using the results from these experiments and from other biophysical techniques.

The structural effects of the fusion peptide of feline leukemia virus (FeLV) on lipid polymorphism of N-methylated dioleoylphosphatidylethanolamine (MeDOPE) were studied using X-ray diffraction. This peptide, the hydrophobic amino terminus of p15E, has been proven to be fusogenic and to promote the formation of highly curved, intermediate structures on the lamellar liquid-crystal to inverse hexagonal phase transition pathway. The p15EK produced marked effects on the thermotropic mesomorphic behaviour of MeDOPE, a phospholipid with an intermediate spontaneous radius of curvature.

X-ray diffraction measurements show that the peptide reduces the d-repeat of the membrane prior to the onset of an inverted cubic phase. This suggests that membrane thinning may play a role in peptide-induced model membrane fusion and strengthens the link between the fusion pathway and inverted cubic phase formation. The results of this study are interpreted in relation to models of the membrane fusion mechanism.

Also included in this thesis is a study that aims to improve lamellar neutron diffraction data collection and analysis.
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<tr>
<td>ACTH</td>
<td>adrenocorticotrophic hormone</td>
</tr>
<tr>
<td>BENSC</td>
<td>Berlin Neutron Scattering Centre</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CMC</td>
<td>critical micelle concentration</td>
</tr>
<tr>
<td>CPP</td>
<td>critical packing parameter</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotrophin-releasing hormone</td>
</tr>
<tr>
<td>DiPoPE</td>
<td>dipalmitoleoyl-sn-phosphatidylethanolamine</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DPC</td>
<td>dodecylphosphocholine</td>
</tr>
<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-sn-phosphatidylcholine</td>
</tr>
<tr>
<td>DOPG</td>
<td>1,2-dioleoyl-sn-phosphatidylglycerol</td>
</tr>
<tr>
<td>DSC</td>
<td>differential scanning calorimetry</td>
</tr>
<tr>
<td>DTA</td>
<td>differential thermal analysis</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>FeLV</td>
<td>feline leukemia virus</td>
</tr>
<tr>
<td>FFEM</td>
<td>freeze-fracture electron microscopy</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>FT</td>
<td>Fourier transform</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>FT-NMR</td>
<td>Fourier transform nuclear magnetic resonance</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
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<td>GHRH</td>
<td>growth hormone-releasing hormone</td>
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<td>GnRH</td>
<td>gonadotrophin-releasing hormone</td>
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<tr>
<td>gp85</td>
<td>FeLV fusion envelope glycoprotein</td>
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<tr>
<td>HA</td>
<td>haemagglutinin</td>
</tr>
<tr>
<td>HA1</td>
<td>$\alpha$-helical coiled coil of haemagglutinin</td>
</tr>
<tr>
<td>HA2</td>
<td>globular head domains of haemagglutinin</td>
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<td>$H_1$</td>
<td>oil-in-water lipid hexagonal phase</td>
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<td>$H_{II}$</td>
<td>water-in-oil (or inverted) hexagonal phase</td>
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<td>Full Form</td>
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<tr>
<td>HFP</td>
<td>hexafluoropropanol</td>
</tr>
<tr>
<td>HT</td>
<td>hydroxytryptamine</td>
</tr>
<tr>
<td>ILA</td>
<td>interlamellar attachment</td>
</tr>
<tr>
<td>ILL</td>
<td>Institut Laue et Langevin, Grenoble, France</td>
</tr>
<tr>
<td>$L_{\alpha}$</td>
<td>lamellar liquid-crystal phase</td>
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<tr>
<td>IMI</td>
<td>inverted micellar intermediate</td>
</tr>
<tr>
<td>IUV</td>
<td>intermediate unilamellar vesicle</td>
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<tr>
<td>LH</td>
<td>luteinising hormone</td>
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<td>LPC</td>
<td>lysophosphatidylcholine</td>
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<td>LPG</td>
<td>lysophosphatidyglycerol</td>
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<tr>
<td>LSC</td>
<td>liquid scintillation counting</td>
</tr>
<tr>
<td>LUV</td>
<td>large unilamellar vesicle</td>
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<tr>
<td>MeDOPE</td>
<td>N-methylated 1,2-dioleoyl-sn-phosphatidylethanolamine</td>
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<td>MLV</td>
<td>multi-lamellar vesicle</td>
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<td>MSH</td>
<td>melanocyte-stimulating hormone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>NK</td>
<td>neurokinin</td>
</tr>
<tr>
<td>NKA</td>
<td>neurokinin A (= substance K)</td>
</tr>
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<td>NKB</td>
<td>neurokinin B</td>
</tr>
<tr>
<td>OCD</td>
<td>orientated circular dichroism</td>
</tr>
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<td>PA</td>
<td>phosphatidic acid</td>
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<td>PC</td>
<td>phosphatidylcholine</td>
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<td>PE</td>
<td>phosphatidylethanolamine</td>
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<td>phosphatidylglycerol</td>
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<td>PI</td>
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<td>palmitoyloleoylphosphatidylcholine</td>
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<td>palmitoyloleoylphosphatidylglycerol</td>
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<td>PS</td>
<td>phosphatidylserine</td>
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<td>$^{31}$P-NMR</td>
<td>$^{31}$phosphorus nuclear magnetic resonance</td>
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<tr>
<td>p15EK</td>
<td>TM polypeptide subunit produced by proteolytic cleavage of gp85</td>
</tr>
<tr>
<td>p15E(1-18)</td>
<td>18 amino acid truncated version of p15EK</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>Q</td>
<td>momentum transfer vector</td>
</tr>
<tr>
<td>RNA</td>
<td>ribose nucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SK</td>
<td>substance K</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>SP</td>
<td>substance P</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicle</td>
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<tr>
<td>TBHA2</td>
<td>thermolysin bromelain HA2</td>
</tr>
<tr>
<td>TH</td>
<td>(L_{\alpha}H_{II}) phase transition temperature</td>
</tr>
<tr>
<td>T1</td>
<td>temperature at which phospholipid isotropic resonances are first observed</td>
</tr>
<tr>
<td>TQ</td>
<td>(L_{\alpha}Q_{II}) phase transition temperature</td>
</tr>
<tr>
<td>TQH</td>
<td>(Q_{II}H_{II}) phase transition temperature</td>
</tr>
<tr>
<td>TFE</td>
<td>trifluoroethanol</td>
</tr>
<tr>
<td>TMC</td>
<td>transmonolayer contact</td>
</tr>
<tr>
<td>TRC-TEM</td>
<td>time-resolved cryo-transmission electron microscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>TRH</td>
<td>thyrotrophin-releasing hormone</td>
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<td>TSH</td>
<td>thyroid-stimulating hormone</td>
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<tr>
<td>Q₁</td>
<td>oil-in-water lipid cubic phase</td>
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<tr>
<td>Qᵢᵣ</td>
<td>water-in-oil (or inverted) lipid cubic phase</td>
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<tr>
<td>UV</td>
<td>ultra-violet</td>
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Chapter 1: Introduction to peptide-lipid interactions
1. Introduction

1.1. Biophysical perspectives

Biophysics is a multidisciplinary subject that employs many techniques in order to understand biological phenomena. The basis of these techniques arise from such subjects as applied mathematics, statistics, engineering, physics and physical chemistry.

Biophysics is not a new science but has been in existence long before the 20th century. For example, Sactorius of Padua (1561-1636) modified Galileo’s (1561-1636) famous invention, the pendulum, into a pulsilogium. This was the first device that was used to measure pulse rate (Hunter, 1990). The pendulum’s chord length was adjusted in order to match the frequency of oscillation to the pulse rate. Thus, measurements in the time domain were transformed into measurements in the length domain. Later, Sir Isaac Newton (1642-1727) illuminated the subjects of mechanics and optics that formed the basis of early microscopy.

After World War II, the development of more sophisticated methods accelerated rapidly. Techniques such as analytical ultracentrifugation could separate molecules according to their size, electrophoresis could separate molecules according to their charge-to-mass ratio and spectroscopy could make both qualitative and quantitative measurements of molecules due to their interaction with electromagnetic radiation. Powerful X-ray and neutron
Diffraction techniques can now solve molecular structures to better than 1Å resolution.

Biophysics is used to study organisms at a number of different levels. For example, at the highest level of organisation, applied mechanics can be used to understand how organisms locomote. The study of individual organs and tissues is important in the medical field of transplantation. A lower level of organisation is the study of single cells. For example, it is possible to investigate how individual cells detect and respond to changing environments. Powerful biophysical techniques can be employed to investigate the specific biomolecules that make up an individual cell and this area of research is the main concern of this thesis. It is interesting to note that the versatile technique, proton-NMR, can be used both medically to detect large structures such as brain tumours as well as biologically to solve molecular structures.

The four main classes of biological macromolecule are nucleic acid (DNA and RNA), proteins (and peptides), sugars and lipids. Performing biophysical investigations on such molecules can give information on their 3-dimensional structure and dynamical properties. The dynamical properties range from a molecule’s gross movement to its fine vibrational spectrum. Thus, the structure-function relation is a central question.

This thesis specifically investigates intimate peptide-lipid interactions. The study of such interactions *in-vivo* or using complex systems in a way to mimic the physiological environment would be extremely difficult. Therefore, more
simple model systems are studied using the chemicals believed to be involved in these biological processes.

1.2. Peptides as chemical mediators

Only in the last 20 years has it been realised that peptides play a crucial role as signalling molecules (Rang et al., 1999). There is a twofold reason for this. Firstly, the molecules of early study tended to be of plant origin in a bid to obtain medicinal products (Sneader, 1986). Few of these substances were peptides. Secondly, it was only recently that technological advances have permitted research into this area. Such advances include the development of solid-phase methods for peptide synthesis and the use of selective antibodies for binding assays (e.g. radioimmunoassay) and immunocytochemical localisation of peptides. More recently, molecular biological techniques have allowed the study of peptide expression. With the advent of the Human Genome Project, and the resulting expansion of the biotechnological industrial sector, peptide and protein research will only increase in momentum.

The first signalling molecule thought to be proteinaceous in nature was substance P. In 1931, von Euler and Gaddum discovered that this chemical had similar physiological properties to acetylcholine, but it could still evoke atropinised tissue responses (Maggio, 1988). However, the chemical expertise of peptide purification was lacking and the first peptide structure to be elucidated was Gramicidin S cyclodecapeptide by Synge in 1945 (Schwyzer,
1996). Organic chemists working in the field at the time regarded peptide synthesis as an independent proof of structure. This proved to be technically difficult but du Vigneaud succeeded in 1954 by synthesising oxytocin and vasopressin (Schwyzer, 1996). The rate of peptide synthesis increased greatly due to the development of solid phase methods, pioneered by R. Bruce Merrifield in 1964. Present day peptide primary structure elucidation and synthesis is rapid. For example, the gene encoding endothelin was cloned and the peptide sequenced within one year (Yanagisawa et al., 1988). Many peptides are now available commercially in kilogram quantities. Figure 1.1 illustrates the length of some of the known peptide primary structures.

Most mammalian peptides are found in the neuroendocrine system. Early biological research on peptides revealed that many acted as neurotransmitters. In common with the classical neurotransmitters (e.g. acetylcholine, 5-HT), peptides are released from pre-synaptic terminal ‘boutons’ following Ca$^{2+}$ influx. All neuropeptides studied to date bind only to proteinaceous receptors. Peptides are released from pre-synaptic terminals in a regulated fashion rather than continuously. Unlike many monoamine neurotransmitters, peptides have a slow onset of action and are regarded as so-called ‘neuromodulators’. In fact, many neuropeptides are co-released with their monoamine counterparts (Campbell, 1987). It is becoming increasingly clear that peptides fulfil many roles within the body. They are involved in diverse physiological systems
such as growth and development, blood pressure regulation and immune system reactivity.

Peptides can also interact with the lipids of cell membranes. For example, the bee venom, melittin, induces cell lysis by forming pores in the cell membrane. The so-called ‘fusion-peptides’ of many enveloped animal viruses can cause two initially distinct membranes to fuse. This mechanism facilitates the unwanted entry of infective viral nuclear material into host cells.

**Biologically Active Peptides**

![Biologically Active Peptides Diagram](image)

*Figure 1.1. Some bioactive peptides. Shown in bold are the peptides studied in this thesis. Adapted from Rang et al., 1999*
1.3. Peptide-lipid biophysical studies

The aim of biophysical studies in peptide research is to elucidate 3-dimensional structures in biologically appropriate environments. Structural results can then be coupled to functional studies in a bid to establish a mechanism of action. For this to be fully accomplished, the dynamical properties of the complete system must be known. However, quantification of fine molecular motions is technically difficult to achieve.

The lipid membrane is important to the activities of many peptides. For instance, take the binding of neuropeptides to their membrane-embedded receptors. The membrane may act both as a solvent, in order to accumulate peptide in the close proximity of the receptor, and as an inducer of peptide secondary structure (Schwyzer, 1995). The 3-dimensional shape each peptide assumes when associated to the cell membrane is crucial for receptor recognition. Accordingly, many of the structural studies to date involve peptide incorporation into organic phases or detergent micelle disperse systems. However, the crystallographic methods that are normally used to study soluble proteins can rarely be applied to soluble peptides and membrane-associated peptides and proteins due to difficulties in crystal growth. This is illustrated in the number of soluble protein structures that have been solved compared to membrane-associated proteins. Approximately 12,000 for soluble proteins compared to 6 for membrane-associated proteins. Additionally, the crystal structure of such species may not be relevant to the
actual biological structure. In the last 20 years, peptide structural information has slowly started to become reality.

Alamethicin is a 20-residue antibiotic polypeptide that can insert into model membranes (Eisenberg, 1984). The crystal structure has been solved to 1.5Å resolution using X-ray diffraction and this structure was the basis for a membrane channel model (Fox and Richards, 1982). This peptide has the ability to self-associate in membranes to form pores, rendering the target cell susceptible to osmotic stress. The pore can be visualized as a 'barrel' in the membrane with a number of alamethicin molecules each representing a component ‘stave’. Alamethicin is thought to be in an α-helical conformation.

Melittin from bee venom is water-soluble yet has considerable affinity for membrane interfaces (Eisenberg, 1984). Mellitin exists as a tetramer in both its crystal and aqueous solution form. The crystal structure has been solved to 2Å resolution (Anderson, 1980). This 26-residue polypeptide was found to be in an α-helical conformation. Melittin possesses a large hydrophobic moment because it has an asymmetric distribution of hydrophobic and hydrophilic amino acids around the helical axis. The unusual extent of its amphiphilicity explains why melittin binds avidly to membrane interfaces. The hydrophobic ‘face’ of the molecule inserts into the apolar hydrocarbon core and the hydrophilic face interacts with the aqueous surface. Melittin may self-associate to form pores in the membranes (Dempsey, 1990). This would account for its lytic action.
The enkephalins are a family of small analgesic pentapeptides which bind to membrane-embedded opiate receptors. NMR studies of enkephalins in the solvent DMSO and in LPC micelles revealed that it had a hydrogen-bonded β-turn conformation (Behnam and Deber, 1984). This type of conformation causes the enkephalins to portray hydrophobic sidedness analogous to melittin. The peptide conformation in detergent micelles may represent an intermediate structure of the peptide prior to receptor binding.

1.4. Hydrophobicity scales

Although investigators have started to examine the 3-dimensional shape of membrane-associated proteins and peptides, their primary structures are much better known. This has led to attempts to predict secondary and tertiary structures from the known primary sequence. In order to achieve this aim, a fundamental knowledge of a particular peptide’s hydrophobicity profile is required.

Hydrophobicity values are a measure of the hydrophobic effect, the necessity for water to form a cavity to accommodate a non-hydrogen-bonding group. The cavity that forms increases the order of the water molecules and hence reduces the entropy of the system. The free energy of the solution would therefore be increased, so the solute partitions into a more hydrophobic environment which reduces the free energy by allowing the water cages to relax to their normal random state. The exclusion of non-hydrogen-bonding
groups from the aqueous phase permits more favourable water-water bonding enthalpies. In addition, hydrophobic molecules are more structurally constrained within water cavities than in apolar solvent. Hence, the entropy of the hydrophobic solute is increased in apolar solvent relative to the aqueous phase due to increased internal freedom (Florence and Atwood, 1988).

Relative, or absolute, individual amino-acid residue affinities for membrane-type phospholipid phases must be established in order to predict hierarchal protein structures. This has led to the publication of a multitude of amino-acid hydrophobicity scales. This has become confusing because each scale represents different properties of amino acids partitioning into different phases. Thus, hydrophobicity scales are difficult to compare.

One particular scale measures the Gibbs free energy of transfer from vapour phase to aqueous solution (Wolfenden, 1981). Here, the vapour phase represents the organic phase. This study is concerned only with the “hydration potentials” of the amino acid side chains. Therefore, it would be inappropriate to study the whole-residue amino acid. If this were done, each side chain would give false favourable ‘hydration potentials’ due to the presence of the free carboxylate and charged nitrogen center in each amino acid. Normally, amino acid side chains in folded polypeptides and proteins are ‘sandwiched’ between extensively bonded amide backbones. The result of covalent peptide bonding will reduce the hydrophilicity of each bonded whole-residue amino acid compared to the free whole-residue amino acids. Therefore, in order to
study the hydrophobicity of only the individual side chains, the α-carbon was replaced with a hydrogen atom. Therefore, glycine became diatomic hydrogen gas (H₂) and alanine became methane (CH₄). The resulting scale covers a hydrophobicity range over 16 orders of magnitude with the glycine side chain the most hydrophobic amino acid followed by the leucine analogue. The aromatic analogues appeared mid-table. Interestingly, all the hydrophilic amino acid side chains appear to have A (adenine) as the second codon letter. Wolfenden postulates a link between the free energy of solvation of amino acids and genetic code evolution.

Other groups have measured the partitioning behaviour of amino acids from water to ethanol (Nozaki and Tanford, 1971). This was done in an attempt to evaluate the protein denaturing properties of certain solvents of which ethanol was one. In this study, ethanol represents the hydrophobic interior of a globular protein but could also feasibly represent a region in the membrane interface of comparable polarity. Tryptophan was revealed as the most ‘hydrophobic’ due to its size but on a component carbon atom free-energy contribution, the amino acids with aliphatic side chains came out on top.

It is important to stress that the nature of the non-aqueous phase is of paramount importance. The fact that tryptophan was more hydrophobic in this scale could imply that this amino acid has an affinity for the membrane interface, rather than the hydrocarbon core. In fact, amino acids with aromatic side chains are often located at the membrane interface in transbilayer helices
and are regarded as being ‘membrane anchors’ (Schiffer et al., 1992). The membrane interface is the most structurally constrained part of lipid bilayers and is composed of “tumultuous chemical heterogeneity” (Wimley and White, 1996). It is, therefore, not surprising that aromatic moieties have an affinity for this region. Geometrically, the flatness of the aromatic ring will allow non-invasive insertion into the interface. The ring system is unpuckered because the carbon atoms comprising it are all sp²-hybridised. Electrostatically, the electron-rich regions above and below the plane are readily available for interfacial non-covalent interactions.

Unfortunately, accurate hydrophobic profiles of peptides do not simply involve summing the hydrophobicities of the component amino-acid residues. Doing this would ignore the conformation factor that takes into account intramolecular non-covalent bonding. Recently, an elegant interfacial hydrophobicity scale was developed that attempts to take secondary structure formation into consideration (Wimley and White, 1996). This scale measures the transfer free energies of whole-residue amino acids. Each amino acid is sequentially placed into a host peptide, namely Ac-Trp-Leu-X-Leu-Leu. The free energy of transfer was then measured from aqueous solution into model membranes composed of DOPC. The host peptide was developed in such a way that it existed as a random coil in both aqueous solution and in the membrane interface, regardless of the guest amino acid. The random coil conformation was confirmed using CD and the interfacial location of a similar
peptide was confirmed by neutron diffraction. As the peptide was in a random coil in both phases, this scale evaluates the partitioning of the covalently bonded peptide bond into account. In effect, this scale uncouples the free energy involved in peptide partitioning and onset of secondary structure. In doing so, the events that occur before the onset of secondary structure are quantified. This type of scale is important when trying to predict the ability of proteins to partition and fold in membranes from their primary sequence. This is illustrated in figure 1.2.

![Figure 1.2](image-url)

**Figure 1.2.** Highly schematic cartoon illustrating of the possible first steps in protein folding. Adapted from Wimley and White, 1996.

The Wimley and White hydrophobicity scale demonstrates that the whole-residue amino acids that have aromatic residues will partition favourably into the membrane interface. Whole residues with small side chains only make
small indifferent contributions. Whole-residues that have charged side-chains and the peptide bond itself make unfavourable contributions. Therefore, it seems unlikely that most peptides will partition into membranes at all. However, certain amino acid sequences have a propensity to form secondary structures within membranes. This process catalyses intra-molecular hydrogen bonding of the peptide backbone, which is known to reduce the free energy of transfer by approximately 4Kcal/mol (Deber and Goto, 1996). Therefore, peptide primary structures confer unique physical properties that allow the molecule to form functionally active conformations in varying physicochemical conditions.

1.5. The tachykinin family

1.5.1. Tachykinin discovery and primary structures

The tachykinin family are a group of small amphipathic peptides that exist in many organisms of the animal kingdom (Maggio, 1984). Five mammalian tachykinins are currently known with NKA and SP being the best characterised. The family name stems from their quick onset of action on gut smooth muscle in comparison to the peptide, bradykinin (brady = slow, kinin = to move). Although SP was the first neuropeptide to be discovered, it was not purified and sequenced until 40 years later (Chang et al., 1971). The first tachykinin to be isolated and purified was eledoisin in 1962 by Espramer and co-workers (Maggio, 1984). Eledoisin was discovered serendipitously in 1949.
when the same investigators were searching for biogenic amines in salivary gland extracts of the Mediterranean octopus *Eledone Moschata*. The novel substance was thought to be proteinaceous in nature and it exerted similar effects to SP. However, the quantity required to exert SP-like effects were markedly different. The very high concentrations of eledoisin found in the octopus salivary gland was the main reason for its early sequencing. The same group then purified the amphibian peptides physalaemin and phyllomedusin in 1964 and noticed that they not only shared the same C-terminal sequence as eledoisin but also had similar biological effects. This led to an accurate prediction of the primary structure of SP before it was sequenced. Other tachykinins have since been discovered. In 1983, Kimura sequenced the cationic tachykinin SK from bovine spinal chord extracts. The letter K stands for kassinin, a structurally homologous amphibian peptide. SK is also referred to as neurokinin A. In the same year came the discovery of an anionic tachykinin, neurokinin B (Kangawa et al., 1983). The primary structures of the tachykinins are shown below.

<table>
<thead>
<tr>
<th>Tachykinin</th>
<th>Primary Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eledoisin</td>
<td>Glu-Pro-Ser-Lys-Asp-Ala-PHE-Ile-GLY-LEU-MET-NH₂</td>
</tr>
<tr>
<td>Kassinin</td>
<td>Asp-Val-Pro-Lys-Ser-Asp-Gln-PHE-Val-GLY-LEU-MET-NH₂</td>
</tr>
<tr>
<td>Physalaemin</td>
<td>Glu-Ala-Asp-Pro-Asn-Lys-PHE-Tyr-GLY-LEU-MET-NH₂</td>
</tr>
<tr>
<td>Phyllomedusin</td>
<td>Glu-Asn-Pro-Asn-Arg-PHE-Tyr-GLY-LEU-MET-NH₂</td>
</tr>
<tr>
<td>SP</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-PHE-Phe-GLY-LEU-MET-NH₂</td>
</tr>
<tr>
<td>NKA</td>
<td>His-Lys-Thr-Asp-Ser-PHE-Val-GLY-LEU-MET-NH₂</td>
</tr>
<tr>
<td>NKB</td>
<td>Asp-Met-His-Asp-Phe-PHE-Val-GLY-LEU-MET-NH₂</td>
</tr>
</tbody>
</table>

The homologous tachykinins vary in length by only one or two amino acids. It is readily observed that the tachykinins have a conserved C-terminal, namely, -Phe-X-Gly-Leu-Met-NH₂. The invariant Phe⁷ residue is probably required
for receptor binding. X is either aromatic (phenyalanine, tyrosine) or a branched aliphatic (valine, isoleucine) side-chain and is thought to be important in receptor selectivity. The more hydrophilic N-terminal varies markedly in amino-acid composition and net charge.

1.5.2. Tachykinin physiology

Two genes encode the three mammalian tachykinins. The gene preprotachykinin A encodes substance P and neurokinin A (Regoli et al, 1994). Differential gene splicing allows the cell to control the amount of each peptide produced. Preprotachykinin B encodes neurokinin B. The C-terminal methionine residues of the preprotachykinin precursors are all followed by glycine that donates its amine group, thus amidating the peptide.

The tachykinins are stored in secretory granules with other peptides (Regoli et al, 1994). Following an action potential, they are released into the synaptic cleft where they interact with post-synaptic, membrane-embedded NK receptor subtypes or diffuse to other sites. There is no evidence for an active re-uptake system. The NK receptors are coupled to G-proteins that act as transducers. SP can activate both the adenylyl cyclase and phospholipase C second messenger systems whereas NKA and NKB can only activate the latter system (Maggi and Schwartz, 1997).

Tachykinins and tachykinin receptors are found in a wide range of tissues including the central and peripheral nervous system, salivary gland and gastro-
intestinal tract. It has been more difficult to locate the distribution of each tachykinin in specific tissues. This is because the type of antisera used has been polyclonal and directed at the conserved tachykinin C-terminal. Also, there does not seem to be a clear correlation between SP innervations and the associated number of binding sites in specific regions of the brain. For example, the highest density of SP is found in the substantia nigra but this area has very few SP binding sites (Maggio, 1988). The inverse situation occurs in the cerebral cortex.

As the tachykinins have a wide distribution in the body, they have many physiological implications. These include vasodilatation, intestinal smooth muscle contraction, salivation and pain transmission. Pathologically, substance P is thought to be involved in aberrant nociceptive pathways and NKA is reputed to cause bronchoconstriction in chronic asthma sufferers (Regoli et al., 1984).

The tachykinins exert their effects via complex interactions with G-protein-coupled neurokinin receptor subtypes. Initially, a “three-agonist-three-receptor-dogma” (Maggi and Schwartz, 1997) was proposed. SP bound preferentially to the NK₁ receptor, NKA to the NK₂ receptor and NKB to the NK₃ receptor. However, the NK receptor family portray a typical 65% shared homology. Usually, this infers that the same ligand binds to the all receptor subtypes (e.g. only 5-HT binds to 5-HT receptor subtypes). In the case of the structurally homologous tachykinins, receptor cross-reactivity is therefore
exhibited. Recently, homologous binding assays have shown that NKA affinity for the NK1 receptor is comparable to that of SP (Hastrup and Schwartz, 1996). Strikingly, the ability of NKA to displace radiolabeled SP is weak but SP can easily displace the other tachykinins (Wijkhuisen et al., 1999). Non-peptide antagonists of SP behave competitively for SP but not for NKA. This has led to the suggestion that the NK1 receptor has either two distinct tachykinin binding sites or a conformational dual nature that prefers SP recognition (Maggi and Schwartz, 1997).

The exact location of the tachykinin binding site(s) for all three NK receptors is still unknown. Small ligands such as the catecholamines, which also bind to receptors that belong to the G-protein-coupled receptor superfamily, appear to bind within the cell membrane. However, peptidic ligands are significantly larger than the classical neurotransmitters and the location of their receptor binding domains remains ambiguous. Chimeric receptor binding studies utilising both the NK1 and NK2 receptor indicate that both the trans-membrane segments and the extra-cellular domains of the receptor may be involved (Yokota, 1992). However, these types of experiments are never unambiguous because the chimeras are assumed to have the original receptor conformation.

The NK receptor portrays significant trans-membrane sequence similarity compared to the extra-cellular domains. This may lend support to an argument for tachykinin binding within the membrane as it indicates that the trans-membrane domain is a functionally important part of the receptor protein.
However, the transmembrane domains may have sequence homology primarily due to structural considerations. Empirically, the more 'hydrophobic' amino acids are selected to comprise trans-membrane helices. This selection process is not as stringent for the extra-cellular domains, which consist of more divergent amino acids. The energetic cost of transferring a hydrophilic residue into apolar solvent is far higher than transferring a hydrophobic residue into aqueous solution (Wolfenden et al., 1981). Indeed, section 1.4 explained that many hydrophobic residues are located near surfaces.

In contrast, there is increasingly strong evidence that binding may occur on the extra-membranous side of the lipid bilayer. Elegant structure-function studies have shown an inverse relationship between lipid binding and receptor affinity (Seelig et al., 1996). In order to isolate some of the physico-chemical properties involved in the binding processes, these investigators synthesised three different SP analogues. Two of the SP analogues had Gly⁹ substituted by arginine or leucine thereby making the molecule more hydrophilic and hydrophobic respectively. A third analogue replaced the Arg¹ with an acetyl-proline moiety and also Gly⁹ with arginine. Increasing the hydrophobicity of the C-terminal, as in (Nle⁹)-SP, or increasing its charge, (Arg⁹)-SP, was found to increase membrane binding. However, both analogues had a reduced affinity for the NK₁ receptor when compared to SP. In addition, it was demonstrated that two SP analogues that had either a charged or polar C-
terminus, which is unlikely to insert into the hydrophobic core of membranes, still show SP N\textsubscript{K\textsubscript{1}} receptor agonist activity (Seelig, 1992, Seelig et al., 1996). Therefore, it appears that the N\textsubscript{K\textsubscript{1}} binding site is not within the hydrocarbon core.

One molecular recognition theory predicts that peptide-protein interactions can occur if the amino acids possess inverse hydropathy values (Bost and Blalock, 1989). Predictions for the N\textsubscript{K\textsubscript{1}} receptor and the conserved NKA C-terminal calculated that residues 193-197 at the end of the receptor’s second extracellular loop could be involved in binding. Subsequent point mutagenesis within this region abolished NKA binding but did not alter SP binding (Wijkhuisen et al., 1999). The same program predicted that residues 20-26 located at the N-terminus of the receptor could be involved in SP binding. This agrees well with binding studies that used mutant N\textsubscript{K\textsubscript{1}} receptors (Fong et al., 1992a) and chimeric N\textsubscript{K\textsubscript{1}} receptors that had N\textsubscript{K\textsubscript{3}} extra-cellular loops (Fong et al., 1992b).

Therefore, recent studies imply that the N\textsubscript{K\textsubscript{1}} receptor has two distinct binding sites for the tachykinins; a specific SP binding site and a general tachykinin-binding site. The putative general tachykinin-binding site, located at the end of the second extra-cellular loop, is in close proximity to the interface of the cell membrane. The amphipathic nature of the tachykinin primary sequence has led to the postulation that the membrane itself may have the capacity to mediate receptor selectivity and affinity. This has stimulated a number of
structural biologists to investigate the lipid-induced structure of the tachykinins.

### 1.5.3 Tachykinin-lipid interactions

The study of tachykinin-lipid interactions may provide insights into their 'biologically active conformation' assuming that such peptides have an important interaction with the post-synaptic cell membrane prior to receptor binding. The lipid matrix acts as a solvent for the receptor protein and is an important medium with regards to the conformational development of the receptor and ligand (Gysin and Schwyzer, 1984, Gremlich et al., 1984). Tachykinin binding studies using chimeric and mutant receptors have been carried out due to the lack of high-resolution structural data. Although the binding site(s) could be located extra-cellularly, tachykinin studies in the presence of lipid is still biologically relevant. All the structural data so far involve a simpler ternary system. That is, studying tachykinin-lipid interactions in the presence of solvent water but in the absence of any receptor domain. Thus, the peptide structures solved are likely to be intermediates that probably exist prior to receptor binding. These intermediate structures are therefore subject to receptor-induced non-covalent forces that may further alter their 3-dimensional shape. Nevertheless, there is a relationship between similar intermediate membrane-associated structures and receptor affinity (Schwyzer, 1995, Seelig et al., 1996).
As mentioned in section 1.3, it is difficult to solve or predict 3-dimensional structures for peptides. The tachykinin family is no exception. In aqueous solution, this difficulty is primarily due to peptide flexibility. Thus, any attempt to crystallise the peptide may isolate only one unimportant conformer. Further, the structure of such a conformer may be induced by lattice packing constraints. The development of CD and $^1$H-NMR techniques has yielded more information on tachykinin structure.

SP has a solubility of approximately 10mg/mL. CD and FTIR experiments of SP in aqueous solution show the peptide to exist as a series of inter-converting conformers due to the free rotations around carbon-carbon $\sigma$-bonds (Chassaing et al., 1986, Choo et al., 1994). The random coil conformation observed is predicted for the other family members as well. However, $^1$H-NMR spectroscopy results indicate some folding at the C-terminal (Chassaing et al., 1986). This is likely to be a small sub-population that may increase when associated to the membrane (Woolley and Deber, 1987).

Although the solution structure is highly flexible, the biologically active conformation of the tachykinins (and other peptides) is thought to be fixed and unique (Milner-White, 1989). It is thought that the cell membrane plays a part in this ligand-receptor interaction (Schwyzer, 1987). If the tachykinins were to insert into the anisotropic confines of a membrane, their structural flexibility would diminish. But in the isotropic medium of the aqueous phase, the tachykinins would have the ability to rotate and translate in all directions.
However, membrane-inserted tachykinins would experience discrete rotational and translational symmetry. This would increase the opportunity for ligand-receptor binding site collisions because both interacting components are now structurally constrained. This hypothesis becomes important if there are a low number of NK surface receptors. The chances of a highly flexible solution-state peptide binding in a biologically active conformation to a fixed active site would be low.

Originally, an ‘address-message’ model had been proposed for the tachykinins (Schwyzer, 1987). This involved the flexible cationic N-terminal region, or “address domain”, being responsible for receptor selectivity and the structurally extended and conserved hydrophobic C-terminal delivering the “message”. It was thought that once the tachykinin had inserted into the membrane the peptide would then laterally diffuse (random walk) within the bilayer plane. This would increase the chance of a receptor collision. This message-address model may be valid but is probably an oversimplification. The model is based on the primary structure of each tachykinin and does not take into account the conformational factor. It is likely that the peptide interacts with the membrane lipids and receptor protein simultaneously resulting in the formation of initial binding domain contacts. A process of induced-fit would result where the receptor, tachykinin molecule(s) and possibly even specific lipid molecules undergo transient, short-lived conformations. This type of dynamic interaction, which may also involve
inorganic ions and water molecules, is presently impossible to detect or measure.

The extent of peptide binding to membrane surfaces (and hence peptide accumulation in the receptor microenvironment) is governed by two main physical phenomena.

One phenomenon is electrostatic in nature and may be explained by the Gouy, Chapman and Stern electrical double layer (Florence and Atwood, 1988, Shaw, 1992). Briefly, a charged micelle or liposome surface will attract counter-ions in order to reduce the electrostatic repulsions between individual surfactant head-groups and neutralise the surface charge. This phenomenon creates an electrical double layer. The inner Stern layer is about the width of a hydrated ion radius from the particle surface. This layer is very compact and is where tightly bound counter-ions are located. The outer layer is more diffuse and contains more appreciable concentrations of co-ions. It is possible that charged peptides could bind to oppositely charged surfaces in the same manner thereby increasing their local surface concentration relative to the bulk solution phase. The chemical environment within the Stern layer can elevate the pKₐ values of accessible side chains by 3 pH units because of the higher proton concentration observed at an anionic surface compared to the bulk phase. This is significant for amino acids such as histidine, which are normally uncharged at physiological pH but could express a charge at an anionic lipid surface.
Hydrophobic interactions could also be involved due to the inherent amphiphilicity of the peptide. Here, amphiphilic peptides bind (non-specifically) to surfaces in order to protrude their hydrophobic amino acid side chains into the hydrocarbon core, thereby avoiding the aqueous phase.

Langmuir film studies have demonstrated that the interaction of SP with monolayers seems to be governed by electrostatic properties rather than hydrophobic forces (Seelig and MacDonald, 1989). This technique has revealed that SP will insert into monolayers composed of anionic POPG but not into monolayers composed solely of zwitterionic POPC. SP binding to monolayers composed solely of POPG was relatively fast, reaching equilibrium after 10 minutes. However, it should be emphasised that the extent of peptide insertion into monolayers was a function of the lateral pressure applied. This study covered a lateral pressure range of between 21-31mN/m. A lateral pressure of 32mN/m is regarded as being physiological (Seelig and MacDonald, 1989). At this physiological pressure, the POPG monolayer expansion value was barely measurable and extrapolated from the observed points. These results must be viewed with caution because this technique employs monolayers rather than bilayers, by definition. The hydrocarbon chain packing in monolayers at the air-water interface will not be as tight as in the bilayers of the cell membrane that contain such lipids as cholesterol. Nevertheless, these results clearly discriminate between SP binding to POPG and POPC. Unlike POPG monolayers, SP could not insert
into POPC monolayers at any lateral pressure. A SP binding constant of $3.2 \times 10^{-4}\text{M}$ was calculated for monolayers composed solely of POPG at 20mN/m. Therefore, this study suggests that lipid head-group net charge controls peptide binding to the monolayer surface rather than non-specific hydrophobic interactions.

CD studies have also indicated that surface charge is important. SP can change conformation when anionic lipid species such as SDS or LPG are introduced to the solution (Woolley and Deber, 1987, Seelig et al., 1996). The concentration of detergent added was slightly above each respective surfactant’s cmc. The conformation did not change as a function of increasing detergent concentration. No such conformational changes were seen if the same amount of zwitterionic LPC is added. Only when LPC concentrations were one order of magnitude higher than that of SDS was similar SP conformational changes observed. Correlating the CD spectra with the protein predictive methods of Chou and Fasman, detergents are thought to increase the amount of $\alpha$-helical structure within the peptide. More recent CD studies attempted to quantify this system further. The low helical content of SP was approximately 12% in SDS and 10% in DPC (Keire and Fletcher, 1996). Therefore, both the monolayer expansion and CD studies indicate that SP will insert into anionic monolayers or micelles and portray a weak helical nature.

$^1$H-NMR studies have shed greater light on the SP structure. The detergent-induced conformational changes observed in CD studies were also observed in
$^1$H-NMR studies (Woolley and Deber, 1987). Both SDS and LPC caused a marked change in the proton chemical shift assignments of SP relative to the solution state. This is indicative of the peptide’s protons residing in a different magnetic environment. These investigators observed the existence of only one, possibly helical, conformer that was in the all-trans configuration. SP was monomeric when associated to detergent, as its subsequent CD spectra was completely different to that of the aggregated peptide in solution (Choo et al., 1994). SP does not change conformation with respect to increasing lipid concentrations, which is in agreement with previous CD results (Young et al., 1994). The chemical shifts were also much broader in nature inferring a significant reduction of SP mobility. The weaker binding of SP to LPC micelles was exploited in order to resolve some of the chemical shifts. The chemical shifts of the C-terminal amino acids were shifted upfield indicating that this part of the peptide interacted with the micelle. There was no chemical shift change in the N-terminal lysine residue inferring a surface location for this part of the peptide. The structure of SP in SDS and LPC was remarkably similar.

Recent high-resolution $^1$H-NMR studies have also confirmed these findings (Keire and Fletcher, 1996). It appears that the structure of SP is similar regardless of lipid head-group type but there is a difference in exchange rates. Determination of the chemical equilibrium constant for each micelle system has revealed that SP undergoes fast chemical exchange in DPC but not in SDS.
Therefore, SP interacts at the SDS micelle surface to a greater extant than at DPC micelle surfaces in agreement with SP not inserting into POPC monolayers (Seelig and MacDonald, 1989).

The exchange-averaged chemical shifts of SP when associated to SDS or DPC micelles do not change after the addition of 150mM NaCl indicating that a change in the ionic conditions of the solution does not alter the SP binding constant. CD results have also revealed no change in SP secondary structure under the same circumstances (Keire and Fletcher, 1996).

The addition of Na\(^+\) ions was an attempt to compete with SP for the SDS and DPC surface and, in doing so, diminish any electrostatic interactions present. In the case of DPC, the lack of effect signifies that hydrophobic forces dominate the SP binding. The same argument can be applied to the SP-SDS system but a dominant electrostatic attraction also exists which accounts for the longer binding times. Hypothetically, it may be possible to quantify the strength of the electrostatic interaction by measuring the chemical shift exchange rates in the presence of other salts. NaCl is regarded as neutral in the Hofmeister series (Collins and Washabaugh, 1985). That is, NaCl is neither a water structures maker (polar kosmotrope) nor a water-structure breaker (polar chaotrope). The addition of a polar kosmotrope such as ammonium sulphate may alter both the binding constant and structure of SP. Ammonium sulphate is excellent at ‘salting-out’ soluble proteins by competing avidly for binding sites with water molecules. In the SDS system, the sulphate ion of the salt will
compete with the detergent head-group for the N-terminal positive charge of SP. Additionally, the ammonium ion will neutralise the micelle surface charge. Recording the change in chemical shifts of SP as a function of the salts of the Hofmeister series may give a handle on the strength of electrostatic interaction between the peptide and micelle surface.

The $^1$H-NMR studies to date indicate a surface or interfacial location for SP. It is important to note that the micelle interface is not a fine boundary line but a complex and diffuse layer. It is proposed that the N-terminal Arg$^1$-Pro$^2$-Lys$^3$-Pro$^4$- is flexible and located within the electrical double layer (Keire and Fletcher, 1996, Keire and Kobayashi, 1998, Young et al., 1994). The N-terminal has a net formal charge of +3. This implicates a micelle surface location for N-terminal and the formation of ionic bonds with the detergent sulphate groups. It is known from protein catalysis that the chaotropic guanidino moiety of arginine always makes an irreversible ligand-carboxylate interaction when the carboxylate is not to be transformed during the enzymatic reaction (Wigley et al., 1987). In contrast, arginine is never present when the carboxylate has to be transformed. This suggests functional importance for the arginine of SP regarding binding to the anionic lipid surface and possibly to the NK receptor itself. It is interesting to note the loss of Arg$^1$ in a potent SP analogue led to a loss of specificity for the NK$_1$ receptor (Seelig et al., 1996).

The C-terminal –Gln$^5$-Gln$^6$-Phe$^7$-Phe$^8$-Gly$^9$-Leu$^{10}$- is thought to interact more intimately with the micelle interface. A helical twisted-turn structure has been
proposed where the Phe\textsuperscript{7}, Phe\textsuperscript{8} and Gly\textsuperscript{9} insert their side chain protons into the micelle interface while Gln\textsuperscript{5}, Gln\textsuperscript{6} protons are located on the surface (Keire and Fletcher, 1998). The locations of the Met\textsuperscript{11} protons were ill-defined indicating a more general surface location. In addition, the structure of the biologically active SP fragment, -Gln\textsuperscript{6}-Phe\textsuperscript{7}-Phe\textsuperscript{7}-Gly\textsuperscript{8}-, was also found to be spatially very similar to the same part of the SP parent molecule (Keire and Fletcher, 1996).

The previously mentioned SP analogues of Seelig also portrayed helical content in SDS micelles. This was calculated to involve approximately four residues for the SP analogues and three for SP itself. Both (Arg\textsuperscript{9})-SP and (Nle\textsuperscript{9})-SP stabilised the helix by increasing its length but this was to the detriment of NK\textsubscript{1} binding. There seems to be a fine balance between SP’s structure-activity relationship.

The presence of Phe\textsuperscript{7} seems to be functionally significant. A binding study where each amino acid residues was systematically replaced by L-alanine demonstrated that Phe\textsuperscript{7} was the most important (Couture \textit{et al.}, 1979). However, the exact role of the aromatic ring is still debatable. For instance, the addition of strong electron donating or withdrawing groups onto the para position did not affect SP potency (Couture \textit{et al.}, 1979). It is possible that restricted rotation about the phenyl nucleus is functionally important.

The role of the variable amino acid at position 8 is unclear. Early classification of the tachykinins was based upon their ability to stimulate certain tissues. The
biological responses seemed to correlate well with the nature of the amino acid in this position (Buck and Burcher, 1986). A group of tachykinins that were potent in certain peripheral tissues also had an aromatic residue at position 8. The receptors that they acted on were then classified as SP-P receptors. Here, the letter P stands for physaleamin, which was the most potent agonist. The second tachykinin group had more affinity at other sites and had an aliphatic side chain at position 8. The receptors that they acted on were then classified as SP-E receptors, the letter E standing for eledoisin. However, swapping aromatic Phe\textsuperscript{8} of SP for aliphatic Ala, and Ile\textsuperscript{8} of eledoisin for Phe did not reverse the tissue selectivity as expected. Therefore, this type of point substitution is too simplistic and cannot explain tachykinin tissue selectivity.

The SDS-associated structure of NKA and NKB is similar to that of SP (Whitehead et al., 1998). Both peptides display helical nature as the predominant secondary structure. For NKA this helicity is located around Phe\textsuperscript{6}-Val\textsuperscript{7}-Gly\textsuperscript{8}-Leu\textsuperscript{9}- but for NKB the helicity is present throughout the molecule.

The two adjacent phenylalanine ring systems of SP can be exploited in order to gather structural information. Both chromophores will absorb UV radiation at a specific wavelength that is dependent on their environment. In solution the maximum UV absorbance is ‘red-shifted’ when SDS is added (Woolley and Deber, 1987). Empirically, this is correlated to a change from a hydrophilic environment to a more hydrophobic environment, for example the micelle
hydrocarbon core. This is in agreement with the NMR results. However, the finding that the free acid of micelle-bound SP had an identical CD spectrum to that of SP itself suggests that the peptide lies on the surface and only 'dips' its aromatic residues into the more hydrophobic micelle interface.

A molecular dynamics simulation of SP in bilayers composed of DMPC also calculated that the phenylalanine residues face the hydrophobic core (Kothekar, 1996). The calculation revealed that the lipid bilayer immobilised the peptide and induced a C-terminal helical conformation. Furthermore, one of the DMPC molecules altered its conformation in order to allow SP penetration. SP also lowered the number of water molecules associated with each lipid head-group indicating interfacial dehydration. Displacement of water from the interfacial region may explain the earlier observed red shifts in the UV spectrum.

X-ray or neutron diffraction techniques on highly aligned, phospholipid multibilayer stacks can also be employed in order to determine lipid-associated peptide structures (Worcester, 1976, Franks and Leib, 1981). Peptide depth of insertion observations can also be achieved using recent magic angle spinning $^{13}$C NMR technologies (Grobner et al., 1999). The diffraction techniques can be regarded as being a more physiological method than many of the other structural techniques mentioned because phospholipid bilayers are employed rather than monolayers. However, the lipids are not quite fully hydrated.
Phospholipid hydration states have been investigated in some considerable depth (Rand et al., 1988). With regards to species of lipid headgroup, PC has a higher hydration level than PE (Yeagle, 1993). The quaternary nitrogen of PC can only weakly H-bond to an adjacent phosphate anion due to steric hindrance of the methyl groups. Therefore, water molecules can compete well with the charged centres contained in a PC headgroup and the complete PC hydration shell is between 16-25 waters per lipid. PE surfaces are regarded as being dehydrated. There is no steric hindrance preventing a strong interaction between the nitrogen lone pair and phosphate anion. In fact, there is evidence of trans-bilayer adhesion between PE containing membranes (McIntosh and Simon, 1996). Therefore, water molecules are largely precluded from H-bonding and must bond with themselves in an ordered fashion. Water ordering increases the free energy status of the system and is another explanation why lipids that contain PE will readily form inverted phase structures.

Neutron diffraction has determined the location of the C-terminal leucine in bilayers composed of DOPC and bilayers composed of DOPC:DOPG (50:50 mol) to an accuracy of 1Å (Bradshaw et al., 1998). This study revealed that the location of Leu⁹ was very similar, regardless of lipid composition. This agrees entirely with previous ¹H-NMR work (Woolley and Deber, 1987, Keire and Fletcher, 1996). The diffraction results also revealed that there were two populations of label associated with the phospholipid bilayer, one 6-8Å from the bilayer center and the other 22Å from the bilayer center. This implies that
SP can take up two different membrane-associated conformations. One orientation is thought to be approximately parallel to the membrane surface while the other involves C-terminal insertion approximately perpendicular to the surface. These results are in agreement with the message-address hypothesis where the C-terminal must submerge into the phospholipid hydrocarbon core in order to activate the receptor but do not entirely agree with the solution NMR results. This is discussed further in chapter 3.

In conclusion, the functional and structural results regarding the tachykinins do not illustrate a clear mechanism of action. Functional studies have revealed that there is a possibility that the NK₁ receptor may exist as two conformers, which may result in re-classification of this receptor. Structural studies have mainly employed ternary monolayer systems in order to obtain results. It is easy to think of the tachykinins as molecules composed of two physicochemical halves. The fact that the tachykinins are so obviously amphipathic in primary structure can be misleading. This has been fuelled by the observation that the C-terminus retains some biological activity in peripheral tissues whilst the N-terminal fragment is inactive. Much attention has centred on the C-terminus with regards to receptor selectivity. This is because of its ease of structure determination. In particular, the variable amino acid at position 8 has come under close scrutiny. Although most of the structural studies have attempted to resolve the C-terminal conformation, less is known about the divergent flexible N-terminal. It is likely that the orchestrated action of each
tachykinin requires the intimate cooperation of each of its component amino acids.

Finally, the tachykinins have been implicated in other biological circumstances. One has been that the peptide is hydrolysed in the synaptic cleft by proteases, resulting in active and inactive fragments (Regoli, 1994). It is known that the C-terminal is active in the periphery whilst the N-terminal is active in rat brain (Sandberg and Iverson, 1979). Tachykinin fragments have been found in malignant tissue (Theodorsson-Norheim et al., 1987). If tachykinin fragments are physiologically important then their inherent amphipathicity may ensure efficient packing/folding profiles when the peptide is in the highly concentrated confines of the secretory vesicles.

Another suggested involvement of the tachykinins is in amyloid plaque formation. It has been demonstrated that the functional domain of amyloid β protein is contained in the sequence β 25-35 (Yankner et al., 1990). This sequence shares 73% homology to eledoisin and at least 56% homologous to the other tachykinin family members. Furthermore, the region of greatest homology was the conserved C-terminus. It was found that the tachykinins reversed amyloid toxic effects but tachykinin antagonists mimicked them.

1.5.4. Aims of the project

The goal of this study is to elucidate the structure and conformation of NKA in lipid-based systems. The results of the study are to be used in a direct
comparison with the better-characterised SP. Two biophysical approaches are used to characterise the NKA structures. These are circular dichroism and neutron diffraction.

Far-UV CD spectrophotometry gives information on a peptide’s secondary structure. The secondary structure of NKA is evaluated by incorporating the peptide into a range of increasingly hydrophobic alcoholic solvents and also micelles composed of DPC and SDS. In some samples a couple of different salts from the Hofmeister series are introduced and the effects recorded. An attempt is also made to incorporate NKA into liposomes composed of DOPC:DOPG so that a direct comparison can be made with the peptide’s conformation in SDS. The conformational results of NKA are used in a final model once the neutron diffraction measurements had been carried out.

Lamellar neutron diffraction gives information on a bilayer’s unit cell size and the distribution of water between them. By adding NKA, any change in the original bilayer structure can be noted. Also, by selectively deuterating part of the NKA molecule, it is possible to calculate its location relative to the bilayer normal to a resolution of 1Å. Therefore, neutron diffraction results can help clarify whether tachykinins bind on the membrane surface or within the hydrocarbon core. This type of structural information is not available using NMR.

In order to normalise neutron diffraction data it is sometimes necessary to determine the average number of waters associated to each lipid molecule.
This experiment is also useful in its own right as it gives information on the peptides ability to hydrate or dehydrate the membrane surface.

1.6. Biomembrane fusion

1.6.1. Introduction to biomembrane fusion

Biomembrane fusion is a ubiquitous process that plays a crucial role in such fundamental events as fertilisation, intra-organelle trafficking, endo- and exocytosis and mitosis. Despite this fact, the structural changes of the participating protein and lipid molecules and the precise kinetic events involved are still unknown. This is because the fusion event is extremely transient and involves only local, isolated patches of lipid.

Biomembrane fusion is a protein-regulated event (White, 1990). Enveloped virus particles exploit the fusion pathway in order to introduce their infective nuclear material into the host cell. The most widely studied membrane ‘fusion protein’ is the HA of the influenza A virus (Hughson et al., 1994). This virus, like all animal enveloped viruses, utilises a specialised, extra-membranous glycoprotein ‘spike’ as a fusion catalyst.

Nature has chosen to develop this intricate process because of a cell’s need to compartmentalise in order to function. They do this by employing a semi-permeable barrier of low dielectric bulk. This is the lipid bilayer, which is now well established as the main structural motif of the cell membrane (Singer and Nicholson, 1972). The partitioning behaviour of a molecule into a lipid bilayer
is governed by its size and hydrophobicity profile. Small hydrophobic molecules generally partition readily. Small, uncharged molecules such as water and urea will also partition with ease. Polar molecules such as glucose do not have a tendency to cross the lipid bilayer by passive diffusion but certain small ions such as protons and hydroxyl ions will cross readily. Macromolecules such as viral nuclear material will not be able to passively diffuse through membranes into the host cell and other mechanisms of transport have, therefore, been developed of which biomembrane fusion is one. Membrane fusion can be defined as the formation of one continuous membrane from two, originally distinct, planar bilayers. This is illustrated in figure 1.3.

![Diagram of biomembrane fusion](image)

**Figure 1.3.** Cartoon for the biomembrane fusion reaction. The viral particle is shown to be spherical but merging membranes are actually planar in nature. The question mark represents the presently unknown lipid structural intermediates involved. Modified from Hughson, 1995.
Although liposomes are regarded as being spherical, close contact between membrane bilayers is geometrically planar. An exponentially increasing repulsive force hinders their close approach from approximately 20Å inwards (Leiken et al, 1993, Israelachvili and McGuiggan, 1988). This is because close-approaching membranes require the expulsion of tightly bound water molecules from each membrane interface (Hughson, 1995). The extramembranous proteins may catalyse the fusion event by dehydrating the membrane surface thereby circumventing the hydration force. This would increase the opportunity for close membrane approach and, therefore, fusion.

The reaction can be measured by doing fusion assays that employ fluorophores such as fluorescein isothiocynate or rhodamine (Hoekstra and Klappe, 1993). Generally, these fluorophores are attached to either the fusion protein or participating lipid membranes. Upon addition of the fusogenic components into a solution of unlabeled liposomes, a change in intensity is measured as a function of time. A fusion reaction typically lasts for between two and five minutes depending upon experimental conditions. Varying the conditions, for example lowering the temperature, can help dissect the fusion process.

Figure 1.4 shows one way that an animal virus can enter a host cell. This mechanism of entry is characteristic of Semliki Forest virus and influenza A virus.
Figure 1.4. This highly schematic cartoon illustrates the unwanted entry of a virus particle via receptor-mediated endocytosis. The fusion event of interest occurs when the virus particle is in the endosome. Adapted from Simons et al., 1995)

The fusion behaviour of influenza A virus is the best documented mainly because the crystal structure of the ectodomain of HA has been solved (Wilson et al., 1981). Therefore, HA-induced biomembrane fusion is the model to which most fusion processes are related.

The HA spike protein of influenza A binds to sialic acid (N-acetyl neuraminic acid) that is expressed on the glycoproteins and glycolipids of the host cell membrane (Stegmann et al., 1986). The cell-surface receptors are located in specialised areas of the plasma membrane called coated pits. The binding is irreversible and affinity depends on the influenza strain (Wiley and Skehel, 1987). Upon binding, the virus particle is endocytosed into the coated pit which then transforms into a coated vesicle. The coated vesicle then fuses with an endosome. The lower pH within the endosome triggers irreversible
conformational changes in HA. These changes cause the exposure of the so-called ‘fusion peptide’ to the endosomal membrane (Wiley and Skehel, 1987). The fusion peptide catalyses the merging of the viral and endosomal membrane leaflets (White, 1990). During the fusion event there is an orchestrated change in the structure of both the fusion protein and in the membrane lipids. The end result is the creation of a fusion pore that could allow the passage of the viral genome into the target cytoplasm (Spruce et al., 1989, Spruce et al., 1991).

Although the fusion activity of influenza A virus and a number of other viridians require a low pH, others will catalyse biomembrane fusion at physiological pH. Here, the fusion ‘trigger’ is the initial virus-cell binding process rather than low pH activation. For example, the binding of the HIV glycoprotein spike, gp120, to host CD4+ and CD8+ receptors and co-receptors will cause gp41 to promote biomembrane fusion (Weissenhorn, 1997).

1.6.2. The role of proteins in fusion

Biomembrane fusion is a protein-controlled event. As explained in section 1.6.1, HA-mediated fusion is the best characterised and, therefore, presents a model to which other fusion mechanisms are based. This section describes the structure of HA at neutral pH and at pH5, where HA becomes fusion competent. It is important to have an appreciation of these structures because they will be related to the final protein structures that form at the end-stage
fusion pore. The structure of the HA at the site of the fusion pore has yet to be experimentally demonstrated. Fusion proteins of other viridians may behave similarly.

The most abundant protein on the viral surface of influenza is HA. Since the crystal structure of HA was solved in 1981 (Wilson et al., 1981) much information has evolved revealing the "unprecedented" molecular rearrangements the glycoprotein undergoes at pH5.

A type I integral membrane glycoprotein, HA is a trimer of 70kD identical subunits, each of which contain a fusion peptide (figure 1.5). Each subunit is manufactured from a single mRNA and is cleaved post-translationally to form HA1 and HA2 glycopolypeptides that are linked by a disulfide bond (Wiley and Skehel, 1987). HA can be proteolytically cleaved ten residues from its transmembrane domain by bromelain. This gives the soluble ectodomain of HA, termed bromelain hemagglutinin (BHA), and it is this crystal structure that has been determined.
Figure 1.5. Ribbon structure of monomeric BHA at pH7. A = HA1 globular head domains, B = HA2 fibrous stem and C = location of the hydrophobic fusion peptide. Modified from Hughson, 1995.

X-ray crystallography has revealed that the HA2 chain is anchored in the viral membrane near its carboxy-terminus, which forms a short tail in the lumen of the particle. The glycoprotein has a very extended structure where the first 63 amino acid residues of HA2 project 96Å from the viral membrane (Wiley and Skehel, 1987). The three HA2 subunits form a triple stranded α-helical coiled coil with some help from HA1. This forms a stem from which the three...
globular HA1 head groups can branch. A depression in the distal tips of each of the three HA1 subunits forms the highly conserved binding site for sialic acid (Wiley and Skehel, 1987). Apart from the transmembrane sequence of HA2, there is one other hydrophobic sequence per subunit and it is located next to the post-translational modification site.

This is the “fusion peptide” and is the most conserved region of the glycoprotein (Hughson, 1995). They are located in the fibrous stem of each HA2 approximately 35 Å from the transmembrane domain and 100 Å from the distal tips (Hughson, 1995). At neutral pH, the fusion peptides act as ‘hydrophobic glue’ within the interfaces of the α-helices of HA2 (Carr and Kim, 1993). This gives the molecule stability at neutral pH. Low pH treatment of HA results in an insoluble product. This is due to the exposure of the hydrophobic fusion peptides that mediates lipid binding. The fusion peptides can be removed from the trimers by thermolysin leaving a water-soluble product, thermolysin bromelain hemagglutinin (TBHA2) (Hughson, 1995). X-ray crystallographic analysis of this soluble fragment, TBHA2, at pH 5 has given insights into the conformational changes that occur in the stem region (Bullough, 1994). The structure of TBHA2 may be the active structure of HA2 immediately prior to the fusion event.
Figure 1.6. Cartoon for the HA fibrous stem region that undergoes molecular rearrangements upon acidification. The horizontal bold lines at the top and bottom represent the target and viral membranes respectively. Modified from Hughson, 1995.

The major structural feature of the stem region is the hairpin loop between the two α-helices (Figure 1.6). Only thirty residues in the middle of the stem (C) retain their neutral pH conformation (Hughson, 1995). At pH 7, the fusion peptide is attached to a "buttressing" helix (A) which is attached to an extended loop (B). The loop bends over the stem facilitating the fusion peptide’s location into the central coiled coil. Upon activation, the helical
region adjacent to and below the stem centre (D) "splays out like a tripod leg" into the centre of the coiled coil displacing the fusion peptide. The extended loop jack knives back onto the middle stem region onto which the buttressing helix also stands. These molecular rearrangements project the fusion peptides by 100Å to the distal tips of the molecule.

At neutral pH, HA facilitates the juxtaposition of the viral and target membrane. HA can achieve this due to its fixed viral transmembrane 'anchor' and its sialic acid binding domains. But at this stage, the fusing membranes are approximately 100Å apart, separated by the long coiled-coil of HA2. For the fusion reaction to proceed, the planar membranes must come into close apposition, the unfavourable hydration forces removed and the two bilayers form highly-bent intermediates. This energetically unfavourable process is catalysed by the participating fusion protein, which allows fusion to proceed at kinetically acceptable rates. The exact structure and mechanics of HA during these events are unknown.

Somehow, the HA molecule must move out of the way and allow the membranes to merge (figure 1.7). One model proposes a "spring-loaded" trap mechanism where HA undergoes further conformational changes in order remove the HA1 globular binding domains (Carr and Kim, 1993, Carr et al., 1997). This would facilitate the exposure of the fusion peptide to the target membrane and avoid possible HA1 steric hindrance. This model emphasises the importance of the kinked-loop region of the HA2 sub-unit, which is in-
between domain C and D in figure 1.6. Another study demonstrated that sequentially substituting the acidic amino acids in this region dramatically reduced the rate of fusion of liposomes (Epand et al., 1999). Importantly, this demonstrates that the protonation state of the loop region is required for the HA2 domain to be fully active. The loop region may also be involved in the clustering of HA trimers (Kim et al., 1998) which is able to promote a tilting of the HA2 stem domain relative to the membrane surface (Tatulian et al., 1995).

The β-sheet domains that are close to the viral membrane are flexible and may permit a large degree of tilting after fusion peptide insertion into the target membrane.

There is ongoing research into many aspects of the fusion event including protein structure determination, lipid- and aqueous-contents mixing fusion assays, molecular modelling and electrical conductance studies. This thesis concentrates on fusion peptide interactions with model membranes rather than the study of protein-induced fusion. A body of evidence has already suggested that the N-terminal region of certain specialised viral glycoproteins play a role in membrane fusion (White, 1990, White et al., 1991, Horth et al., 1991). The fusion peptides have the task of perturbing the target membrane in such a way as to promote fusion. The fusion peptides of a number of viridians can promote liposome-liposome fusion (Lear and DeGrado, 1987, Martin et al., 1991).
Figure 1.7. A highly schematic model for the haemagglutinin-induced biomembrane fusion event. The first picture illustrates close membrane approach, where the HA molecule is anchored in the viral membrane. The next picture shows HA in a fusion active state with the fusion peptide inserted into the target membrane. At this point, the trimer must move away in order to allow membrane apposition and fusion pore formation. Modified from Hughson, 1997.

The fusion peptide sequence is conserved within but not between virus families (White, 1990). They range in length from 24 to 36 amino acids, have a termination point as the first positively charged residue and are rich in glycine and alanine residues. Some fusion peptides have a tendency to form amphipathic helices whilst others show a tendency to form sided helices, bulkier residues on one face and the smaller apolar glycines and alanines on the other (White, 1990).

Two fusion peptides are studied in this thesis; the fusion peptide of SIV and the fusion peptide of FeLV. Their primary sequences are shown overleaf.
Fusion peptides

SIV     Gly-Val-Phe-Val-Leu-Gly-Phe-Leu-Gly-Phe-Leu-Ala
FeLV    Glu-Pro-Ile-Ser-Leu-Thr-Val-Ala-Leu-Met-Leu-Gly-
         Gly-Leu-Thr-Val-Gly-Gly-Ile-Ala-Ala-Gly-Val-Gly-
         Thr-Gly-Thr-Lys

Both fusion peptides are the hydrophobic N-terminus of a transmembrane region of a viral envelope glycoprotein spike. In the case of the SIV fusion peptide, this is the N-terminus of gp32, which itself is cleaved from gp160 (Bosch et al., 1989). The fusion peptide of FeLV is the hydrophobic N-terminus of p15E, the putative transmembrane region of the viral envelope glycoprotein spike gp85. Fusion assays have demonstrated that the most active version of SIV peptide is only 12 amino acids long (Martin et al., 1991). The p15EK is also known to be fusogenic (Davies et al., 1998) and is thought to have an active truncated form (Brasseur et al., 1990).

Although the molecular mechanism of action is not known for any fusion peptide, an elegant model has been proposed that has been supported experimentally. The model predicts that the fusion peptide will insert into target membranes at an oblique angle relative to the bilayer normal (Brasseur et al., 1990). The oblique angle of insertion calculations is based on the peptides being α-helical and having quantified helical hydrophobic moments, a measure of the peptide's amphiphilicity (Eisenberg et al., 1986). The contributed hydrophobic moment of each amino acid is projected perpendicular to the idealised helical axis. Here, hydrophobic residues have positive values and hydrophilic residues have negative values. Although a
generalisation, the assumption that the fusion peptides form helices is not unreasonable because previous studies have shown fusion peptides to gain helicity when membrane associated (Lear and DeGrado, 1987, Martin et al., 1991). Both the degree of peptide hydrophobicity and distribution of hydrophobic residues along the helical axis are important for fusion competence. For instance, it has been demonstrated that changing the hydrophobicity of fusion peptides by adding polar residues will reduce fusogenic potential (Bosch, 1989). Also, rearranging the primary sequence, so as to maintain the global hydrophobicity, but change the theoretical peptide tilt reduces rates of fusion. Here, peptides that had a bilayer insertion perpendicular to the bilayer surface were fusion incompetent. In contrast, point mutations that were designed to maintain the global hydrophobicity and the optimal insertion angle did not alter fusogenicity (Martin et al., 1994). This hypothesis was tested using SIV gp160 expressed on a virus vector. The glycoprotein spike had mutations in the fusion peptide designed to maintain or change its angle of insertion (Horth et al., 1991). The results were in full agreement with the previous studies.

It is thought that the oblique insertion of the peptide will facilitate the positioning of bulky hydrophobic residues in the hydrocarbon core and the smaller amino acid residues at the interface (Epand et al., 1992). This orientation will locally disrupt the phospholipid in terms of hydrocarbon chain packing but not interfacially. Peptide precession around its helical axis would
cause bilayer perturbation that in turn may cause the lipids to express negative curvature strain (Gruner, 1985). In so doing, the lipid molecules may form non-bilayer structures, a prerequisite for fusion pore development.

Although specialised proteins control the biomembrane fusion, it is clear that there is another parameter that plays a crucial role in this dynamic event. That is the role of 'non-bilayer' forming lipids.

1.6.3. The role of lipid in fusion

Biomembrane fusion requires the orchestrated movement of lipid molecules as well as fusion proteins. There are over 100 different species of lipids and these molecules display immense polymorphism (Luzatti et al., 1968). Although the biological membrane is classically represented as planar, a large percentage of phospholipids, when purified from biomembranes and then hydrated, spontaneously form the 2-dimensional HII phase, not a lamellar phase (Gruner, 1985). Such phospholipid species are now termed 'nonbilayer' lipids. The exact relationship between bilayer composition and biological function remains enigmatic. However, it is thought that nonbilayer lipids play a role in biological membrane fusion. Figure 1.8 shows a variety of different inverted phases that phospholipid can form.

The study of inverted phase structures and the factors that influence their development is important in our understanding of biomembrane fusion. This is because the initial lipid intermediate structures that form from the lamellar
phase, either during a phase transition or the fusion event, are thought to be similar. In order to understand why nonbilayer lipids will readily form inverted structures, greater understanding of their physical properties is required.

Figure 1.8. Phospholipids display polymorphism. The 2-dimensional inverted hexagonal phase is particularly well characterised (Seddon, 1990). Here, side-by-side lipid molecules roll up into cylinders with monolayer walls and the polar phase resides within the tubes. This phase is a water-in-oil phase. Phospholipids also form a number of structures that exhibit cubic symmetry. At least seven cubic (Q) phase space groups have so far been characterised. There is a strong link between the structure of certain cubic phases and the biomembrane fusion pore. Adapted from Seddon, 1990.
Lipid molecules are amphipathic where the polar head groups face the aqueous phase and the hydrophobic hydrocarbon chains oppose each other in the bilayer. The lipid bilayer can be described as a tail-to-tail bimolecular sheet that forms because of the hydrophobic effect. Phospholipids spontaneously form closed-bilayers at exceedingly high dilutions (10^{-10} to 10^{-14} M) and this phenomenon supports the fact that the lipid bilayer is the major structural motif of the cell membrane.

<table>
<thead>
<tr>
<th>Lipid species</th>
<th>Phase</th>
<th>Molecular Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysophospholipids</td>
<td>Micellar</td>
<td>Inverted Cone</td>
</tr>
<tr>
<td>Detergents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>Bilayer</td>
<td>Cylinder</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidyserine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylethanolamine (unsaturated)</td>
<td>Hexagonal</td>
<td>Cone</td>
</tr>
<tr>
<td>Phosphatidic acid - Ca++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiolipin-Ca++</td>
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</table>

Figure 1.9. Illustration of the molecular shapes of some surfactants. Adapted from Cullis and DeKruijff, 1979.
The chemical diversity of phospholipids molecules is the prime factor that governs the bulk lipid phase type. Lattice effects such as the surface hydration status and other external parameters such as temperature also modulate phospholipid phase behaviour. Phospholipids differ chemically due to head group type, length of hydrocarbon chain and its degree of unsaturation (figure 1.9).

One explanation for the behaviour of nonbilayer forming phospholipids relates chemical structure to dynamic molecular shape (Cullis and DeKruijff, 1979). Molecular shape depends on the relative size of the polar head group relative to the hydrocarbon chain. Phospholipids such as DPPC can be regarded as cylindrical and readily form lamellar structures. Sodium dodecyl sulphate (SDS) has a large ionic head group relative to a short hydrocarbon tail and could be modelled as an inverse cone shape that enables the molecule to pack into micelles. Finally, DOPE has a small head group and long hydrocarbon chain that may form a cone shape. This species of phospholipid is thought to be involved in biomembrane fusion and will readily form inverted micelles.

The dehydrated head group of PE and its unsaturated hydrocarbon chains will further promote $H_{II}$ phase generation. This model is not without its flaws. For example, at body temperature, the phospholipids should possess enough thermal energy to be inherently flexible and, therefore, not be of fixed shape.
Juxta-positioning of a phospholipid species with a well-defined molecular shape introduces the concept of spontaneous (intrinsic) monolayer curvature (figure 1.10) and aids the prediction of interfacial curvature under specific conditions (e.g. temperature).

**Mean curvature**

![Diagram](image)

**Figure 1.10.** Diagram illustrating the expression of spontaneous monolayer curvature. Adapted from Seddon, 1990.

The membrane interface possesses two principle radii of curvature which are mutually perpendicular to each other. The average of these curvatures is defined as the mean curvature and the product of the curvatures gives the Gaussian curvature. The Gaussian curvature determines the type of interface
present. Thus, the lamellar phase and $H_{II}$ phase have zero Gaussian curvature because one the principle radius of curvature is zero. A micelle always has a positive Gaussian curvature value as both curvatures have the same sign. However, if the principle curvatures have an opposite sign then the surface exhibits a negative Gaussian curvature. Such surfaces include saddle surfaces (Seddon, 1990) and the fusion pore itself (Chernomordik et al., 1999).

A centrosymmetric membrane that is composed of any phospholipid species has no tendency to curve. Any curvature would destabilise the bilayer, generating smaller vesicles that are not as thermodynamically stable (Epand et al., 1995). In addition, monolayer separation would be energetically costly due to terminal methyl group exposure to polar solvent. Although bilayers composed solely of DOPE and those composed solely of DPPC are geometrically similar, they possess markedly different physical properties (Seddon, 1990).

Each DOPE monolayer experiences negative intrinsic curvature strain due to its unsaturated tails and small, tightly packed ‘hydrophobic’ head groups. The PE head groups spontaneously want to curl away from the planar bilayer surface into tight cylindrical tubes. This would allow greater intra-molecular H-bonding between the head group nitrogen and phosphate and reduce the exposed surface area to water. However, cylindrical packing into an $H_{II}$ lattice results in ‘free interstitial space’ between the tubes. This is energetically unfavourable because the hydrocarbon chains must extend to fill the space,
which reduces the entropic term. Therefore, below the $T_H$, the lamellar phase dominates but the bilayer is structurally frustrated. Curvature strain gives an indication of what shape the monolayer surface would like to form if the polar heads were hydrated and the hydrocarbon chains exposed to nonpolar solvent. In contrast, DPPC bilayers have little tendency to curve. The PC headgroup H-bonds extensively with water because the three methyl groups sterically hinder intermolecular bonding between the quaternary ammonium group and phosphate. The $H_B$ tubes composed of DPPC would have a larger radius than those composed of DOPE. This would result in a larger interstitial free volume between the tubes, which is energetically unfavourable.

Bilayers composed of phospholipids that possess a spontaneous tendency to curve have lower melting points than those bilayers that are not inherently frustrated. This is because less energy, in the form of thermal energy, is required to overcome the balance of forces between head group curvature and hydrocarbon chain packing. Phospholipids with PE head groups have a greater desire to form highly bent surfaces. It follows, therefore, that concentrated populations of nonbilayer phospholipids within a target membrane could also facilitate biomembrane fusion.

Fusion assays have demonstrated that the lipid composition of the fusing vesicles is important. Fusion either only occurs (Martin et al., 1991) or the rate enhanced (Lorge et al., 1986) when liposomes are composed of nonbilayer lipids such as DOPE.
It has been proposed that, although the initial triggers of the fusion event show great diversity, the actual macromolecular rearrangements of the lipid molecules are similar for many fusing systems (Chernomordik and Zimmerberg, 1995). The multi-step fusion process involves the merging of two distinct, planar bilayers to form highly curved fusion intermediates (Papahadjopoulos et al, 1977) and there has been much evidence to support this hypothesis. Studies by Ellens et al. revealed increased liposome fusion rates in the same temperature range in which Gagné et al. observed isotropic $^{31}$P-NMR resonances for the same lipid. Isotropic resonances are produced by lipid structures of high curvature in which lateral diffusion of the component phospholipids can motionally average the chemical shift anisotropy. These findings also correlate with the presence of lipidic particles, seen by electron microscopy (Gagne et al., 1985, Hui et al., 1983) and with amorphous and inverted cubic phases (Qn), measured by X-ray diffraction (Gruner et al., 1988). These highly curved, isotropic lipid arrangements may be fusion intermediates. The fusion peptide of simian immunodeficiency virus (SIV) promoted the formation of isotropic structures, while a non-fusogenic mutant of this peptide did not (Epand et al., 1994). Similar results were also seen in a study of the pH-dependent fusion peptide of influenza virus HA (Epand and Epand, 1994).
Figure 1.11. The modified-stalk theory of membrane fusion and inverted phase transitions. A = close-approaching planar bilayers, B = quasi-hourglass low-energy stalk, C = transmonolayer contact, D = interlamellar attachment site (fusion pore). The ILA is thought to decay into the cubic phase. The physical properties of C determine the final lipid phase formed. Adapted from Siegel, 1999.

The formation of isotropic lipid mesomorphs also occurs during the lamellar liquid-crystal\inverted cubic (L_{a}Q_{II}) phase transition and the lamellar liquid-crystal\inverse hexagonal (L_{a}H_{II}) phase transition (Ellens et al., 1989).
correlation has been found between the ability of certain additives to promote fusion, for example diacylglycerol (Epand, 1985, Siegel et al., 1989, Basáñez et al., 1996), and their ability to lower the $L_\alpha H_{II}$ transition temperature ($T_{H}$). Similarly, some fusion inhibitors raise $T_{H}$ (Epand, 1986). Although the $Q_{II}$ and the $H_{II}$ phases, which are kinetically stable, are unlikely to exist at the site of a developing fusion pore, knowledge about the topology of the interface as these phases begin to form may have implications in biological fusion pathways.

The exact structures of the intermediates involved in the $L_\alpha Q_{II}$ and $L_\alpha H_{II}$ phase transitions have been widely debated. Studies using freeze-fracture electron microscopy (Verkleij, 1984) or cryo-electron microscopy (Frederick et al., 1989, Frederick et al., 1991, Siegel et al., 1989) have been unable to describe transition intermediates uniquely.

Experimental data (Lucy, 1970, de Kruijff et al., 1979, Verkleij et al., 1979, Verkleij et al., 1992, Sen et al., 1982) and theoretical free-energy calculations of intermediate lipid structures (Siegel, 1986, Siegel, 1993) have led to the proposal of two fusion mechanisms, one involving inverted micellar intermediates (IMIs) (Siegel, 1993), the other utilising a stalk-like structure (figure 1.11) (Markin et al., 1984). However, the energy required to form stalk-like structures has been shown to be much less than that required for the formation of IMIs (Siegel, 1993). Additionally, studies using temperature-jump cryo-electron microscopy (Siegel et al., 1994) have demonstrated that the
most likely pathway is the formation of low energy stalk-like structures and ‘hemifusion’ intermediates (also known as transmonolayer contacts, TMCs).

The initial structural intermediates first observed were ‘lipidic particles’ (Verkleij et al., 1979). Miller defined these as intermembrane attachment sites. Lipidic particles are intimately involved in bilayer/nonbilayer phase transition mechanisms as has been reviewed by Verkleij (1984).

Studies on lipid phase transitions using large unilamellar vesicles (LUVs) composed of dipalmitoleoyl phosphatidylethanolamine (DiPoPE) using cryotransmission electron microscopy have shown that intermembrane connections appear some 22°C below the $T_H$ when the pH of the sample is jumped from pH9 to pH5 (Siegel and Epand, 1997). It is inferred that intermembrane connections evolve to form isolated TMCs, then aggregated TMCs (figure 1.11).

In DiPoPE, aggregated TMCs, which have a lower free energy than isolated TMCs (Siegel, 1993), then form a basis for $H_{II}$ phase growth via the formation of quasihexagonal phase domains (figure 1.12). The integrity of the TMC structure is the committing step in membrane fusion. TMCs comprising N-methylated dioleoyl phosphatidylethanolamine (MeDOPE) have a lower rupture tension than diaphragms comprising DiPoPE. Ruptured TMCs form interlamellar attachments (ILAs) or fusion pores. Many ILAs in close proximity will nucleate to form $Q_{II}$ phases. However, X-ray diffraction (XRD) and electron microscopy have not detected TMC-aggregates, even though they
are believed to have long-range order. There is evidence to suggest that specialised fusion proteins also catalyse the formation of similar lipid intermediates (Chernomordik and Zimmerberg, 1995, Chernomordik et al., 1993, 1995, Vogel et al., 1993). Therefore, studying the structural effects of fusion peptides in the proximity of inverted phase boundaries provides instructive evidence about the fusion mechanism.

**Figure 1.12.** The aggregation of TMCs may provide a mechanism for the development of the inverted hexagonal phase. The clustering of TMCs in B reduces the amount of curvature observed in the monolayers of A. Aggregated TMCs are thought to form quasi-hexagonal domains (C) then the inverted hexagonal phase (D). Adapted from Siegel and Epand, 1997.
1.6.4. Aims of the project

The main goal of this project is to understand better the intimate fusion peptide-phospholipid interactions and resulting peptide and lipid conformational changes that may occur in the early stages of a developing fusion pore. The fusion protein of the particular fusion peptide under study is not present; therefore, some specificity and co-ordination will be lost. Nevertheless, the model systems to be described allow the complete control of lipid composition and maximise intimate lipid-peptide interactions.

Chapter 3 describes the membrane location of the SIV fusion peptide in the lamellar phase. This was achieved using neutron diffraction. The results of this chapter may relate to how the peptide inserts into the planar membrane after the fusion protein has undergone its conformational change. This technique also monitors any change in unit cell dimensions and change in the water distribution within and between stacked bilayers when the peptide is present. Thus, this technique provides a test for the oblique insertion theory of Brasseur et al.

Previous studies have shown that the fusion peptide from feline leukemia virus (FeLV) has the ability to reduce the $T_\text{II}$ of both DiPoPE and MeDOPE and increase the amount of $^{31}\text{P}$-NMR isotropic resonance (Davies et al., 1998). For MeDOPE, these resonances were observed at temperatures as low as 40°C in the presence of fusion peptide. This evidence indicates that the fusion peptide destabilises the $L_\alpha$ phase and promotes the formation of non-bilayer structures.
by increasing negative curvature strain. XRD measurements did not reveal the production of any isotropic intermediates by the p15EK on the L\textsubscript{a}H\textsubscript{II} transition pathway for DiPoPE. Three reasons may have accounted for this: DiPoPE has a small spontaneous radius of curvature, R\textsubscript{0}, (Gruner et al., 1988) the isotropic structures observed by NMR are isolated and lack long-range order; and the temperature scan rate employed was too rapid. However, a shoulder on the 2\textsuperscript{nd} and 3\textsuperscript{rd} diffraction orders of the H\textsubscript{II} phase indicated a possible novel, peptide-induced H\textsubscript{II} lattice.

Chapter 5 and 6 describe the effect of the FeLV fusion peptide on the thermotropic lipid phase behaviour of MeDOPE-composed multilamellar vesicles (MLVs). Lipid mesomorphism was measured by \textsuperscript{31}P-NMR and XRD, which are quantitatively and qualitatively direct methods of characterising lipid phase transitions. In the case of the \textsuperscript{31}P-NMR study a truncated version of the FeLV fusion was used. This version is thought to be the fusion domain of the peptide.

These chapters describe the effect of this peptide on lipid structural parameters. Based on the results of this technique, peptide-induced lipid polymorphism is related to biomembrane fusion and the experimental data is discussed in terms of theoretical models.
Chapter 2. Circular dichroism (CD)
Chapter 2

2.1. General introduction

Biomolecular functionality is utterly shape-dependent. The spatial arrangement of each atom in a molecule confers its chemical and physical properties. It is now known that biomolecules assume a huge array of characteristic shapes so that they can carry out a variety of thermodynamically efficient functions.

Peptide secondary structure is governed by its amino acid sequence and its local physico-chemical environment. Peptide secondary structure (or conformation) is the spatial arrangement of its component amino acid residues along the peptide main-chain. A polypeptide gains secondary structure mainly because the peptide bond is rigid and planar and because of H-bond requirements (figure 2.1). This forbids unlimited conformational freedom. The partial double-bond character of the amide carbon-nitrogen prevents free rotation around this bond. Consequently, cis and trans stereoisomerism is possible. The all trans configuration is generally regarded as the most stable as it confers the least steric hindrance. The carbonyl carbon-α carbon bond and the peptide nitrogen-α carbon bond are purely single in nature. This allows a large degree of rotation either side of the peptide bond. The angles of rotation can be calculated to give fine regions of secondary structure (Ramachandran plot). However, this rotation is also limited to the type of
amino-acid side chain present. The combination of a rigid peptide bond and the type of amino acid residue present profoundly influences polypeptide secondary structure.

Two of the best characterised polypeptide secondary structures are the α helix and β sheet. The α helix is a rod-like structure where the peptide backbone spirals to form a tight coil and the amino side-chains project in a helical array (figure 2.2a). The main chain backbone is intra-molecularly H-bonded. "The NH group of one residue H-bonds with a CO group of another that is four amino acids away in the linear sequence. Each residue is related to the next one by a rise of 1.5Å along the helical axis and a rotation of 100°, which gives 3.6 amino acids per helix turn" (Stryer, 1995). Thus, the biological α helix is analogous to a right-handed corkscrew.

The β pleated sheet was the second periodic protein structure to be proposed (hence β) (figure 2.2b). Unlike the α helix, the polypeptide chain in a β pleated
sheet is fully extended, called a β strand. The distance between adjacent amino acids is 3.5 Å. Each β strand is stabilized by H-bonding with NH and CO groups from a different polypeptide chain. The adjacent polypeptide chain can run in the same direction (parallel β pleated sheet) or opposite direction (antiparallel β pleated sheet).

Figure 2.2. Illustration of the α-helix (a) and the β-sheet (b). Full details can be found in the text. Modified from Doolittle, 1985.
Circular dichroism can be used to gain information on a polypeptide's secondary structure. This method measures the ability of a molecule to absorb differentially right and left circularly polarised light as a function of wavelength. A curve fitting procedure can be used to elucidate secondary structural components from an observed spectrum. A greater knowledge of a polypeptide's structure will aid our understanding of its molecular mode of action.

2.2. Theoretical background to circular dichroism

"Circular dichroism refers to the differential absorption of the left and right circular components of plane polarized radiation" (Price, 1996). The CD spectroscopic effect is observed when plane polarised UV radiation passes through a chiral (asymmetric) environment. The chromophore may be either intrinsically chiral or placed in an asymmetric environment. The basic CD experiment involves placing a sample in a beam of plane polarised radiation and recording the differential absorption of the left and right circular components. To understand the theory of the CD experiment in more detail, a description of the polarization of light has to be appreciated.
Figure 2.3. Unpolarised light (left) vibrates in all directions orthogonal to the direction of propagation. In this diagram, light is traveling into the plane of the page. Special filters of an appropriate length can absorb the beam in all directions apart from the waves that vibrate parallel to the filter's optical axis. This results in plane polarised light.

Light is an electromagnetic wave that consists of an electric vector (E) and a magnetic vector (H). Polarisation of light deals only with the electric component and is, therefore, different to the study of NMR, which is concerned with the magnetic component. In unpolarised light, the E vector vibrates in all directions perpendicular to its direction of propagation and will rotate every $10^8$ seconds. If the unpolarised light is passed through a special crystal, only electric vectors vibrating parallel to the optical axis of the crystal will pass through. Such crystals include the Nicol prism (calcium carbonate) and polaroid sheets. The light is now plane polarised and the vibrations of the E vector is confined to one plane, orthogonal to the direction of travel (figure 2.3).
A Cartesian frame is useful in describing a plane polarised beam. If the z-axis is regarded as the direction of propagation, the E vector can be regarded as the vector sum of two component vectors in the x-z and y-z axis (figure 2.4). It is important to note that there is only one E vector wave train even although figure 2.4 shows two orthogonal waves. The two orthogonal waves are only a mathematical representation of the physical phenomenon.

In figure 2.4, each vector component is in phase. If the vector components are not in phase, the beam is no longer plane polarised. If one of the vector components is exactly $\pi/2$ out of phase, circularly polarised light is produced (figure 2.5).
Figure 2.5. The vector components that comprised plane polarised radiation in figure 2.4 are no longer in phase but are out of phase by $\pi/2$ radians. Simple graphical addition shows that the beam would now be helical with respect to the z-axis. Modified from Warren, 1987

Again, it is mathematically useful to describe circularly polarised light on the Cartesian frame. Here, the wave train vibrates in a helical trajectory around the z-axis (figure 2.6).

Figure 2.6. Visualisation of the helical trajectory of circularly polarised light.
Looking end on, the plane polarised radiation can be represented as a line in at an oblique angle to each axis whereas circularly polarised waves trace out a circle (figure 2.7). The circular polarized beam is right-handed if the vector rotates clockwise and left-handed if it rotates anti-clockwise.

\[ \text{Figure 2.7. End on view of plane polarised light (left) and circularly polarised light (right). Modified from Warren, 1987.} \]

Plane polarised light can be considered as the vector sum of right and left circularly polarised light of equal amplitude (figure 2.8). If the amplitudes are not equal, then elliptically polarised light is produced (figure 2.9).
Figure 2.8. Plane polarised light can be regarded as the sum of left and right circularly polarised components. In the CD experiment itself, a modulator splits the plane polarised radiation into its component halves. Modified from Warren, 1987.

Figure 2.9. Elliptically polarised light can be regarded as the sum of left and right circularly polarised components. Modified from Warren, 1987.

This is what a typical CD experiment measures. The asymmetric sample absorbs the right and left circularly polarised vector components of plane polarised light to different extents. In practice, plane polarised waves are reduced to its left and right circular components by passing the beam through a modulator (for example, a piezoelectric crystal such as quartz) that is subjected
to an alternating (50KHz) electric field (Kelly and Price, 1997). When the beams recombine after passing through the sample, elliptically polarised light is produced. Dichroism is then expressed as the ellipticity in degrees, θ, where θ is the angle whose tangent is the ratio of the minor and major axes. The CD produced is dependent on the concentration of the sample and ellipticity is therefore standardized into molar ellipticities.

\[
[\theta]_{\text{MRW}, \lambda} = \frac{\theta \cdot \text{MRW}}{10 \cdot d \cdot C}
\]

where MRW is the mean residue weight (the molecular weight divided by the number of peptide bonds) of the dichroic solute, θ is the observed ellipticity, C is the concentration in g mL\(^{-1}\) and d is the optical path length in centimeters. The units are deg.cm\(^2\).dmol\(^{-1}\).

In practice, the observed ellipticities are of the order of 10 millidegrees, a photo multiplier is employed to amplify the signal.

A CD spectrum is obtained when dichroism is plotted as a function of wavelength. In order for dichroism to be observed, the two circularly polarised components must pass through an asymmetric medium. As both the circularly polarised components are inherently asymmetric as well, they will interact differently with chiral chromophores.

In order for CD to be observed, the sample medium must possess an intrinsic chromophore and exhibit chirality. In the case of polypeptides, the chromophore is the peptide bond. The electronic absorbance of the peptide bond ranges from 180-240nm. There is a weak but broad n → π* transition
centered at 210nm and an intense $\pi \rightarrow \pi^*$ at 190nm. In contrast to the narrow bands seen with atomic spectra, the bands resulting from molecular excitations are broad. As well as the electron within the molecule being excited to a higher electronic level, it is also subject to a number of rotational and vibrational states. The number of these states increase on the interaction with solvent. In a CD spectra, the absorption bands at each observed wavelength merge to form a smooth overlapping curve.

The unique CD spectra of a polypeptide is caused by the spatial orientations of the chromophore around chiral centers. The $\alpha$ carbon of the naturally occurring amino acids, with the exception of glycine, is asymmetric. Amino acids are, therefore, enantiomers. Thus, the peptide bond has a non-superimposable mirror image and is optically active. The chiral centers in a polypeptide give rise to an asymmetric secondary structure.

The amount of differential absorption at each wavelength depends on the electronic structure and geometry of the biomolecule involved. Periodic polypeptide secondary structures give rise to characteristic CD spectra in the far UV range.

The data analysis of far UV CD spectroscopy assumes little or no involvement from secondary chromophores such as the aromatic side chains. Aromatic chromophores and auxochromes will absorb in the lower energy region of the UV range (260-320nm). This gives rise to near-UV CD spectroscopy, which
yields information on tertiary structure. Any change in the micro-environment around a chromophore will result in a change in absorbance.

The theory connecting the CD spectra to molecular structure is not yet fully developed. The procedures that are presently used to interpret the secondary structures of all the biomolecules are empirical. One of the most popular methods used to analyse CD data is CONTIN analysis (Provencher and Glöckner, 1981). In this approach, the CD spectrum of 16 known protein crystal structures are recorded over a wavelength spread of 240-190nm. The reference proteins differ in secondary structure from being highly α-helical (myoglobin 79% α-helical, 0% β-sheet by X-ray) to more β-sheet (concanavalin A 2% α-helical, 51% β-sheet by X-ray). The spectrum is then related to the structural components of the protein. This method assumes that the solution structure of the protein is the same as the type of crystal used in the X-ray structure determination. The CD spectra from the 16 protein samples give a set of standard curves. The unknown secondary structure of the sample can then be calculated from simple graphic addition of the standard curves. This is done using complicated curve-fitting features and only rarely is a perfect fit obtained. For example, the curve fitting procedures take little account of peptide length. An empirical factor is added to take into account helix length (Chen et al., 1974) and β-sheet dimensions (Chang et al., 1978). More reliable results are obtainable if the sample can be related to a structurally defined reference.
2.3. Determining the conformation of NKA in alcoholic solvents and lipid-based systems

2.3.1 Aims of the experiment

Peptide and protein secondary structure is not solely dependent on its primary sequence. The surrounding micro-environment is an important parameter with regards to a peptide’s conformational status (Waterhaus and Johnson, 1994). After biosynthesis, the tachykinins experience a number of different physico-chemical environments. They are stored with other peptides in secretory vesicles, released into the synaptic cleft and interact with membrane-embedded receptors. This CD study attempts to determine the conformation of NKA in a range of biologically relevant systems that models in-vivo conditions, particularly the NKA-cell membrane interaction. Therefore, NKA was analysed when incorporated into LUVs composed of DOPC. However, the sample preparation of this experiment proved unsuccessful. NKA was then incorporated into simpler zwitterionic and anionic micelles composed of DPC and SDS respectively. The concentration of SDS used ranged 1-60 mM, thereby passing through its cmc value. Doing this is thought to promote peptide secondary structure. The conformation that the NKA takes up in progressively higher SDS concentrations may be similar to the conformation adopted during the initial membrane contacts. However, it is doubtful if the exact environment can be reproduced in-vitro. Analysing NKA secondary structure in SDS micelles was done to mimic an anionic membrane surface.
Another way to promote peptide secondary structures is to place them in progressively hydrophobic solvents. This also gives a 'second opinion' with regards to the SDS samples. Placing membrane-interactive peptides into solvents of reduced polarity and calculating the resultant secondary structures is biologically useful because the cell membrane possesses a polarity gradient: greatest polarity at the surface to the lowest polarity in the hydrocarbon core. The gradient is at its steepest at the glycerol backbone region. However, the results must be viewed with caution as the membrane is an anisotropic medium whereas the solvent systems are isotropic.

2.3.2. Materials

NKA was synthesized by Albachem (26 Craigleith View, Edinburgh, EH4 3JZ, Scotland, UK) using solid-phase methods. Analytical high pressure liquid chromatography (HPLC), Maldi-Tof mass spectrometry and amino acid analysis showed that the peptide was of acceptable purity (>99%). The peptide was stored below 0°C in a Pyrex container with an aluminum foil-lined plastic top. The peptide is a white, low-density powder which is slightly hygroscopic. DOPC was purchased from Sigma and used without further purification (>99% pure).

The buffer used for all the biophysical studies described in this thesis was 20mM PIPES, 1mM EDTA, 150mM sodium chloride and 0.002% sodium azide at pH7.4. Buffer ingredients were all reagent grade.
SDS and DPC were purchased from Sigma and were both approximately 99% pure.

The alcoholic solvents were purchased from Sigma and were all analytically pure.

All measurements were carried out at the CD facility in the Department of Biological Sciences, The University of Stirling. The instrument used was a Jasco J-600 spectropolarimeter that had a Windows™ interface. The source of illumination was a xenon arc lamp. Once the beam has passed through the sample and CD has been produced, it is amplified by a photomultiplier. The elliptically polarised beam will have a periodic variation of polarization due to the periodic variation in the extent of absorption. This manifests itself as a periodic change in photon intensity reaching the photomultiplier. The photomultiplier produces a small ac current that is proportional to the differential absorption of left and right circularly polarised light. The sample cell path-length was 0.02cm and the wavelength scan rate was 10nm/min.

It is common practice to take a reading of the blank cuvette and controls and then subtract the spectra from the sample signal. In this series of experiments, the controls were the solvent systems without the presence of NKA. Each solvent blank exhibited no CD. However, there remains one caveat. The peptide itself may alter the structure of the lipid and this change in structure may contribute to the spectrum. NMR spectra of the lipid would be able to
distinguish if there has been any change in lipid packing due to the introduction of peptide. All experiments were carried out at room temperature.

2.3.3. Sample preparation

An unsuccessful attempt was made to incorporate NKA into DOPC-composed liposomes. The method used was as follows. The appropriate amount of NKA powder was measured into a Pyrex test tube. A mixture of DOPC dissolved in chloroform was then added. It was observed that the peptide had not fully dissolved so a small quantity of methanol was added. The solution then became clear and homogenous. This observation hints that the peptide is not soluble in non-polar environments. The peptide mixture was then thoroughly vortexed to ensure adequate mixing. The volatile solution was then ‘dried down’ under a stream of inert N₂ gas until lipid films could be seen. The resultant lipid films were placed under vacuum overnight in the presence of the desiccant, phosphorous pentoxide (P₂O₅), to remove trace solvent. Each film was reconstituted in buffer and vortexed rigorously for 10 minutes at room temperature. The lipid dispersions were then subjected to 5 freeze-thaw cycles by immersing them into liquid nitrogen for 2 minutes followed by plunging them into a water bath at ~25°C until they thawed. Repeated freeze thawing to below the chain-melt temperature ensures that the lipid is in the fully hydrated L-α phase regardless of the thermal history of the lipid and ensures adequate distribution of salt between lamellae. At this stage, the dispersion looked
white and turbid in nature. The MLVs were then passed through two stacked polycarbonate membrane filters using a high-pressure extruder (Lipex Biomembranes Inc., Vancouver, Canada). The filter pore size was 0.1µm (Nucleopore Corp., Pleasington, CA, USA) and the pressure used was 600 lb/in². After the extrusion of pure buffer the pure DOPC control was extruded a total of ten times. The starting white, turbid dispersion changed in appearance to a final blue, clear, homogenous solution. This is indicative of the appearance of IUVs although there is probably still some size variation (New, 1990). The filters were then changed and the procedure repeated for the NKA samples. However, the samples refused to pass through the filters, even at higher pressures. This can occur if the peptide binds to the polycarbonate material. Due to lack of peptide and limited CD time, studies on the simpler SDS and alcoholic solvent systems were initiated.

The sample preparation of these systems is easy, requiring only accurate measurements of peptide added to the particular solutions. Some of the CD reviews in the literature encourage the use of the SO₄²⁻ ion instead of the Cl⁻ ion as the buffering salt. This is because the Cl⁻ strongly absorbs below 200nm (Kelly and Price, 1997). However, as mentioned in section 1.5.3, the type of anion present may influence peptide secondary structure, particularly at a surface. Therefore, the Cl⁻ ion was still used in the buffer and an attempt was made to see if there was any change in secondary structure due to the addition
of various salts in the Hofmeister series. This is at the expense of a clearer CD signal.

A variety of samples of NKA incorporated in increasingly hydrophobic alcoholic solvents were made up. NKA was soluble in each solvent system under study and this included pure octan-1-ol, the only solvent that forms an interface with water. The NKA concentration was 1mg/mL although the net NKA concentration is 0.75mg/mL (0.66mM) taking into account the weight contribution of the salt.

2.3.4. Results and discussion

Figure 2.10 shows the CD spectrum of NKA in buffer and a range of alcoholic solvents that are in a 1:1 ratio with buffer at pH 7.4. Each spectrum indicates that NKA is in a random coil conformation. This type of CD spectrum for NKA in buffer implies a monomeric status for this peptide. It follows that no aggregation has occurred over the time period of the experiment.

Table 2.1 summarises the calculated secondary structure components. The calculated structures should be viewed with caution as the data is noisy below 200nm. Also, the CONTIN procedure is usually applied to protein analysis rather than the study of small membrane-interactive peptides. Table 2.1 also shows the calculated secondary structure components of NKA in undiluted solvents (figure 2.11). Again, NKA appeared to be soluble in each sample.
Figure 2.10. CD spectra showing NKA (0.66mM) in a variety of alcoholic solvents. Each solvent has been diluted 1:1 volume-in-volume with buffer.

Some of the observed spectra of NKA in figures 2.8 and 2.9 are markedly different. However, the conformation of NKA in butan-1-ol and octan-1-ol appears very similar. The conformation of NKA in methanol, TFE and HFP resembles the random coil structures observed in figure 2.10. The CONTIN analysis suggests that NKA is relatively unconstrained in each solvent, portraying very weak helicity at the utmost. This is not surprising given the short length of the peptide and the isotropic nature of the system. CD is not a tremendously reliable measure of β sheet structure or β turns. The CD technique is much more accurate in determining helical contributions to the spectrum. Therefore, it would be unwise to give too much weight to the large β sheet contributions in the analysis. The solvents that caused the greatest
helicity in NKA were TFE and HFP. These solvents may have a polarity similar to that of the membrane interface.

Figure 2.11. CD spectra showing NKA (0.66mM) in variety of alcoholic solvent systems.

<table>
<thead>
<tr>
<th>NKA (0.75mg/mL) in:</th>
<th>% secondary structures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α helix</td>
</tr>
<tr>
<td>buffer</td>
<td>0</td>
</tr>
<tr>
<td>methanol:water 1:1</td>
<td>2</td>
</tr>
<tr>
<td>methanol</td>
<td>1</td>
</tr>
<tr>
<td>TFE:water 1:1</td>
<td>6</td>
</tr>
<tr>
<td>TFE</td>
<td>6</td>
</tr>
<tr>
<td>HPP:water 1:1</td>
<td>3</td>
</tr>
<tr>
<td>HFP</td>
<td>0</td>
</tr>
<tr>
<td>butan-1-ol</td>
<td>0</td>
</tr>
<tr>
<td>octan-1-ol</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.1. Percentage of NKA secondary structure in a range of solvent systems, as calculated by the methods of Provencher and Glockner, 1981.
Further observations were made with micelle-based systems. Firstly, the addition of SDS at concentrations below its cmc value caused the mixtures to become unstable. Solutions of SDS monomers should be clear or opalescent in nature. However, NKA caused the SDS solutions to precipitate into a white mass, phase separating from the water. This has also been observed with SP (Woolley and Deber, 1986) (Young et al, 1994). Both tachykinins probably form an insoluble macro-molecular salt with the detergent. It is known from the surface chemistry of disperse systems that the addition of a molecule (drug or surfactant) of opposite charge to that of the stabilising emulsifier can facilitate system instability and coalescence (Aulton, 1988). This occurs because of the electrostatic attraction between the two ionic surfactants form an insoluble salt. The original surfactant is now no longer employed as an emulsifier and the oil and water phase separate. This may result in cracking of an emulsion or caking of a suspension.

In this experiment, there is insoluble salt formation when the system is ternary (no disperse phase present). NKA is acting like a cationic surfactant peptide that binds to SDS forming an insoluble precipitate.

The addition of SDS at concentrations above its cmc value gave opalescent solutions that exhibited a clear CD signal. Two SDS concentrations were used: 14mM and 60mM giving an NKA mole percent of 4.5 and 1.1 respectively. Both gave very similar spectra. This is in agreement with the
finding that SP did not change conformation under the same experimental circumstances (Woolley and Deber, 1986).

The DPC system also exhibited clear CD spectra. These spectra are shown in figure 2.12 and the CONTIN analysis in table 2.2.

Unfortunately, only the samples that contained the salts NaCl and Na₂SO₄ gave acceptable quality spectra. The samples that contained the polar chaotrope, NaSCN, were too noisy below 240nm. The SCN⁻ ion strongly absorbs in this region (Kelly and Price, 1997). No attempt was made to use a different anion with similar physical properties.

![Figure 2.12. CD spectra showing 1.1mol% NKA added to SDS (60mM) and DPC (60mM) micelles.](image)
The most striking feature of the analysis is the increased amount of α helical content. This is still a small amount of secondary structure but it may represent a non-ideal helical turn structure, as proposed by Keire and Fletcher. Helicity occurs most when NKA is incorporated into DPC with NaCl in the buffer. This is reduced when Na₂SO₄ is present. Therefore, it can be argued that Na₂SO₄ competes with NKA for the DPC micelle. Again, this demonstrates the importance of the micelle surface region. Here, Na₂SO₄ must interfere with the weak hydrophobic binding of NKA to the DPC micelle. The reverse situation is true regarding the SDS micelles although the difference in helicity is probably too small to explain. However, the lack of a trend may be explained by the stronger electrostatic interactions between NKA and the SDS micelle surface. Therefore, these results do not argue against the CD and NMR results published to date.

<table>
<thead>
<tr>
<th>NKA (1.1mol%) in:</th>
<th>% secondary structures</th>
<th>α helix</th>
<th>β sheet</th>
<th>remainder</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS + NaCl</td>
<td></td>
<td>14</td>
<td>46</td>
<td>40</td>
</tr>
<tr>
<td>SDS + Na₂SO₄</td>
<td></td>
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<td>47</td>
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</tr>
<tr>
<td>DPC + NaCl</td>
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</tr>
<tr>
<td>DPC + Na₂SO₄</td>
<td></td>
<td>9</td>
<td>63</td>
<td>28</td>
</tr>
</tbody>
</table>

**Table 2.2.** Percentage of NKA secondary structure in a range of disperse systems, as calculated by the methods of Provencher and Glockner, 1981. Each disperse system contained 60mM detergent.
2.4. Circular dichroism study of FeLV fusion peptide in DOPC-composed MLVs

2.4.1. Aims of the experiment

An unsuccessful attempt was made to study the FeLV fusion peptide in LUVs composed of DOPC. Conformational studies of various fusion peptides (Martin et al., 1993, 1994) including the FeLV (Davies et al., 1998), and molecular modelling studies (Callebaut et al., 1994) have shown that these peptides exhibit structural flexibility when exposed to changing physicochemical conditions. Possession of this physical characteristic may be crucial to the function of fusion peptides. As the dynamic fusion reaction proceeds, the local chemical environment encountered by the fusion peptide will change. Such a change in the micro-environment is clearly demonstrated later in chapter 4. In chapter 4, the FeLV fusion peptide causes the lipid bilayer to thin and readily form highly bent inverted structures. Each different lipid structure represents a different environment for the peptide to inhabit. Therefore, the FeLV fusion peptide may carry out a number of different tasks until the fusion reaction (lipid and aqueous contents mixing between viral and target membrane) is complete. If indeed fusion peptides play not just one, but many roles in the fusion process because of their structural flexibility they may be regarded as molecular acrobats. The goal of this experiment was to monitor any change in peptide conformation with temperature by employing CD spectroscopy. Attempts were made to incorporate the FeLV fusion peptide
into IUVs composed of DOPC, DOPC:DOPG (1:1) and DOPC:DiPoPE (2:1). Using different species of lipid may instigate a number of possible outcomes. No change in secondary is expected in the DOPC-composed liposomes. DOPC will not readily form inverted structures with increasing temperature. The peptide may have a different conformation in the DOPC:DOPG mixture due to surface charge. The presence of DiPoPE was used to encourage the formation of inverted structures. However, it is acknowledged that there exists a discrepancy between the optical technique employed and the type of liposomes required to form inverted phases. Basically, unilamellar liposomes will not form inverted structures without prior formation of a multi-lamellae system (Siegel, 1999). Multi-lamellar structures will strongly absorb the polarised beam and damage the quality of the CD signal. Nonetheless, this would be experimentally useful to observe.

2.4.2. Materials
Albachem (26 Craigleith View, Edinburgh, EH4 3JZ, Scotland, UK) synthesised the FeLV fusion peptide using solid phase methods. The peptide sequence was EPISLTVALMLGGLTVGGIAAGVGTGTK (Swiss Protein Data Bank, 1996; release number 34). This peptide is sparingly soluble in most solvent systems suitable for phospholipid research. Therefore, a lysine residue was added onto the C-terminus to increase solubility in chloroform/phospholipid mixtures and phospholipid/aqueous-phase
dispersions. Lysine is the next, naturally occurring, amino acid in the protein sequence. Even with such a modification, the peptide was still difficult to deaggregate in a number of solvents it was introduced to.

Analytical high-pressure liquid chromatography (HPLC), Maldi-Tof mass spectrometry and amino acid analysis showed that the peptide purity was greater than 95%. The peptide is difficult to synthesise and even more difficult to purify. The peptide was stored below 0°C in a Pyrex container with an aluminum foil-lined plastic top. The peptide is a white, low-density powder that is not hygroscopic.

2.4.3. Sample preparation

As mentioned earlier in section 2.3.2, the physical properties of some peptides govern the type of liposome preparation used. Earlier attempts in the laboratory to incorporate this fusion peptide in DOPC-composed IUVs using high-pressure extrusion were not fruitful. The same scenario occurred with the NKA peptide. Therefore, a different method was tried based on dialysis and is described as follows.

The appropriate lipid films were produced as described in section 2.3.2. The lipid:peptide mole ratio was 12:1 and the peptide concentration was 1mg/mL. This ratio is known to maximize the signal-to-noise ratio and the peptide concentration will give adequate CD (Davies, Ph.D. thesis, University of Edinburgh, 1998). The appropriate amount of buffer was added that
contained the non-ionic detergent octyl glucopyranoside (cmc = 23mM) (New, 1990) at concentrations four times that of the lipid but still less than its cmc value. The solutions were vortexed for 10 minutes and placed in dialysis tubing (Medicell, International Ltd). The dialysis tubing was placed in 5L of buffer and weighted down. A magnetic stirrer was used to ensure adequate convection across the outer membrane surface. The buffer was renewed after 6, 12, 24 and 48 hours giving a total of 90 hours dialysis. The final solutions were then centrifugated in order to separate the LUVs (supernatant) from any MLVs present.

The detergent used acts as an intermediary, or screen, allowing the phospholipids intimate contact with the aqueous phase. This facilitates LUV formation. The dialysis tubing permits the diffusion of detergent molecules and free peptide molecules but not phospholipids. However, this technique cannot ensure that every detergent molecule is expelled from the sample. OG was chosen because it has a high cmc value and is not optically active.

Each sample was analysed in the far-UV region at specific temperatures from room temperature to 60°C.

2.4.4. Results and discussion

Every CD spectra containing peptide was too noisy to be analysed. The control samples did not exhibit CD. Therefore, it appears that samples containing the FeLV fusion peptide are of a poor quality. This may be
explained by peptide aggregation. The sample may have contained a membrane-bound and also a peptide population that aggregated in solution. Although dialysis may have removed much of the free peptide, any aggregates present would remain within the dialysis tube. Ways of filtering the membrane-bound form and the aggregated form are required. This can be achieved by centrifugation but was not attempted here. Further studies on this system were not possible due to the prohibitive cost of the peptide (~£2,000 for 25mg).
Chapter 3. Neutron diffraction
Chapter 3

3.1. General introduction

The discovery of the neutron by Chadwick in 1932 and the subsequent large-scale production of neutron beams from reactors have allowed investigators to study matter in a greater depth. The requirement of a nuclear reactor, or spallation source, to produce neutron beams means that neutron scattering centers are few and far between. Providing the ‘beam-time’ is granted, scientists travel to foreign countries in order to investigate their samples. Investigators vary from astrophysicists to biologists. Biologists utilise neutron beam sources as another form of super-microscope that is employed to solve biological structures.

There are many biological techniques used that are under the ‘umbrella’ of neutron scattering. These include small (solution) angle neutron scattering (SANS) (gross structural size and shape), neutron spectroscopy (structural dynamics) and neutron reflection (surface topography). This chapter is concerned with orientated small angle neutron diffraction (membrane crystallography). This type of liquid crystallography involves the study of lipid bilayers and how various substances modify them.
3.2. Historical perspectives and neutron properties

The neutron is a fundamental, uncharged particle of which all matter is composed. It is a constituent of all atoms with the exception of hydrogen.

The British physicist Ernest Rutherford, as well as Australian and American scientists, predicted that neutrons existed in the 1920s. However, proof of the neutron’s existence was difficult due to its uncharged nature. Seventy years ago there was tremendous excitement all over Europe as the ‘neutron’ was waiting to be discovered. In 1932, Sir James Chadwick at Cambridge proved that neutrons did exist by correctly interpreting experiments carried out by Irène and Frédéric Joliot-Curie and other scientists. Chadwick recognised that the Joliot-Curies had produced free neutrons by the interaction of alpha particles with beryllium nuclei. When this newly discovered radiation was passed through paraffin wax, detectable protons were produced. Based largely on the conservation of mass, Chadwick realised that this new radiation was the neutron.

\[ \alpha + ^7\text{Be} \rightarrow ^{12}\text{C} + \text{n} \]

In 1934, Fermi developed a detailed account of the neutron-nuclear interaction and the diffraction of neutrons (from radium-beryllium sources) was achieved in 1936. Halpern and Johnson also discovered nuclear fission at this time, and thereby converted ‘neutron diffraction’ from a physical phenomenon into a real scientific technique. The first neutron diffractometer was established in 1945 at Argonne National Laboratory, Illinois.
The neutron has a mass slightly greater than that of the proton. This results in the de Broglie wavelength of thermal neutrons being approximately 0.5Å-10Å, similar to inter-atomic space separations. Therefore, thermal neutron beams can be diffracted by crystal structures and the resulting interference fringes can give information on the spatial distribution of its component atoms.

The physical properties of the neutron give neutron scattering both advantages and disadvantages when compared to X-ray scattering. Some of the advantages are as follows: -

1) The coherent scattering amplitudes do not decrease with angle, as is the case with X-rays. In neutron scattering, the neutron beam scatters from atomic nuclei rather than the circulating electron cloud. The nuclei (scattering centers) are in fixed positions and their dimensions are much smaller than the wavelength of the primary beam.

2) The resultant coherent scattering amplitudes are isotope-dependent. Neutron scattering is dominated by resonance scattering. When a free neutron collides with atomic nuclei, a compound nucleus is temporarily formed. The compound nucleus has a higher energy level that the ground state nucleus. The resultant coherent scattering amplitudes are related to the energy level of the compound nucleus. This explains why atomic scattering 'strength' does not relate to the periodic table of the elements. The coherent scattering amplitudes of most biological
atoms vary by a factor of 2 or 3. Therefore, the signal is not ‘swamped’ by the presence of heavy atoms as occurs with X-rays.

3) The isotope-dependent scattering of neutrons is the main driving force behind thermal neutron scattering of biological molecules. Isotopic substitutions provide a non-invasive and subtly effective way of applying the X-ray technique of isomorphous replacement. This is best exploited when hydrogen is replaced by deuterium. Most atomic nuclei induce a 180° phase shift on a scattered neutron with hydrogen being a notable exception. Therefore, hydrogen nuclei are said to scatter neutrons ‘negatively’. Its massive incoherent cross-section means that hydrogen will only contribute to the isotropic background of the resulting spectrum. Conversely, deuterium has a large coherent cross-section and will contribute to the appearance of discrete interference spots. Therefore, hydrogen\deuterium exchange can be used to construct difference Fourier maps. This can reveal the location of hydrogen atoms (or water) within a sample. Hydrogen is effectively transparent to X-rays because it has only one electron. This makes the detection of water in any biological sample more difficult with X-rays.

4) The neutron can probe deeper into a sample core because it is uncharged (although the neutron has an extremely small, unknown dipole moment). There are no electrostatic forces to overcome. In fact, approximately 98% of the primary beam will pass through the sample
undeviated. This explains the extensive shielding that is required for the beam-stop. It follows that there is little ionising radiation damage to the sample.

There are also a number of disadvantages with neutron scattering. These are:-

1) Neutron flux is very low compared to modern X-ray sources. Presently, the neutron flux is equivalent to the X-ray flux of the 1940s. This makes for long measuring times and increased demand from researchers to use the facilities.

2) The interaction of neutrons with matter is weak. Therefore, large samples are required, milligram quantities for single crystals and gram quantities for powder diffraction.

In practice, the two techniques are complementary and crystallographers make use of both neutron and X-ray properties in order to answer their questions.

3.3. Diffraction theory applied to model membranes

In 1912, Max von Laue discovered that X-rays could be diffracted in an orderly manner from a crystal (Bragg, 1968). Diffraction may be defined as “the deviation of a wave from its natural direction of propagation when it encounters an object or a medium with spatially varying transmittance” (Warren, 1987).

A barrier or an opening (aperture) will cause a wave to bend or diffract. This occurs maximally when the wavelength is of a similar size to the aperture size.
If a number of regularly spaced apertures are encountered by a wave train, the diffracted waves can interfere with each other (figure 3.1).

Figure 3.1. Incident neutron waves are scattered by the atomic nuclei. This gives rise to interference patterns. The higher orders of diffraction are observed at greater angles with respect to the primary beam. The zeroth order cannot be measured experimentally as it is combined with the undeviated primary beam.

Waves that are in phase will give rise to constructive interference while waves that are out of phase will give rise to destructive interference (figure 3.2 and 3.3).

If a photographic plate is placed in front of diffracted X-rays, a series of interference fringes will be observed. This phenomenon is exploited in crystallography. Instead of a series of apertures obstructing the wave’s path, a crystal is used as the optical grating. The intensity of the interference spots observed at the screen will depend on the diffraction angle and the type of...
crystal. Measuring the intensity as a function of angle will give information on the substructure of the crystal's unit cell.

\[
\text{Figure 3.2. Waves that are in phase will give rise to constructive interference.}
\]

\[
\text{Figure 3.3. Waves that are } \frac{\pi}{2} \text{ out of phase will produce destructive interference.}
\]

If white light (X-rays or neutrons of many wavelengths) is used as the primary beam, then interference effects will be seen at many angles, regardless of the orientation of the crystal. However, monochromatic beams will only diffract at discrete angles called the Bragg angle (equation 3.1). This can be derived from figure 3.4. At all other angles only isotropic background is observed.

\[
n \lambda = 2d \sin \theta \quad 3.1
\]
where \( n \) is the order of diffraction, \( \lambda \) is the beam wavelength, \( d \) is the repeat spacing of the unit cell and \( \theta \) is half the diffraction angle of the Bragg reflection relative to the primary beam. There is an inverse relationship between angle and repeat spacing. This means that large structures will diffract closer to the beam stop than smaller structures.

![Diagram of Bragg angle geometry](image)

**Figure 3.4.** Demonstration of the Bragg angle geometry. The path length between scattering centers must equal an integral multiple of the wavelength to give rise to constructive interference.

Only at the Bragg angles will the path-length of the scattered waves equal an integral multiples of wavelength (figure 3.4). This will give rise to constructive interference. If the scattering centers are progressively misaligned, then destructive interference occurs and no diffraction is observed. This is not immediately intuitive since adjacent scattering centers will not scatter neutrons whose waves are \( \pi/2 \) out of phase. Therefore, adjacently
scattered waves can interfere to produce a wave of some arbitrary amplitude although this amplitude will be less than the maximum. The reason for the lack of diffraction is subtler. The crystal lattice contains many hundreds, if not thousands of unit cells. Therefore, if each unit cell is progressively out of alignment then for every wave that that is scattered from a center, there is another wave that is exactly $\pi/2$ out of phase with it. This emphasizes the importance of a highly aligned quality crystal. If only a couple of scattering centers are misaligned, then diffraction is still observed at the Bragg angle but the peak has a greater mosaicity. In the case of neutron diffraction, a counting detector measures the diffracted wave intensity. Initially, the scattered waves are weak and locally spherical (figure 3.5). It is highly unlikely that diffracted waves will further interfere with the transmitted primary beam. Far away at the detector, the added waves are effectively planar.
Figure 3.5. The scattering of neutrons from the scattering centres is weak and spherical. Relative to the detector, the diffracted waves appear planar.

This chapter is concerned with 'orientated' neutron diffraction from 1-dimensional model membrane liquid crystals. Such experiments were first carried out in the 1970s. The experiment requires the formation of highly aligned multi-bilayer stacks. A more detailed explanation of sample preparation is given in the materials and methods section of this chapter.

Diffraction from a single bilayer will produce continuous scattering (molecular transform) of very weak intensity that cannot be measured. This is because the component atoms that make up phospholipid molecules have weak coherent cross-sections. As neutrons (and X-rays) interact weakly with such atoms, many layers of model membranes are required in order to observe good diffraction. The lattice effectively amplifies the signal. Figure 3.6 illustrates a side-view of the reflecting planes of the diffracting lattice and figure 3.7 shows
the unit cell’s diffracting substructure that gives rise to the molecular transform.

Figure 3.6. Side-on view of the 1st order reflection planes in a phospholipid multi-bilayer stack. One lipid bilayer is contained between each dotted line. The unit cell size is equal to a distance, d. This gives rise to the lattice transform of the diffractogram.

The multi-bilayers display a high degree of order only in the direction perpendicular to the membrane surface. Therefore, the final neutron scattering density maps are 1-dimensional and describe the position of membrane components relative to the bilayer normal.
Figure 3.7. The internal structure of the unit cell that accounts for the variation in diffracting intensity as a function of angle. The unit cell may be classified as the equivalent distance from one bilayer center to the next. The unit cell comprises both the lipid bilayer and an interfacial water layer. Again, the 1st order of diffraction is shown where the scattering centers are a distance, d, apart.

Figure 3.7 also shows the diffraction geometry of model membranes. The figure shows the Bragg condition for the 1st order of diffraction. That is, a path length equal to one wavelength occurs between scattering centers that are a distance, d, apart. Although only two scattering centers are shown, the neutron beam samples every atomic nucleus within the unit cell. The resulting intensity of the diffracted wave, therefore, is directly related to the coherent cross-sections of each atom within the unit cell. If the sine of the angle is then doubled, there will now be a path difference of two wavelengths between the
original scattering centers. Incoming waves will still constructively interfere when scattered by the original centers but there now exists a scattering center halfway between the original centers. This ‘new’ scattering center also fulfills the Bragg condition and contributes towards the second order diffracted wave intensity. The scattering centers are now every $1/2d$ apart, therefore the resolution of the experiment is increased by a factor of two. Higher orders of diffraction progressively increase the resolution of the experiment. This is analogous to increasing the magnification of an ordinary microscope.

![Diagram](image)

**Figure 3.8.** Cartoon illustrating a diffractogram obtained from orientated neutron diffraction of stacked multi-bilayers. In reality, each order is recorded separately and the detector counts the number of neutrons that collide with the pixels. The higher orders are of a reduced intensity and tend to be smeared out into an arc.
A typical diffractogram of an orientated sample is shown in figure 3.8 and the intensities of each order can be portrayed graphically as a function of reciprocal space (figure 3.9).

Relating the geometry of the experiment to the Bragg reflections observed at the detector can yield useful initial information. The spacing ratio of the reciprocal space reflections will give information on the type of bulk lipid phase present. Diffraction spots that have an equal spacing are indicative of a lamellar phase signature which is expected anyway for phospholipid multibilayer stacks of known melting point before the experiment commences. The distance between each spot gives the inverse spacing \( (1/d) \) of the sample which is equal to \( Q \), the momentum transfer vector. Its inverse, therefore, yields the d-repeat (real space) of the unit cell.

**Figure 3.9.** The integrated intensities from the diffractogram can be plotted as a function of reciprocal space. Shown are the Bragg from the first three orders of diffraction. Each order 'sits' on the isotropic background noise caused by the hydrogen content in the sample.
Any off-meridional smearing of the diffraction spots gives an indication of the amount of order in the sample. Ideally, no smearing will occur if the bilayers are perfectly aligned (perfectly imperfect crystal). However, there are usually a number of bilayers that are randomly orientated in the sample. This gives rise to powder diffraction and the diffracted intensity spreads out into an arc over $2\pi$ radians (Debye-Scherrer rings). This is most noticeable when measuring the higher orders as these weaker reflections have a poorer signal-to-noise ratio. If the sample has a large powder component, the higher orders of diffraction may not surface above the incoherent background noise caused by the hydrogen content within the sample. As the diffraction intensity from orientated crystals is concentrated onto a smaller detector area, better resolution is obtained than with powder diffraction, where the intensity is effectively ‘diluted’ over $2\pi$ radians. The neutron diffraction experiment is very similar to simple light microscopy in that the wave trains of the main beam are scattered by the sample. However, there are two major differences apart from the difference in respective wavelengths. Firstly, light microscopy generally uses polychromatic light, whereas neutron diffraction tends to use monochromatic waves. Therefore, in neutron diffraction the sample must satisfy the Bragg geometry in order for the scattered neutrons to interfere constructively. Secondly, the light waves scattered by a biological sample can be recombined and focused as an image. Presently, neutrons (and X-rays) cannot be focused. Therefore, the generation of an image that represents the
diffracting structure has to be done some other way. This can be achieved by mathematically focusing the diffraction spots in a process known as Fourier analysis.

**Figure 3.10.** Fourier synthesis. Each component sinusoid can be summed to generate the original diffracting structure. In this case, a square wave function represents a real life object of identical dimensions but other structures can be constructed in the same manner. The parameters of each component wave can be measured from the diffractogram. Adapted from Warren, 1987.

The diffractogram represents the multiplication of the 1-dimensional periodic lattice transform (delta function) with the non-periodic molecular transform (continuous transform). The calculated amplitudes contained within the diffractogram, therefore, are the Fourier transform (FT) of the convolution of
the original crystal with the unit cell. The sample and the diffractogram are termed FT pairs. The unit cell structure can be elucidated solely from the information provided in the diffractogram.

Fourier’s theorem states that ‘any mathematical function can be considered as the sum of a series of sinusoidal frequency components’ (Bracewell, 1989). This can be visualized in figure 3.10. If the diffracting unit cell is relatively featureless and shaped like a book, then an infinite number of added waves can represent the original structure. The waves can be added since they can be described by first order differential equations (Gough et al, 1996). This allows the principle of superposition of waves to be applied. Thus, a square wave function is described only if the high frequency components are included in the sum. If they are not included then an under-resolved structure is generated. The component waves must be summed in order to relate to a real-life object. Individually, the component waves are meaningless.

The periodicity of the multi-bilayer sample facilitates the use of Fourier’s theorem in our studies. The definition of a periodic structure (or mathematical function) is one that repeats over an infinite distance (or time). Realistically, this is impossible but a mathematical approximation of this is very useful. The number of unit cells in our sample (approximately one thousand) represents the periodic structure and gives rise to series of delta functions at each integral multiple of the fundamental frequency (1st order spatial frequency). Two delta
functions either side of the line x=0 represent the FT of a cosine wave of duration equal to the number of unit cells that comprise the lattice. As the number of unit cells is many, the cosine wave is regarded as periodic. Each individual unit cell is a non-periodic structure. It does not repeat itself and has a fixed period equal to the d-repeat. The waves that describe the unit cell are truncated and their FT gives rise to a continuous band spectrum instead of discrete line functions. This is illustrated in figure 3.11.

Multiplication of the delta function at each spatial frequency with the molecular transform gives the parameters of the component wave function that will describe the bilayer profile. This is a trivial process and is arithmetically the same as multiplying the structure factor amplitude at each respective spatial frequency by ±1, depending on the phase. Each recorded order of diffraction represents a cosinusoid term in a Fourier series that describes the bilayer profile. An expression for a Fourier series is given in equation 3.2 and is dealt with in more detail in the next section.

\[ \rho(x) = \rho_0 + \sum_{h=1}^{h_{\text{max}}} F(h) \cos\left(\frac{2\pi hx}{d}\right) \]  

3.2

Where \( \rho(x) \) is the mathematical function that describes the transbilayer profile, \( \rho_0 \) is the mean value of \( \rho(x) \), \( F(h) \) is the structure factor amplitude of each diffraction order and \( d \) is the d-repeat.
Figure 3.11. Plot showing the multiplication of the lattice transform with the molecular transform. A sinc function \( \frac{\sin(x)}{x} \) can be used to fit the observed points and create a pseudo-continuous transform. The structure factor values between the delta functions are meaningless, as they do not represent measured points. A sinc function that fits well to the observed points can be useful in phasing the data.

As the unit cell is centro-symmetric, the wave functions used in the Fourier synthesis are cosinusoids (even functions). The amplitude of the wave is known as the structure factor amplitude and is simply the square root of the intensity of the diffraction spot. The frequency of the wave is the spatial frequency of each diffraction spot. In the case of periodic structures such as phospholipid multi-bilayer stacks, each frequency component is an integral multiple of the fundamental (1st order) frequency. The scattering centers of the 1st order of diffraction are a distance, \( d \), apart so the spatial frequency is \( 1/d \). That is, the wavelength of the first order wave is exactly the same as the period.
of the unit cell, the d-repeat. The 2\textsuperscript{nd} order of diffraction has scattering centers a \( \frac{1}{2} d \) apart. Since the frequencies of the gratings are now twice as many, the spatial frequency is \( 2/d \). Therefore, the wavelength of the 2\textsuperscript{nd} order is \( \frac{1}{2}d \), and two complete wave cycles are observed within the d-repeat. Thus, the relationship between the component waves and the final bilayer profile is analogous to the individual notes (or harmonics) that make up a musical chord. The difference is that the component waves of a bilayer profile have different amplitudes whereas the harmonics that comprise a chord are all struck with the same hardness and so have equal amplitudes.

The correct addition of each component cosinusoid will give a mathematical function (Fourier series) that describes the image of the original diffracting structure in terms of neutron scattering density parallel to the bilayer normal.

The correct definition of structure is important. The structure is ‘the image of the membrane that consists of the average spatial distribution of the submolecular groups projected onto the line normal to the plane on the membrane from which the relative intergroup distances can be measured’ (Wiener and White, 1991). For neutron diffraction, if the bilayer were perfectly crystalline, then the projections of each atom along the line normal to the bilayer surface would be a series of delta functions. However, the massive thermal fluctuations within a liquid crystalline sample prevent this. The delta functions become broad overlapping functions and the contributions of individual atoms to the bilayer profile are lost. Instead, the bilayer is
represented by a sum of cosinusoid waves. The peaks and troughs of the final structure represent the average distribution of the principle molecular fragments that make up the lipid bilayer (figure 3.12). As the neutron scattering lengths of the principle molecular fragments are known (figure 3.13), a real-space model of the bilayer can be constructed. In fact, the bilayer profile of DOPC at 66% rh has been reduced to a series of Gaussian functions that describe the average spatial projections of these principle molecular fragments (Weiner and White, 1992).

![Figure 3.12](image_url)

**Figure 3.12.** Typical neutron scattering density profiles across a DOPC bilayer. The inter-bilayer water compartment is at the outer region of the graph. A pair of phospholipid molecules is also shown, to assist with interpretation of the profile. The peaks at 8Å and 42Å represent the phosphate head groups. The trough at 25Å represents the negative scattering of the terminal methyl groups.
The correct addition of each component wave is not uncomplicated. This is because the phase of the wave is unknown. The phase is the relative horizontal position of each wave in the Fourier series. The process of phasing the data is analogous to finely focusing a light microscope in order to resolve the structure better. Diffracted beams from centro-symmetric unit cells, such as the phospholipid multi-bilayer stacks described in this study, are always in phase. This greatly simplifies the problem but does not eliminate it. The diffractogram represents the intensities of each order at a particular point in reciprocal space. The square root of the intensity gives the structure factor amplitude. This is the value needed in order to solve the structure. However, the value of the square root can be positive or negative. This is the same as choosing wave phases of 0 or \( \pi \) radians. Therefore, in the process of converting from intensities to amplitudes, vital phase information is lost.

The phase problem in membrane crystallography is made worse by our inability to accurately measure the structure factors. Only when the structure factors are accurately determined does the investigator stand the best chance of phasing the data. Phospholipid multi-bilayers are very sensitive to the slightest humidity gradient in a sample can. This can change the d-repeat of the lipid bilayer whilst the experiment is underway. For example, if the d-repeat remains constant whilst collecting all orders except for the 3rd order, then the 3rd order will not have an integral multiple spatial frequency of the fundamental frequency. Although the actual measurement of the diffracted
intensity may be highly accurate, the phase difference of the 3\textsuperscript{rd} order is not exactly 0 or $2\pi$ radians with respect to the other component waves in the series. This area of data analysis is somewhat esoteric and can be better explained using the power of analogy. The experimental situation is analogous to playing a musical chord where one of the notes requires tuning. Just as the sound of the musical chord is of a poorer quality, so is the final bilayer profile. In the same way that sound engineers investigate the properties of musical notes in order to improve the quality of a chord, the next section of this thesis describes a more accurate method to determine the structure factor amplitudes of the component waves that comprise the bilayer profile. This technique also aids in phasing lamellar neutron data.
Figure 3.13. Bar chart showing the coherent scattering amplitude densities for the principle molecular fragments that comprise the unit cell. Note the large difference between hydrogen and deuterium for neutrons and the lack of difference for X-rays. The mean scattering of the unit cell is equal to the sum of the atomic coherent scattering amplitude densities divided by the molecular volume. The calculated mean scattering is also equal to the scattering amplitude of the zeroth order. Adapted from Bradshaw, 1995
3.4. Real-time swelling series method improves the accuracy of lamellar neutron diffraction data

3.4.1. Introduction

The so-called swelling series method has been widely utilised in X-ray diffraction measurements of lamellar phospholipid preparations as a technique for the determination of structure factor phases. The process consists of recording structure factor amplitudes at a range of points in reciprocal space which, when scaled to each other and plotted, trace out the continuous transform of a single bilayer. In practice this is achieved by the use of a number of samples, each prepared to a different lamellar spacing by controlling the humidity of the atmosphere or the osmotic pressure of the solution (King and Worthington, 1971).

One study has described an adaptation of the swelling series method, in which changing the relative humidity of a sample caused oscillations of the observed diffracted intensity (Bradshaw et al., 1998). This experiment was performed in situ by swelling dehydrated orientated stacks of phospholipid in the sample can of a neutron diffractometer whilst several consecutive \( \theta-2\theta \) scans were conducted. It was demonstrated that this method, when applied to lamellar phospholipids, has the potential to improve both the accuracy of measurement of structure factors and their phase assignment. Such an approach is only feasible with neutron diffraction where the low levels of radiation damage
allow a single sample to be scanned repeatedly. Each programmed scan gives one structure factor value for each order of diffraction when the Bragg geometry for that order is satisfied. A number of scans are conducted as the bilayers swell by taking up water. In each scan the position of any structure factor will have shifted slightly from the previous scan, thereby sampling a different region of reciprocal space and having correspondingly different amplitudes. The net result is that a family of structure factor measurements is obtained for each order.

A previous paper presented data from a sample of DOPC collected under real-time conditions of increasing relative humidity of pure water (Bradshaw et al., 1998). The term pure water means that the water contained no salts to hold the rh of the sample environment below 100%. The isotopic composition of the pure water was 100% $^1$H$_2$O (therefore 0% $^2$H$_2$O). It was demonstrated that, at this isotopic composition of water, the observed structure factors did not lie on a single continuous transform. This result is to be expected because the scattering density of the unit cell changes as more water is incorporated into the sample. Incorporation of this, negatively scattering, water affects the bilayer structure in two ways. The mean scattering density is reduced and the neutron scattering contrast is altered. Therefore, the observed structure factors do not lie on the same continuous transform because the scattering structure is effectively different even if the physical structure of the bilayer remains unchanged. It is common practice however, to take calculated structure factor
values from the continuous transform and use these values in Fourier subtractions if the d-repeat of the samples differs by approximately 1Å (Büldt et al., 1979). Our report highlighted the potential errors in subtractions by doing this.

This chapter presents a swelling series of structure factors collected from DOPC at 8.06% ²H₂O and is compared to an earlier set collected at 0% ²H₂O. At 8.06% ²H₂O, water has a net neutron scattering density of zero. It is demonstrated, as speculated earlier, that under these conditions the measured structure factors do indeed lie on a single continuous transform. This indicates that there is no change in the neutron scattering of the unit cell and also infers little or no change in the physical structure of the bilayer at the resolution of our measurements. The two data sets, at 0% and 8.06% ²H₂O are used in calculations to quantify the errors caused by subtracting structure factors from samples that do not have exactly the same d-repeat. These findings reinforce our assertion that the novel adaptation of the swelling series method contributes substantially to improved accuracy in neutron diffraction experiments.

3.4.2. Sample preparation

DOPC was purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. 20 mg samples were dissolved in chloroform. An artist’s airbrush, using nitrogen as propellant, was used to deposit the lipid onto
quartz microscope slides (75 mm by 25 mm). The slides were placed in a vacuum, over the desiccant phosphorous pentoxide ($P_2O_5$), for twelve hours. The slides were then hydrated before being placed back in the vacuum desiccator for a further twelve hours to remove as much water as possible. Since some water molecules tightly bound to the phosphoryl head-group may have remained, samples for measurement with 8.06% $^2H_2O$ were hydrated in a humid atmosphere with water at this isotopic composition, before being dried again under vacuum for the second time. The samples were protected from light whenever possible in order to reduce any chance of lipid peroxidation. The sample preparation mentioned here is very similar to the other diffraction experiments described later in this chapter.

3.4.3. Experimental setup and data analysis

Neutron diffraction measurements were carried out on the D16 membrane diffractometer at the ILL, Grenoble, France. Neutrons were produced from a fission reaction of $^{235}$uranium. Such neutrons have a range of wavelengths generally below 1Å. This wavelength is too short for diffraction studies so a moderator ($H_2O$ or $^2H_2O$) 'cools' them until they obtain thermal equilibrium. The resultant ‘cold’ neutrons are collimated then monochromatised. A lead-graphite crystal monochromator deflects the incident neutron beam out of the guide. The beam divergence was ~1%, which is acceptable for neutron
scattering. This means that the mean of the wavelength spread was 4.516Å ± 0.02Å.

The membrane diffractometer controls the positions and orientations of the sample and the detector. This set up allows both the sample and detector to be rotated around a common axis until the Bragg condition is satisfied. The reciprocal space axis was then calibrated. The sample-to-detector distance was 1m. A crystal of known unit cell size will give discrete Bragg reflections at known points in reciprocal space. This allows the unit cell dimension to be calculated according to a simple trigonometrical relationship (equation 3.3). This is illustrated in figure 3.14.

$$y = Y \tan(\theta)$$  \hspace{1cm} (3.3)

where y is the unknown distance from the primary beam to the reciprocal space reflection, Y is the sample-to-detector distance and $\theta$ is the Bragg angle.

**Figure 3.14.** The trigonometrical relationship between the sample and the detector. Further details can be found in the text.
The sample environment was a standard aluminium can, in which temperature control is achieved by circulating water through an integral water jacket, and humidity control by changing the solution in a teflon water bath at the base of the can. Aluminium and silicon are commonly used materials in neutron scattering experiments because they have a negligible incoherent cross-section and so contribute little background noise to the spectra. Each anhydrous DOPC sample, on its quartz slide, was quickly transferred straight from its vacuum desiccator into the D16 can together with a bath of pure water, at either 0% or 8.06% $^{2}$H$_{2}$O. A series of continuous $\theta$-2$\theta$ scans was immediately initiated. Each scan (from $\theta = 1.5^\circ$ to $\theta = 15.0^\circ$) took approximately three hours to complete. The samples were run at 25°C. The mosaic spread of the second order of diffraction was determined for each sample.

The $^{3}$He gas filled detector consisted of a 2-dimensional array of pixels. The diffracted neutrons collide with the helium isotope to give $^{3}$H and a proton. The integrated intensity is therefore calculated by measuring the resulting $\beta$ emission by scintillation counting. The detector counts for each frame of data were corrected for variations in response. This was achieved by division of a corresponding array of data recorded from pure water ($^{1}$H$_{2}$O). The complete set of frames from each scan were then collapsed into a linear spectrum and combined to generate a pseudo $\theta$-2$\theta$ scan. The D16 instrument software carried out all of the analysis to this stage.
The background around each peak was fitted and subtracted using SigmaPlot (SPSS Inc., Chicago, IL), a commercial spreadsheet and graphing package. Gaussian distributions were then fitted to the Bragg reflections. The intensity of each order of diffraction equals the area under each fitted Gaussian peak. This intensity value is then subject to three experimental correction factors. Firstly, a neutron absorption correction takes into account the removal of neutrons from the diffracted beam. This is related to the amount of hydrogen in the sample and the angle of diffraction. The grazing, low orders of diffraction have a longer neutron path length within the sample and, therefore, have greater absorption corrections. Secondly, a Lorentz factor is applied that accounts for the detector’s inability to measure the intensity of each order of diffraction with equal accuracy. The higher orders of diffraction tend to ‘cut’ through the Ewald sphere at a greater rate than the lower orders. Therefore, the detector measures less diffracting intensity of the higher orders. Thirdly, an angular correction takes into account the difference in sample cross section that is measured for each order. At low diffraction orders, the narrow beam samples most of the silicon wafer. Conversely, at higher diffraction orders the cross-section exposed to the beam is much less. Angular corrections are not required at the membrane diffractometer at the ILL because the width of the beam is greater than the width of the sample.

Once the corrections have been applied, the intensities were then square-rooted to produce arbitrary structure-factor amplitudes.
3.4.4. Results and Discussion

Structure factors for the 0% $^2$H$_2$O samples were calculated from the observed intensities and plotted against their spatial frequencies, as shown in figure 3.15 (a). Reference to this figure shows that the 0% $^2$H$_2$O neutron structure factors, determined at a range of spatial frequencies, do not lie on the same continuous transform. Each order traces out its own curve through the observed points. A quadratic expression was used to join the observed points of each order, in turn.

The constantly changing relative humidity with the sample can was reflected in a constantly changing d-repeat throughout the data collection period. This meant that no two of the observed structure factors, even those within the same scan, indexed on to the same reciprocal lattice. Interpolation between the observed points of each order produced sets of structure factors for a range of d-repeats. These structure factors sets were used to calculate continuous transforms $C(x)$, using equation 3.4:

$$C(x) = \sum_{h=0}^{h_{\text{max}}} F(h) \frac{\sin(\pi dx - \pi h)}{(\pi dx - \pi h)}$$  \hspace{1cm} (3.4)

where $F(h)$ are the observed structure factors, $d$ the Bragg spacing, $x$ the distance along the bilayer normal to the plane and $h$ is the order number. The resulting continuous transforms show a smooth variation in amplitude, reinforcing faith in the phase assignments (figure 3.15a). The continuous transform of many non-periodic functions can be well described by a sinc function ($\sin(x)/x$). The sinc function is a band spectrum that can be used to
represent the molecular transform of a bilayer unit cell. The FT of such a sinc function yields the original real space scattering density profile.

Figure 3.15. Plots of structure factor amplitude versus spatial frequency of data points collected by real-time swelling series from highly aligned bilayers of DOPC at 25°C. The dry DOPC sample was placed in a D16 sample can along with water troughs containing water of 0% (a) or 8.06% $^2$H$_2$O (b). Consecutive $\theta$-2$\theta$ scans were run over a total period of 12 hours (a) or 9 hours (b). Also shown are continuous transforms calculated using Equation 3.3.
The sets of structure factors were also used to calculate a family of trans-bilayer coherent neutron scattering-density profiles, as shown in figure 3.16 (a). Within this family of profiles, no scaling was necessary, since each profile was produced by the same sample. However, each family of profiles was put on a per-lipid scale using the ‘relative-absolute’ method of White (Jacobs and White, 1989, Wiener et al., 1991). The ‘relative-absolute’ scale involves establishing the mean neutron scattering density of the unit cell and applying a factor that correctly scales the bilayer profiles fluctuations around this mean. This procedure is explained further in section 3.5.3.

Scaling was achieved by using previous data collected from the same lipid, on the same diffractometer (Duff et al., 1993; Bradshaw et al., 1994; Bradshaw, 1997). This scaling process also yields the value of $F(0)$, the zeroth order, for each structure factor set, as discussed below.

In the case of the data collected at 8.06% $^2$H$_2$O, all observed points were simultaneously fitted to a continuous transform, using a least-squares minimisation procedure. Sets of model structure factors $F(H)$, each corresponding to a d-repeat of $D'$, were fitted against all observed data points, including the calculated $F(0)$, to satisfy the following equation:

$$F(h) = \sum_{H=0}^{H_{\text{max}}} F(H) \frac{\sin(\pi D'h/d - \pi H)}{(\pi D'h/d - \pi H)}$$

The process was repeated using a number of different values of $D'$ since analytical continuation theory (King and Worthington, 1971) predicts that all the model structure factors should lie on the same continuous transform. The
results are shown in figure 3.15 (b). Sets of model structure factors resulting from this least-squares fitting procedure are shown in table 3.1 and scattering density profiles, calculated from them, are shown in figure 3.16(b).

![Figure 3.16](image)

**Figure 3.16.** Swelling series of neutron scattering density profiles of DOPC calculated by Fourier synthesis, using structure factors from table 3.1. (a) $d = 50\,\AA$; (b) $d = 51\,\AA$; (c) $d = 52\,\AA$; (d) $d = 53\,\AA$; (e) $d = 54\,\AA$. The 8.06% profiles (b), also range from $d = 50\,\AA$ to $d = 54\,\AA$.

One distinct advantage of the swelling series method is the potential increase in the accuracy of intensity measurement. Any change in d-repeat, caused by
temperature or humidity fluctuations, or incomplete equilibration, can result in large differences in intensity of any single order. This effect is seen at its most extreme in the first order of the 0% \(^2\text{H}_2\text{O}\) series, where a change in d-repeat of 2.5 Å (from 50.8 to 53.2 Å) causes a 280% change in amplitude (which is equivalent to nearly an 800% change in intensity). The indexing of each structure factor to its own spatial frequency, rather than assuming that they all fit the same reciprocal lattice, removes this potential source of error. In the case of data collected at 8.06% \(^2\text{H}_2\text{O}\), there is the added advantage that all observed structure factors can be used to define the same continuous transform, even when they all index to different reciprocal lattices.

The trans-bilayer distribution of coherent neutron scattering density can be described as a one-dimensional bilayer profile, which is constructed using Fourier summation. Each order of diffraction contributes a frequency component cosine function that gives a term in the summation. For centro-symmetric phospholipid bilayer structures the Fourier equation is:

\[
\rho(x) = \sum_{h=1}^{h_{\text{max}}} F(h) \cos(2\pi x h / d)
\]  

The mean value of a cosinusoidal wave is zero. Therefore, it follows that the sum of a number of cosinusoidal waves will also have a mean value of zero. This would infer that that the mean value of all bilayer profiles would be zero regardless of the lipid species studied. Clearly this cannot be true so a constant value is added to the series that is related to the total coherent scattering length of a particular lipid:
\[ \rho(x) = \rho_0 + \sum_{h=1}^{h_{\text{max}}} F(h) \cos(2\pi x h / d) \] 3.7

Multiplying \( \rho(x) \) by \( \cos(0) \) and integrating over the period gives the area under the bilayer profile. Every term on the right-hand side, except the constant, has a zero integral value, so \( \rho_0 \) is the mean value of \( \rho(x) \). The value \( \rho_0 \cdot d \) is equal to the area under the bilayer profile and also equals the unobservable zeroth order, \( F(0) \). Its value can be determined by summing the total coherent scattering-length of each atom in the unit cell. If the unit cell is considered to contain two phospholipid molecules, then \( \rho_0 \cdot d \) is equivalent to the total coherent scattering-length of two DOPC molecules plus its associated waters. The number of waters present per lipid must therefore be known from other methods (section 3.5.3).

The Fourier series of the bilayer now becomes:

\[ \rho(x) = \rho_0 + 2 \frac{1}{d} \sum_{h=1}^{h_{\text{max}}} F(h) \cos(2\pi x h / d) \] 3.8

Since the mean scattering density of the unit cell is related to the amplitude of \( F(0) \), an alternative form of this equation, which removes the factor of two by including both sides of the diffraction pattern, is:

\[ \rho(x) = \frac{1}{d} \sum_{h=-h_{\text{max}}}^{h_{\text{max}}} F(h) \cos(2\pi x h / d) \] 3.9

The mean scattering density of the unit cell can be estimated by summing the coherent scattering-lengths of the atoms that comprise the unit cell. So:

\[ \rho_0 = 2 / d \left( n_w b_w + b_{\text{lip}} \right) \] 3.9
where \( n_w \) is the number of waters/lipid, \( b_w \) is the coherent scattering-length of water and \( b_{lipid} \) the coherent scattering-length from a single lipid molecule. The factor of two simply arises from the fact that the bilayer is composed of two monolayers.

\( F(0) \) cannot be determined experimentally, yet an accurate estimate of its amplitude is essential for the construction of continuous transforms from diffraction data. The introduction of extra solvent in the swelling series method normally also changes the mean scattering density of the unit cell, and therefore also changes \( F(0) \). This is demonstrated in table 3.1. Example values of \( F(0) \) for neutron and X-ray studies are given in table 3.2. The change in \( F(0) \) with hydration means that the structure factors from swelling series measurements of stacked bilayers do not fit on a single continuous transform, as shown in figure 3.15 (a). However, in the special case of neutron structure factors of stacked bilayers hydrated with 8.06% \(^2\text{H}_2\text{O} \), \( F(0) \) does not change with hydration because water of this isotopic composition has a net neutron scattering density of zero. With this solvent, the value of \( F(0) \) determined at one hydration can be used for all other hydrations, and the fact that \( F(0) \) does not change removes a large source of potential error when fitting the observed points to a single continuous transform (figure 3.15).
<table>
<thead>
<tr>
<th></th>
<th>0% $^2$H$_2$O</th>
<th>8.06% $^2$H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d = 50$ Å</td>
<td>$d = 51$ Å</td>
<td>$d = 52$ Å</td>
</tr>
<tr>
<td>$F(0)$</td>
<td>7.28</td>
<td>5.56</td>
</tr>
<tr>
<td>$F(1)$</td>
<td>-14.06</td>
<td>-10.96</td>
</tr>
<tr>
<td>$F(2)$</td>
<td>-2.37</td>
<td>-5.28</td>
</tr>
<tr>
<td>$F(3)$</td>
<td>4.46</td>
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<tr>
<td>$F(4)$</td>
<td>-3.26</td>
<td>-2.56</td>
</tr>
<tr>
<td>$F(5)$</td>
<td>-1.38</td>
<td>-1.82</td>
</tr>
<tr>
<td>$\sum_{h=0}^{5} F(h)^2 / d$</td>
<td>5.76</td>
<td>4.19</td>
</tr>
<tr>
<td>$\sum_{h=1}^{5} F(h)^2 / d$</td>
<td>4.72</td>
<td>3.58</td>
</tr>
</tbody>
</table>

Table 3.1. Relative absolute structure factors for DOPC hydrated to various levels with 0% $^2$H$_2$O or 8.06% $^2$H$_2$O, derived from the data shown in Figure 3.15. In the case of the 0% $^2$H$_2$O data, the structure factors were determined by interpolation between observed points. For the 8.06% $^2$H$_2$O data, the structure factors are points on the single continuous transform that best describes all observed points.
<table>
<thead>
<tr>
<th>Lipid</th>
<th>X-rays</th>
<th>Neutrons 0% $^2$H$_2$O</th>
<th>Neutrons 100% $^2$H$_2$O</th>
<th>Neutrons 8.06% $^2$H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC (18:1)</td>
<td>5 w/l</td>
<td>6.2</td>
<td>27.0</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>10 w/l</td>
<td>4.5</td>
<td>46.2</td>
<td></td>
</tr>
<tr>
<td>DOPG (18:1)</td>
<td>267.2</td>
<td>8.5</td>
<td>29.3</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>295.3</td>
<td>6.8</td>
<td>48.4</td>
<td></td>
</tr>
<tr>
<td>DOPE (18:1)</td>
<td>258.8</td>
<td>6.7</td>
<td>27.5</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>286.9</td>
<td>5.0</td>
<td>46.6</td>
<td></td>
</tr>
<tr>
<td>DOPS (18:1)</td>
<td>270.6</td>
<td>11.1</td>
<td>31.9</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>298.7</td>
<td>9.4</td>
<td>51.0</td>
<td></td>
</tr>
<tr>
<td>DPoPC (16:1)</td>
<td>254.3</td>
<td>6.9</td>
<td>27.7</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>282.4</td>
<td>5.2</td>
<td>46.8</td>
<td></td>
</tr>
<tr>
<td>DPPC (16:0)</td>
<td>256.5</td>
<td>3.9</td>
<td>24.7</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>284.6</td>
<td>2.2</td>
<td>43.8</td>
<td></td>
</tr>
<tr>
<td>DPPG (16:0)</td>
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<td>27.0</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>279.5</td>
<td>4.4</td>
<td>46.1</td>
<td></td>
</tr>
<tr>
<td>DPPE (16:0)</td>
<td>243.0</td>
<td>4.3</td>
<td>25.2</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>271.1</td>
<td>2.6</td>
<td>44.3</td>
<td></td>
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<tr>
<td>DPPS (16:0)</td>
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<td>8.8</td>
<td>29.6</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>282.9</td>
<td>7.1</td>
<td>48.7</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. Relative absolute $F(0)$ values for common phospholipids.

Another significant source of error in X-ray swelling series measurements comes from the scaling of the individual data sets to each other. Each point in the swelling series has to be determined from a different sample, since ionising radiation damage precludes the re-use of samples for more than one measurement. The scaling procedure is classically based upon the formula:

$$k = \sum_{h=0}^{\infty} F^2(h) / d$$  \[3.10\]
where $k$ is a constant. However, this summation must include $F(0)$ which, as we have already shown, changes with hydration. The scattering density contrast also changes with humidity (see below). Moreover, the summation should also extend to infinity, which is clearly impractical. The result of carrying out the summation over an incomplete series, and with either no zeroth order or at best, an approximation, is an inaccurate scaling of the data sets to each other. The errors inherent in this process are quantified in figure 3.17.

None of these limitations applies to the neutron adaptation of the swelling series method when used with $8.06\% \, ^2\text{H}_2\text{O}$. The low levels of radiation damage inherent in the use of neutrons means that several swelling series points can be obtained from the same sample. In the real-time swelling approach described here, the sample is not even disturbed between measurements, so that the requirement for scaling between measurements in the same swelling series disappears.
Figure 3.17. Determination of errors introduced into difference subtraction data by subtracting structure factor sets that do not have the same d-repeat. Experimentally determined structure factors (five orders) for DOPC at various d-repeats were subtracted from each other and the difference expressed as a percentage. The points shown are the mean of 5 independent calculations with the error bars showing the maximum spread. (a) 0% $^2$H$_2$O data, each data point represents the mean of 5 calculations of the form:

\[
\text{Percentage} = \frac{100 \sum_{k=1}^{5} |F_{(k)} - F'_{(k)}|}{\sum_{k=1}^{5} |F_{(k)}|}
\]

(b) 8.06% $^2$H$_2$O data, errors calculated as in (a). (c) 0% $^2$H$_2$O data, errors calculated as in (a), except that the continuous transform method (Equation 1) was used to correct one of the data sets before the subtraction. The magnitude of the error increases as the coherent neutron scattering density of water deviates from zero. In other words the errors are lowest at 8.06% $^2$H$_2$O, greater at 0% $^2$H$_2$O, and are therefore predicted to be greatest at 100% $^2$H$_2$O.
The introduction of water at any isotopic composition other than 8.06% $^2$H$_2$O affects not just the value of $F(0)$, but also has an impact on the other structure factors. Figure 3.16 (a) compares the bilayer structure at different points on the swelling series. At 0% $^2$H$_2$O, it is clear that water penetration into the head group region reduces the height of the phosphate-ester peaks, and shifts their center of mass into the bilayer. This demonstrates that the effect of increasing the water content is to reduce the neutron scattering contrast of the system. The reason for this is that the region to which the negatively scattering water is introduced is immediately adjacent to and, indeed, partially overlaps, the region of highest scattering density, namely the phosphates and ester linkages. The change in scattering contrast, the difference between the minimum and maximum scattering density, or the maximum deviation from the mean scattering density $F(0)$, therefore has an impact on all structure factors. This effect is seen most clearly in table 3.1, where $\sum F(h)^2/d$ changes as the d-repeat swells, even when $F(0)$ is included in the summation. Moreover, as the level of hydration rises, the d-repeat increases and the phosphate peaks appear to move further into the bilayer, as the negatively scattering water erodes their hydrated edges. It is not possible, therefore, to use the location of these peaks as a measure of the bilayer thickness, as some authors have chosen to do, since their center of mass shifts position with changes in hydration.

The d-repeat of the 0% $^2$H$_2$O swelling series sample increased from 50.8 to 53.2Å, the 8.06% $^2$H$_2$O sample from 50.4 to 51.8Å, over the period of data
collection. The mosaic spreads of the swelling-series samples were comparable to those from a similar sample measured under more conventional steady-state conditions. For example, the half width at 1/e height \( e = 2.71828 \) of the second order of the 0% \(^2\text{H}_2\text{O}\) swelling series sample was 0.34°, 0.38°, 0.38° and 0.36° for the four scans. This compares with a mosaic spread of 0.38° for a steady-state sample of pure DOPC measured with identical instrument geometry, fully equilibrated to an atmosphere of approximately 100% relative humidity.

These measurements show that there was no noticeable increase or decrease in the sample disorder throughout the swelling series measurements. The low mosaic spread is typical for measurements of (static) unsaturated phospholipids, such as DOPC, but is perhaps not expected in dynamic systems as reported here. In order to minimise disorder in the sample two complementary factors are important in minimising the degree of swelling during the measurement of each single order. Firstly, the sample should equilibrate with the atmosphere inside the sample can faster than the can atmosphere equilibrates with the water at the base of the can. In our measurements this was achieved by using only one water bath, in which the surface area of the water was relatively small (approximately 5 cm\(^2\)) and positioned some 1.5 cm below the rim. Secondly, the time spent in scanning each order should be the minimum consistent with good counting statistics. In
this respect, the high neutron flux of the D16 instrument was advantageous to the study.

A standard procedure in neutron procedure is that of difference calculation, which is most powerful when both data sets in a subtraction have exactly the same d-repeat. However, this is rarely the case; typically one has to be content with a difference up to 2% or so. Figure 3.16 shows a series of bilayer scattering profiles, calculated from points on the lines interpolated between the swelling series points. The difference between profiles that differ by only 1.0 Å is apparent. This error is quantified in figure 3.17. The figure shows that a difference in d-repeat of 1.0 Å in a difference subtraction with 0% $^2$H$_2$O data introduces an error of close to 20% in the result (plot a). This error is approximately halved if the data are collected at 8.06% $^2$H$_2$O (plot b).

Büldt et al. (1979), have proposed that when the two sets of structure factors to be used in a subtraction do not have exactly the same d-repeat, one of the sets can be recalculated using the continuous transform method (equation 3.4). Figure 3.15 demonstrates that this is only possible in the unique situation where the data are collected at 8.06% $^2$H$_2$O, for at 0% $^2$H$_2$O the observed structure factors do not actually trace out a single continuous transform, as shown in the figure. Figure 3.17 (c) quantifies the error inherent in mistakenly using the continuous transform to adjust the d-repeat of data collected at 0% $^2$H$_2$O. Up to a difference in d-repeats of approximately 0.6 Å the 'correction'
has little effect upon the error. Above 0.6 Å difference, the 'correction' procedure actually increases the magnitude of the error.

However, the swelling series method described here allows a degree of adjustment of the d-repeats of lamellar structure factors. At 8.06% $^2$H$_2$O, the continuous transform method can be used. Even at 0% $^2$H$_2$O, the swelling method allows interpolation between the measured values of each structure factor in order to determine the bilayer structure at any d-repeat within the range covered by the measurements.

The above discussion appears to have neglected the suggestion that the bilayer structure might change during the hydration process. This point has been raised by Worcester (1976) who has suggested that the conformation of phosphate-containing head group of DMPC in the L$_\alpha$ phase is dependent upon hydration level. It is possible that the 0% $^2$H$_2$O data are affected by this phenomenon, though it is not certain that the current resolution ($h = 5$) would be sufficient to show this. Similarly, Hristova and White (1998) have reported structural rearrangements of the fatty-acyl chains of a derivative of DOPC in which the double bond of the sn-2 chain had been brominated. It is unlikely that neutron diffraction would be sensitive to these structural changes in undeuterated lipids. Moreover, unless any structural rearrangement occurs over a very small change in d-repeat (the smooth curves through each 0% order in figure 3.15 do not show this to be the case) then calculating an intermediate set of structure factors between the observed points will simply
result in an intermediate structure. This view is further reinforced by the observation that the 8.06% $\text{H}_2\text{O}$ data appear to fit very closely to a single continuous transform, thereby indicating that neutron diffraction is insensitive to any lyotropic structural changes of the lipids that may have occurred during these measurements.

3.5. Revealing the membrane-bound location of NKA

3.5.1. Introduction

This section describes an orientated, small angle neutron diffraction study of the NKA-model membrane interaction. The measurements reveal the location of the both the conserved hydrophobic C-terminus and the flexible hydrophilic N-terminal of NKA within highly aligned model multi-bilayers. This was achieved by selectively deuterating a specific amino acid of NKA at each terminus. The deuterated amino acid represents the neutron scattering equivalent of isomorphous replacement in X-ray diffraction. The location of the deuterated label, relative to the bilayer normal, can be ascertained by Fourier subtraction methods.

This type of experiment is useful in visualizing the depth of insertion of NKA into model membranes. A model is presented that may represent an intermediate membrane-associated orientation for NKA that exists prior to receptor binding. This assumes that NKA associates with the membrane before receptor activation.
The study also describes the distribution of water across the bilayer. This can be achieved by recording several diffraction images of the bilayer that is hydrated using different $^1$H$_2$O/$^2$H$_2$O ratios (table 3.3).

<table>
<thead>
<tr>
<th>Sample content</th>
<th>$^2$H$_2$O (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC:DOPG 50:50 (mol%)</td>
<td>0, 50, 100</td>
</tr>
<tr>
<td>DOPC:DOPG + NKA (3-mol %)</td>
<td>0, 50, 100</td>
</tr>
<tr>
<td>DOPC:DOPG + $^2$H$_{10}$-Leucine NKA (3-mol %)</td>
<td>0, 50, 100</td>
</tr>
<tr>
<td>DOPC:DOPG + $^2$H$_8$-Lysine NKA (3-mol %)</td>
<td>0, 8, 50</td>
</tr>
</tbody>
</table>

Table 3.3. The multi-bilayers and their respective hydrating solvents that were measured at the V1 membrane diffractometer at BENSIC.

The bilayer profiles that are used in Fourier subtractions to give structurally meaningful results are as follows:

1) \[(\text{DOPC:DOPG (50:50mol%) 100% }^2\text{H}_2\text{O}) - (\text{DOPC:DOPG 0% }^2\text{H}_2\text{O})\]

   = water distribution between the pure lipid bilayers. A high ratio of DOPG was used in order to make a direct comparison of the effects of NKA with a previous SP study (Bradshaw \textit{et al.}, 1998a).

2) \[(\text{DOPC:DOPG + NKA 100% }^2\text{H}_2\text{O}) - (\text{DOPC:DOPG + NKA 0% }^2\text{H}_2\text{O})\]

   = water distribution between bilayers containing peptide. This water distribution should be the same for the two deuterated peptide samples. The increased scattering caused by the deuterium label will cancel out in the subtraction. If the water distributions of each deuterated peptide subtraction is identical to the water distribution of
the protonated peptide subtraction then isomorphous replacement has been achieved.

3) \((\text {DOPC:DOPG + } _{2}^{3}\text {H}_{10}\text {-Leucine NKA}) - (\text {DOPC:DOPG + NKA}) =\)
distribution of deuterium label. This enables depth of insertion calculations to be carried out. No \(1\text {H}_2\text {O}/2\text {H}_2\text {O}\) ratio is given. The distribution of the deuterium label should be the same at any \(1\text {H}_2\text {O}/2\text {H}_2\text {O}\) ratio. If the deuterium label distribution is the same at each \(1\text {H}_2\text {O}/2\text {H}_2\text {O}\) ratio then it is highly likely that that the phase assignments for each diffraction order are correct.

4) \((\text {DOPC:DOPG + NKA 8.07\% } 2\text {H}_2\text {O}) - (\text {DOPC:DOPG 8.07\% } 2\text {H}_2\text {O}) =\)
transbilayer distribution of whole protonated peptide. However, this subtraction is usually too complex to explain. Adding a single component like a peptide greatly complicates the system. There are now too many parameters that makes the subtraction illegible. For example, there now exists three different types of lipid population. That is, lipid that has bound to the peptide, interfacial lipid and unbound bulk lipid. The distribution of water is also likely to be different, hence the subtractions at 8.07\% \(2\text {H}_2\text {O}\). There may also be more than one peptide orientation. This all makes for a subtraction profile that is difficult to interpret.
3.5.2. Experimental setup and data collection

Neutron diffraction measurements were carried out on the VI membrane diffractometer at the BENS, Germany. The wavelength of the neutron beam was 5.618Å. The VI beam is very narrow compared to D16 at the ILL. Only a small portion of the sample slide is exposed to the beam. In contrast, the D16 beam is wider than the sample.

DOPC and DOPG were purchased from Avanti Polar Lipids (Alabama, USA) and used without further purification (99% pure). Alachem (Edinburgh, UK) synthesised the peptide to the following sequence: HKTDSFVGLM-NH₂ (>99% pure). NKA was synthesised in an undeuterated form, and with the N-terminal lysine and the C-terminal leucine replaced by deuterated analogues that contained a total of 8 and 10 deuterons respectively. Sample preparation was very similar to the method described in section 3.4.2 except that a highly polished silicon wafer was used instead of the quartz slide. The silicon wafer has a flatter, smoother surface thereby facilitating greater alignment of the model membranes. The wafer should, therefore, keep the powder component of the sample to a minimum.

Each peptide was accurately weighed out to give a peptide concentration of 3-mol% with respect to the lipid. Each peptide was then added singly to the lipid/chloroform solution. The resultant solution was not homogenous so a couple of drops of co-solvent (methanol) were added making the solution go
clear. The mixture was then sprayed onto the silicon wafer and the sample handling described in the swelling series was carried out.

Three separate periods of beam time was allocated to this experiment. This was just enough time to carry out multiple diffraction for each sample at three different $\text{H}_2\text{O}/\text{H}_2\text{O}$ ratios. The sample can at the VI membrane diffractometer has a massive volume. This means that equilibrium times are long (> 12 hours) and the slightest thermal gradient within the sealed aluminium can results in rh fluctuations. This will cause the Bragg reflections to move in reciprocal space. Therefore, the sample was measured in an environment of 92% rh. It was found that equilibration times were slightly faster and more stable at this humidity. The rh was controlled at 92% by a saturated KNO$_3$ solution in the water baths at the base of the sealed can.

Rather than carry out 0:20 scans a series of rocking curves were executed. This involved fixing the detector position at each 20 position and rotating the sample through the Bragg angle. This was done for each diffraction order. Four scans were executed for each of the 5 diffraction orders measured in order to check for any movement of the Bragg reflections. This is a good check for sample equilibration.

An attempt was made to measure a 6th order but it was either to weak or had a zero value on the continuous transform. No attempt was made to measure the stronger 7th or 8th orders of diffraction as Bradshaw et al achieved for their SP samples due to time restraints.
As only 5 orders of diffraction are measured, each neutron scattering density map was under-resolved. This means that the water distribution profile across the bilayer is not accurate because the procedure involves subtracting under-resolved profiles from each other. However, this does not affect the accuracy of deuterium label determination. Only three orders of diffraction are required to determine the label positions relative to the bilayer normal.

3.5.3 Data analysis

Only two hours of equilibration time was allowed for each sample. This length of time was too short and the sample swelled as the data was collected. However, the intensities were accurately measured at each point in reciprocal space and a swelling series was used to analyse the data.

The intensity of each order of diffraction was calculated as mentioned previously and then subjected to the correction factors appropriate to the VI membrane diffractometer. The intensities were then square-rooted to produce arbitrary structure factor amplitudes.

As in the swelling series, a family of structure factors were recorded at a number of spatial frequencies as the sample swelled in real-time. The four structure factor amplitudes were plotted against their spatial frequencies. A quadratic expression was used in order to fit the observed points. It is argued that it is possible to interpolate structure factors between the observed points. This procedure was carried out for all diffraction orders of each sample.
Unfortunately, the sample swelled uncontrollably and there was not one common spatial frequency (or integral multiple of the spatial frequency) that satisfied every family of structure factors. However, very few of the scans came into this category and a spatial frequency was chosen that was common to all the 1st and 2nd order scans. This is important because the strong first and second orders make the biggest impact on the transbilayer profile. The spatial frequency chosen that best fitted the swelling series was 0.0198Å⁻¹, which corresponds to a d-repeat of 50.51Å.

At this stage, all the structure factor amplitudes at their respective spatial frequencies were known. However, each structure factor amplitude had only an arbitrary value. The structure factors were then normalized using the ‘relative-absolute’ scale. The scaled structure factors were then Fourier transformed into component cosinusoids. Each cosinusoid has to be correctly Fourier synthesised in order to construct the transbilayer profile. This requires knowledge each structure factor phase. Recording each sample at three different isotopic compositions of water (0%, 50% or 100% ²H₂O) serves two functions. The ¹H₂O/²H₂O exchange method is utilised to scale structure factor amplitudes and it can also assist with phase assignment.

For centro-symmetric structures, progressively increasing the ²H₂O content by a constant amount will similarly change the value of each structure factor. Therefore, a plot of structure factor amplitudes against ¹H₂O/²H₂O ratio
produces a good straight-line fit. The straight-line fits of each data set are shown in figure 3.18 and 3.19

![Graph](image)

**Figure 3.18.** Portrayal of the $^1$H$_2$O/$^2$H$_2$O exchange method used to phase the orders of diffraction. Plot (a) shows the structure factors of DOPC:DOPG (50:50 mol) and plot (b) with 3-mol% NKA added. Each straight-line function has been obtained by least squares fitting to the observed points.

Any line that passes through the line $y = 0$, indicates a phase change from $^1$H$_2$O to $^2$H$_2$O. The slope of the straight line should be negative for the odd diffraction orders and positive for the even orders if the inter-lamellar water distribution can be described as a single Gaussian. The gradient technique can break down for the higher orders of diffraction and did so in this analysis. This indicates that the water profile cannot be described accurately by a single Gaussian at higher resolution. Simultaneous least squares fitting to the
straight-line functions provide a set of scale factors. The scale factors were applied to the appropriate structure factors. Each structure factor was now consistently internally scaled with respect to their $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ ratios.

Figure 3.19. Plot (a) shows the fitted structure factors for DOPC:DOPG (50:50 mol) with 3-mol% $^2\text{H}_{10}$-leucine NKA added and plot (b) shows a similar graph with $^2\text{H}_8$-lysine NKA added to the lipid.

The samples that contain peptide were then scaled relatively to each other. Again, this can be achieved through simultaneous least squares fitting to straight-line functions. This is possible because the water distribution between each peptide-containing sample should be the same at any given $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ ratio, assuming isomorphous replacement. Therefore, the difference ($^2\text{H}_2\text{O}$ -
\(^{1}\text{H}_{2}\text{O}\) structure factor amplitudes of all peptide-containing samples must be the same. Again, the scale factors are applied to the appropriate data sets.

At this stage, all the structure factors were scaled internally and the peptide containing samples were also scaled relatively to each other. The structure factors and corresponding bilayer profiles were then normalised \textit{absolutely} using the technique mentioned in section 3.3.4. Again, the \(^{1}\text{H}_{2}\text{O}/^{2}\text{H}_{2}\text{O}\) exchange method was used to scale sets of the structure factors to each other.

This procedure assumes that the water distribution can be described by a Gaussian and that this function has a value of zero neutron scattering density at specific regions of the unit cell. In the case of the water distribution, the hydrocarbon core of the bilayer represents such a region. However, this hypothesis is not quite true on two levels concerning this data and an absolute normalisation is only approximated. Firstly, in any experiment of this type, \(^{1}\text{H}/^{2}\text{H}\) exchange occurs. It is also known that water can diffuse through the membrane at a rapid rate. Therefore, it is unlikely that the ‘tails’ of the Gaussian would be exactly zero. Secondly, Fourier termination error was evident at the extremes of each Gaussian distribution. The procedure used to normalise the data is described as follows.

A value was added to each water distributions so that the ‘tails’ of the Gaussian function were close to zero neutron scattering density. This added value gives the arbitrary mean scattering density of the water distribution across the unit cell and is used to calculate the instrument constant. In order to
scale the water distributions absolutely, the true neutron scattering density of the unit cell must be known. This requires the full atomic composition of the unit-cell to be known. Although this is readily available for the peptide to lipid ratio (3 peptides for every 100 phospholipids), the number of water molecules associated to each lipid can only be known from other techniques. It is likely that the number of waters associated with each lipid will change when peptide is incorporated into the sample.

It is possible to calculate the number of water molecules which hydrogen bond to each lipid "head-group" by using radioactive labels. $^{14}$C-labeled DOPC and tritiated water (both isotopes from Amersham International) were used so that scintillation counting could be employed to determine the water to lipid mole ratio at 92% rh. By preparing control samples containing each one of the labels and variable samples that contain both, the scintillation counts recorded can be correlated to a water:lipid ratio. This can be done because known quantities of radioactive lipid and water have been used in a manner that mimics the original experimental conditions.

Radioactive lipid films (with and without peptide) were deposited on quartz microscope slides and hydrated at 92% rh. The hydrating solvent was tritiated water of known concentration and was placed in a sealed glass hydration chamber. The hydration chamber was in turn placed in a sealed perspex isolator, also fixed at 92% rh. The isolator contained two openings that allowed manual sample transference. After 48 hours each slide was transferred
from the hydration chamber to scintillation tubes that contained the liquid scintillation cocktail. The isolator, therefore, was employed to prevent dehydration of the sample when removing them from the hydration chamber into the tubes. If this is not done the calculated number of waters per lipid will be a gross underestimate. This is due to dehydration effects caused by taking the coverslips from the hydrating atmosphere (92% rh) to the outside atmosphere (~30% rh). Using the isolator prevents this. In this experiment, underestimates can only occur due to $^1\text{H}_2\text{O}/^3\text{H}_2\text{O}$ exchange with the atmosphere over a transference time period of ~3 seconds. This is probably minimal. Counting was done on a Beckman LS6500 liquid scintillation counter.

The experiment was duplicated giving very similar results each time. A total of $10 \pm 0.46$ (standard error) waters were found to bind to the pure DOPC:DOPG bilayers reducing to $7.8 \pm 0.16$ waters in the presence of 3-mol% NKA.

Now that the atomic composition of each unit cell was known, the total coherent scattering length densities were calculated. This value was simply divided by the d-repeat to give the mean scattering density of the unit cell. This value was then divided by the arbitrary mean to give the instrumental constant. This value was used to multiply all the structure factor amplitudes to put them on the finalised ‘relative-absolute’ scale.
Model fitting was also employed in real and reciprocal space in order to completely phase the data sets. In practice, model fitting and data scaling occurs concomitantly. Many different phase combinations were tried. For these data, only a unique set of structure factor phases could account for the distribution of water across each bilayer and the consistent distribution of each deuterium label at all $^{1}\text{H}_{2}0/\check{2}\text{H}_{2}O$ ratios. Fourier differences in real-space are very simple to undertake because, at high resolution, a pair of Gaussians can accurately describe the distribution of deuterium along the bilayer normal. However, the resultant profiles suffer from Fourier termination error. Therefore, a modelling procedure is used that can remove this problem. The Gaussian distributions only have a few parameters that require fitting (height, position and standard deviation). A range of these parameters can be estimated from the real-space difference profile. The estimated real-space parameters can then be Fourier transformed back into reciprocal space. A least-squares fitting procedure calculates the best diffractogram that fits the real-space Gaussian distribution. The resultant real-space Gaussian distribution represents the mean distribution of the deuterium label projected along a line parallel to the bilayer normal. Applying the Central Limit Theorem to this result means that this modelling technique permits the determination of label distribution to ~1Å resolution (Crawshaw and Chambers, 1994, Hristova and White, 1998). The Central Limit Theorem is useful because it can be applied to any statistical distribution, not just Gaussians.
3.5.4. Results and Discussion

The complete sets of scaled structure factors are shown in table 3.4. Figure 3.20(a) displays the neutron scattering density profile across a DOPC:DOPG bilayer. The inter-bilayer water compartment is at the outer region of the graph. The peaks represent the phosphate head group region. Notice, that the profile contains a ‘bump’ at the bilayer centre. This is Fourier termination error that arises because the bilayer profile is under-resolved. If the profile was fully resolved, this region of the profile should have a lower scattering density as it represents the negative scattering terminal methyl groups.

Figure 3.20. Neutron scattering density profiles of bilayers containing DOPC:DOPG 50:50 (mol) through 5 orders of diffraction (a) and with 3-mol% NKA added (b).
<table>
<thead>
<tr>
<th>Sample</th>
<th>F(1)</th>
<th>F(2)</th>
<th>F(3)</th>
<th>F(4)</th>
<th>F(5)</th>
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<td>DOPC + DOPG 50:50 0% 2H2O</td>
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<td>-5.6</td>
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<td>±0.10</td>
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<td>0</td>
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<td>±0.17</td>
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<td>2.16</td>
<td>5.14</td>
<td>1.63</td>
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<td>±1.98</td>
<td>±0.61</td>
<td>±0.07</td>
<td>±0.16</td>
<td>±0.05</td>
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<td>DOPC + DOPG 50:50 + NKA 0% 2H2O</td>
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<td>3.24</td>
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<td>DOPC + DOPG 50:50 + NKA 50% 2H2O</td>
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<td>±0.15</td>
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<td>DOPC + DOPG 50:50 + 2H10-Leu NKA 0% 2H2O</td>
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<td>-7.9</td>
<td>-8.32</td>
<td>-2.37</td>
<td>2.86</td>
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<td>±0.12</td>
<td>±0.03</td>
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</tr>
<tr>
<td>DOPC + DOPG 50:50 + 2H10-Leu NKA 50% 2H2O</td>
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<td>4.69</td>
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<td>-3.58</td>
<td>2.66</td>
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<td>±0.27</td>
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<td>±0.04</td>
<td>±0.05</td>
<td>±0.04</td>
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<tr>
<td>DOPC + DOPG 50:50 + 2H10-Leu NKA 100% 2H2O</td>
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<td>4.34</td>
<td>1.52</td>
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<td>DOPC + DOPG 50:50 + 2H8-Lys NKA 0% 2H2O</td>
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<td>-3.21</td>
<td>1.77</td>
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<td>±0.02</td>
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</tr>
<tr>
<td>DOPC + DOPG 50:50 + 2H8-Lys NKA 50% 2H2O</td>
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<td>±0.02</td>
<td>±0.02</td>
<td>±0.02</td>
<td>±0.01</td>
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Table 3.4. Fully corrected and scaled structure factor amplitudes for bilayers composed of DOPC:DOPG (50:50 mol) and with 3-mol% NKA, 3-mol% 2H10-Leu NKA or 3-mol% 2H8-Lys NKA added. The errors were determined by least squares fitting to straight-line functions using the 1H2O/2H2O exchange method.
Figure 3.20(b) portrays the bilayer profile when 3-mol% NKA is added and figure 3.21(a) and (b) represents the bilayer profiles when the deuterated NKA analogues are incorporated. Although both figures in 3.21 look similar, they are markedly different to figure 3.20(b). This is discussed below.

Figure 3.21. Neutron scattering density profiles of bilayers containing DOPC:DOPG 50:50 (mol) plus 3-mol% $^2$H$_{10}$-leucine NKA through 5 orders of diffraction (a) or with 3-mol% $^2$H$_{10}$-lysine NKA (b).

Figure 3.22(a and b) shows the distribution of deuterium, introduced in the form of $^2$H$_2$O, within and between all samples measured. This is an important result and has a fundamental bearing on the interpretation of the data. Firstly, the shape of the distribution should be exactly the same for the peptide-containing samples. This would infer that that isomorphous replacement had
taken place. This is difficult to ascertain for the presented data. It is assumed that the replacement of one amino acid for a deuterated analogue would not alter the peptide or lipid conformation. This is supported by figure 3.22(b), which shows that the deuterium distribution is the same for both deuterated peptide analogues. However, the deuterium distribution for the protonated peptide has a different shape. This may be explained by Fourier error. There is also the possibility that the deuterium distribution may not be described by a Gaussian at all. A similar shape of deuterium distribution was observed between bilayers composed of SP through higher diffraction orders.

Figure 3.22. (a) The distribution of deuterium, introduced in the form of $^2$H$_2$O, within and between bilayers of DOPC:DOPG 50:50 (mol) (solid line) and with 3mol% NKA added (broken line). The origin is located at the centre of the water layer between two adjacent bilayers. Plot (b) shows a similar profile for the two deuterated NKA analogues.
In bilayers composed of DOPC:DOPG, 10 waters bind to each phospholipid molecule but this is reduced to 7.8 waters per phospholipid molecule in the presence of 3-mol% NKA. Therefore, it appears that NKA displaces water and may dehydrate the bilayer surface. This is in agreement with the SP-DOPC molecular dynamics simulations of Kothekar, 1996. Membrane dehydration suggests a surface orientation for this peptide. Also, NKA-induced water displacement at the membrane surface may explain the ‘red-shifted’ fluorescence studies by Woolley and Deber, 1987. If the membrane surface is dehydrated, then NKA would reside in a more hydrophobic environment.

| Population | Parameter (from bilayer centre) | Position (Å) | Width (FWHH) | Distribution (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Position</td>
<td>9.17 ± 0.0 Å</td>
<td>13.47 ± 0.11 Å</td>
<td>71.91 ± 0.84 %</td>
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<td>2</td>
<td>Position</td>
<td>21.07 ± 0.83 Å</td>
<td>8.92 ± 0.6 Å</td>
<td>28.09 ± 0.84 %</td>
</tr>
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</table>

**Table 3.5.** Location of deuterium label on $^2$H$_{10}$-leucine NKA. The centre, position and width of Gaussian distributions, fitted by least-squares refinement in reciprocal space, are given.

Table 3.5 summarises the difference profile representing the distribution of deuterium label in bilayers containing $^2$H$_{10}$-leucine NKA. The profile was calculated by the difference method. There are two distributions of label in
each sample, one located close to the membrane surface and another located approximately 9Å from the bilayer centre. The profile is shown in figure 3.23.

![Graph showing scattering density profiles](image)

**Figure 3.23.** Difference neutron scattering density profiles calculated using 5 orders of diffraction for NKA. The profiles show the distribution of $^2$H$_{10}$-leucine in 3-mol% NKAbilayers containing DOPC:DOPG 50:50. The Fourier subtractions (solid line) fit well with the Gaussians (broken line). Any deviations in the fit are probably due termination error.

Figure 3.24 illustrates a model for this interaction. It appears that one population of peptide inserts into the membrane and the other lies along the surface. Consistent with the SP monolayer area measurements of Seelig, these neutron data clearly demonstrate bilayer penetration of NKA into anionic lipid bilayers. The depth of penetration of the NKA is remarkably similar to that of SP (Bradshaw *et al.*, 1997, Darkes *et al.*, 1998). The existence of two populations of peptide can explain the difficulties found in phasing the data and the observation that the bilayer profiles containing the deuterated label was
so different to that containing the protonated peptide. Also, the fact that there are two populations of peptide is biologically appreciable. A number of peptides may be required for receptor activation. For example, it is known that two acetylcholine molecules are required to activate the nicotinic receptor.

![Figure 3.24](image.png)

**Figure 3.24.** The proposed locations of NKA in a membrane leaflet of the lipid bilayer. The deuterated leucine label is shown.

However, NMR and modelling studies suggest a surface location for the tachykinins (Kothekar, 1996, Fletcher and Keire, 1996). The exact location(s) of the tachykinins appears ambiguous. It is important to take into account two factors. Firstly, the SDS systems used in the NMR studies would facilitate greater NKA insertion into the hydrocarbon core than a fixed lipid bilayer. Electrostatic repulsion between the SDS headgroups and the short hydrocarbon
monolayers will result in a less well-defined interfacial region. This will increase the opportunity for tachykinin insertion. However, because this is not observed, it seems even more unlikely that the tachykinins will insert into bilayers. The results of Seelig were also based on monolayers at low lateral pressures. Secondly, the tachykinins dissolve well in aqueous systems but do not form clear solutions in only phospholipid/chloroform mixtures. This implies that the hydrophobicity of the tachkinins may not result in spontaneous insertion into the membrane hydrocarbon core.

The ambiguity of the above results are somewhat resolved by the location of the $^2$H$_8$-lysine label. From the model in figure 3.24, it was expected that the distribution of deuterons on the hydrophilic lysine residue would be located solely at the membrane surface. Figure 3.25 shows the actual distribution of deuterium label of $^2$H$_8$-Lysine NKA and table 3.6 describes the distribution numerically.

<table>
<thead>
<tr>
<th>Population</th>
<th>Parameter</th>
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</thead>
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<tr>
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<td>Position (from bilayer centre)</td>
<td>8.43 ± 0.22 Å</td>
<td>65.6 ± 0.6 %</td>
</tr>
<tr>
<td></td>
<td>Width (FWHH)</td>
<td>6.99 ± 0.33 Å</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Position (from bilayer centre)</td>
<td>21.07 ± 0.83 Å</td>
<td>34.3 ± 0.6 %</td>
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<tr>
<td></td>
<td>Width (FWHH)</td>
<td>7.54 ± 0.66 Å</td>
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</table>

Table 3.6. Location of deuterium label on $^2$H$_8$-lysine NKA. The centre, position and width of Gaussian distributions, fitted by least-squares refinement in reciprocal space, are given.
Figure 3.25. Difference neutron scattering density profiles calculated using 5 orders of diffraction. The profiles show the distribution of $^2$H$_{10}$lysine in 3% (mol) NKA in bilayers containing DOPC:DOPG 50:50 (mol). The Gaussians were fitted as mentioned in the text.

Surprisingly, the distribution is very similar to that of the hydrophobic leucine label. At first it was thought that the same labelled peptide had been added to the lipid! When the whole experiment and data analysis was repeated the same results were achieved! Initially, these results imply that both the N-terminal and C-terminal can insert into the membrane figure 3.26.

This hypothesis does not appear to be logical. The mechanism of action of SP and NKA seems to be very similar but it is highly unlikely that the N-terminal of SP could insert into the membrane at any great depth due to the presence of arginine's ionic guanidino group. It is also unlikely that the N-terminal of NKA could insert. The CD measurements on this short peptide in organic
solvent suggest a predominantly unfolded structure. In fact this model can be discarded by examining the percentage distribution of label. If both terminals of the NKA were able to insert in an anti-parallel manner, the percentage distribution of the lysine label would be different. Approximately 70% of the label distribution would be expected at the surface location leaving 30% distributed in the hydrocarbon core. In fact, the reverse is true.

Figure 3.26. The discarded model of the NKA-bilayer interaction where both terminals of the peptide were thought to insert in an anti-parallel manner. Full details can be found in the text.

The neutron results may be explained by the following hypothesis. The model discards the possibility that both terminals of the peptide insert simultaneously into the hydrocarbon core of the membrane. All the multi-bilayer peptide-containing samples were reconstituted from organic solvent. At no time is the peptide introduced into aqueous phase and allowed to ‘approach’ a membrane as occurs in-vivo. Therefore, the use of the word ‘insertion’ is misleading. It
is proposed that there exist two populations of label that both lie parallel to the membrane surface. This is illustrated in figure 3.27. The peptide that is submerged in the hydrocarbon core is kinetically trapped over the time course of the experiment. This population of peptide represents an experimental artefact of the sample preparation. The membrane-embedded label may also be explained by the high mole fraction of peptide used in this study, which was necessary to improve the signal-to-noise ratio. The observation of such multiple orientations may be explained by peptide flexibility and/or aggregation. Performing the same experiment with the arginine group of SP deuterated could test this hypothesis. A membrane-embedded label position would only occur due to this method of sample preparation.

Figure 3.27. Proposed model of the NKA-model membrane interaction as determined by neutron diffraction. 30% of the peptide has a surface or interfacial location.
The population of peptide that lies on the membrane surface, however, is in agreement with the other biophysical studies mentioned. This population (30% of total peptide used) of peptide seems to have partitioned from the hydrocarbon core to the membrane surface. It is thought that this population represents NKA in a monomeric form as the lipid-to-peptide ratio approximates as 100-to-1.

It is not possible to ascertain the exact location of the phospholipid molecule to which NKA associates. The addition of peptide may change the initial lipid structure. This may be investigated further by performing a similar experiment with selectively deuterated lipids. However, the results may be difficult to interpret due to the number of different lipid conformations within the sample. However, these data confirm a bilayer surface or interfacial orientation for NKA. The evidence suggests that, in terms of membrane location, the membrane bound forms of these NKA and SP is rather similar, implying that receptor specificity is controlled by finer levels of structural detail.

3.6 Preliminary neutron diffraction results of the SIV fusion peptide-membrane interaction

3.6.1 Introduction

Valuable structural information can be used to gain insight into the molecular mode of action of viral fusion peptides. It is thought that the fusion peptides
perturb planar target membranes in order to generate inverted lipid structures. In this way, fusion peptides promote the fusion reaction.

One of the important properties of fusion peptides may be their structural flexibility. For example, the fusion peptide of HA at pH7 forms β-turn structures to stabilize the trimer (Wilson et al., 1981) but at pH5, the fusion peptide gains α-helix structure when associated to membranes (Lear and DeGrado, 1987). Another study has shown the fusion peptide of FeLV to change structure when placed in different lipid-based systems (Davies et al., 1998). This emphasizes the point that one fixed peptide structure cannot be used as a model to describe the complete fusion event.

The SIV fusion peptide is thought to assume an α-helical structure when associated to membranes and insert at an angle of 53° to the bilayer normal (Brasseur et al., 1990). This model has been supported by functional and structural analysis using SIV fusion peptide mutants (Martin et al., 1994, Horth et al., 1991). In this way, the SIV fusion peptide is thought to disrupt the parallelism between adjacent phospholipids. This will, in turn, induce (or increase) negative curvature strain within the outer leaflet of the bilayer, increasing the opportunity that the bilayer will form a stalk-like structure (Markin et al., 1984).

Neutron diffraction measurements were carried out in order to define the location of the SIV fusion peptide in bilayers composed of zwitterionic DOPC. This section describes ongoing work. The structure factors collected have
been corrected and normalised but the phases of some of the orders have yet to be determined. Two amino acids were deuterated in order to define the orientation of the peptide within the bilayer.

3.6.2 Experimental set up and data collection

Neutron diffraction measurements were carried out on the C5 membrane diffractometer at Chalk River National Laboratories, Ontario, Canada. The mean of the wavelength spread was 2.37Å. This experiment was very similar in setup to the NKA diffraction study.

DOPC and DOPG were purchased from Avanti Polar Lipids (Alabama, USA) and used without further purification.

Fusion assays have demonstrated that a truncated version of SIV peptide is the most active (Martin et al., 1991). This peptide is only 12 amino acids long and was synthesised by Albachem (Edinburgh, UK) according to the following sequence: GVFVLGFLGFLA. The fusion peptide was synthesised in an undeuterated form, and with the valine-2 and leucine-8 deuterated. Sample preparation was very similar to the method described in section 3.4.2.

The Bragg reflections were recorded by carrying out a series of rocking curves as described in section 3.5.2. Collection of the first seven orders of diffraction was attempted. After the seventh order scan was completed, the first order was re-scanned to check that the membranes were in equilibrium. This was the case
for every sample. The experimental temperature was 25°C and the rh controlled to 92%.

3.6.3 Data analysis

The sample can at C5 has an extremely small volume. Therefore, equilibration times were quick and no movement of the Bragg reflections was observed. The stable relative humidity environment controlled the d-repeat of each sample. The unit cell size was 51.20Å, 50.83Å, 50.83Å and 50.97Å for DOPC, DOPC + SIV, DOPC + $^2$H$_8$-valine-2 SIV and DOPC + $^2$H$_{10}$-leucine-8 SIV respectively. This was calculated from calculating the gradient of a plot of Q against order number (figure 3.28).

From this plot, the detector misalignment could also be calculated. The structure factor sets were corrected using the methods described in 3.5.3.

*Figure 3.28. Plot of the scattering vector, Q, against diffraction order for a typical sample scan at C5. Here Q is equal to $(2\pi \sin \theta)/\lambda$ and has units of inverse Ångstroms.*
3.6.4. Results and discussion

The structure factor values are illustrated in table 3.7. Figure 3.29 and figure 3.30 show the straight-line fits that were used to phase the low orders of diffraction. From these, a bilayer profile of DOPC was constructed (figure 3.31) and a tentative description of the deuterium distribution within and between each bilayer is given in figure 3.32. Each distribution is well represented by a Gaussian function. This shows that isomorphous replacement has been achieved.

![Figure 3.29](image)

**Figure 3.29.** The $^1$H$_2$O/$^2$H$_2$O exchange method was used to phase the low orders of diffraction. Plot (a) shows the structure factors of DOPC and plot (b) with 1-mol% SIV added. Each straight-line function has been obtained by least squares fitting to the observed points.
Figure 3.30. Plot (a) shows the fitted structure factors for DOPC with 1-mol% $^2$H$_8$-val SIV added and plot (b) shows a similar graph with $^2$H$_8$-leu SIV added to the lipid.

Figure 3.31. Neutron scattering density profile of DOPC through five orders of diffraction.
Figure 3.32. (a) The distribution of deuterium, introduced in the form of $^2\text{H}_2\text{O}$, within and between bilayers of DOPC (broken line) and with 1-mol\% SIV added (solid line). The origin is located at the centre of the water layer between two adjacent bilayers. Plot (b) shows a similar profile for the two deuterated SIV analogues.

The radiotracer technique described in section 3.5.3 was used to determine the number of water molecules that bind to each lipid head group at 92\% rh. For pure lipid bilayers composed of DOPC, this value was 11.54 waters ($\pm 0.15$ standard error). The SIV fusion peptide caused this value to decrease to 10.1 waters ($\pm 0.1$). Fusion peptides may have the ability to dehydrate the membrane surface to permit close membrane approach. However, it is difficult to judge if this small difference is relevant. Repeating the experiment using a range of peptide concentrations would ascertain if dehydration was an actual effect. Therefore, at this stage it appears that the fusion peptide does not radically change the distribution of water between bilayers.
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<th>F(3)</th>
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Table 3.7. Experimentally determined, corrected and normalised structure factor amplitudes for each data set. The phases of some of the diffraction orders has yet to be determined.
Chapter 4. X-ray powder diffraction (XRD)
4.1. X-ray diffraction study on feline leukaemia virus fusion peptide and lipid polymorphism

The structural effects of the fusion peptide of FeLV on lipid polymorphism of MeDOPE were studied using X-ray powder diffraction. This peptide, the hydrophobic amino terminus of p15E, has been proven to be fusogenic and to promote the formation of highly curved, intermediate structures on the lamellar liquid-crystal to inverse hexagonal phase transition pathway. The peptide produces marked effects on the thermotropic mesomorphic behaviour of MeDOPE, a phospholipid with an intermediate spontaneous radius of curvature. Measurements show that the peptide reduces the d-repeat of the membrane prior to the onset of an inverted cubic phase. This suggests that membrane thinning may play a role in peptide-induced model membrane fusion and strengthens the link between the fusion pathway and inverted cubic phase formation. The results of this chapter are interpreted in relation to models of the membrane fusion mechanism.

4.2. Theoretical background to X-ray powder diffraction

The theory behind diffraction using X-rays is very similar to neutron diffraction mentioned in section 3.1. However, for X-rays the scattering centres are a particular atom’s circulating electrons, not atomic nuclei.
Therefore, there is a correlation between atomic coherent scattering amplitudes and the periodic table.

An orientated crystal will give rise to a 1st order diffraction spot a distance Q from the centre of the detector. If the crystal is ground into a powder, there will now exist a number of smaller crystals that take up random orientations with respect to the incoming X-ray beam. Instead of a 1st order diffraction spot being seen on the detector, a series of Debye-Scherrer rings are present where the intensity is smeared over 2π radians. The distance of each ring from the centre of the detector remains the distance, Q. This effect is analogous to recording diffraction whilst spinning the orientated crystal through 2π radians. For powder diffraction, all the observable Bragg reflections are seen simultaneously because “all Bragg angles are equally probable” (Franks and Leib, 1981).

As explained in section 3.3, diffraction can be used to obtain information about a structure’s dimensions and atomic architecture. The aim of this experiment is to record a series of Bragg reflections for each sample under study as a function of increasing temperature. The ratio of all the reciprocal space reflections relative to the origin gives information on the type of phospholipid phase present. The reciprocal space distance between the diffraction spots allows the determination of unit-cell size. This experiment is not concerned with the molecular substructure of the diffracting object. Indeed, this is extremely difficult with unorientated, dilute lipid dispersions. The aim of this
experiment is to attempt to observe any non-bilayer structures that may form during heating towards $T_H$. The FeLV fusion peptide may induce such structures as determined by $^{31}$P-NMR (Davies et al., 1998).

4.3. Materials and Methods

The amino acid sequence of the fusion peptide of FeLV, p15E, is EPISLTVALMLGGLTVGGIAAGVGTGT. p15E is sparingly soluble in most solvent systems suitable for phospholipid research a lysine residue was added onto the C-terminus to increase solubility in chloroform/phospholipid mixtures and phospholipid/aqueous-phase dispersions. The peptide is, therefore, named p15EK. Lysine is the next, naturally occurring, amino acid in the protein sequence (Swiss Protein Data Bank, 1996; release number 34). Albachem (26 Craigleith View, Edinburgh, EH4 3JZ, Scotland, UK) synthesised p15EK using solid-phase methods to greater than 95% purity.

Lipid films were made according to sections 2.3.2 and 4.3.

The X-ray diffraction experiments were performed at station 2.1 of the Synchrotron Radiation Source at Daresbury Laboratory, UK. The synchrotron-derived X-rays are produced from a three-stage machine that accelerates electrons close to the speed of light. Firstly, free electrons are injected into a linear accelerator (an electron gun) before being passed into a booster synchrotron. The electrons travel through a number of hairpin bends under the action of bending magnets that cause them to be greatly accelerated. The electrons are then passed into a larger storage synchrotron. The electrons
travel in a vacuum in a polygonal orbit that covers a distance of ~100m. The bending magnets cause the electrons to change path and, by definition, accelerate. As the electrons are traveling close to the speed of light, electromagnetic radiation called synchrotron radiation is emitted. The emitted radiation is comprised of photons that have a wavelength spectrum from the infrared range to gamma rays. The radiation is emitted at each bending magnet. Radiofrequency waves are fed into the storage ring to allow the same electron to emit radiation for 20 hours or so. By far the most intense radiation emitted is X-rays. For this experiment, X-rays were focused in both horizontal and vertical directions by means of a triangular Ge111 monochromator and an uncoated plane quartz mirror respectively.

The specimen-to-detector length was 1.5 meters. A quadrant detector was used, which uses ‘delay-line’ technology. The diffracted photons collide with gas within the counter tube and the resultant ionisation collides with evenly spaced anode wires that send a pulse to the cathode delay line. The pulse from the cathode delay line takes a finite time to reach the histogramming memory. This time corresponds to the position the photon collided with the detector.

A teflon-lined brass chamber with mica windows was used as a sample holder and an external circulating water bath was used to control the sample temperature. A thermocouple fixed to the sample chamber monitored the temperature continuously. The temperature increased in a linear fashion at a rate of 30 K/hr. Each frame of data collection lasted for 30 seconds. The
effect of thermal radiation from X-ray beams of this kind was minimal. The
XOTOKO program was used to correct the raw data. Corrections for sample
thickness and variations in detector response were applied and background
counts were subtracted. Detector response was determined by measuring a
fixed source, $^{59}$Fe, overnight both before and after data collection. Calibration
of the x-axis for small angle scattering was achieved by using rat-tail collagen
as a standard (Fraser and MacRae, 1981). This calibration was repeated prior
to the exposure of each new liposomal sample. The corrected data were then
plotted using PeakFit™ v4 software (Jandel Scientific, Chicago, IL), a
commercial graphics package. The area under a fitted Gaussian distribution
gave the intensity of each order of diffraction.

4.4. Results

4.4.1. MeDOPE control

This type of experiment may be run as a series of temperature jumps, each one
followed by a pause whilst the sample equilibrates to the new temperature
before a single frame of data is recorded. Whilst this approach ensures
equilibrium across the sample, information is lost during heating. Therefore,
the approach chosen for these measurements was a continuous temperature
scan, with consecutive frames of X-ray data recording the whole transition
process. In this way, it is unlikely that the sample ever reaches equilibrium,
but the chance of observing intermediate structures is maximized. This is only possible with bright synchrotron X-ray sources.

![Graph showing change in repeat spacings (Å) for the Lα and H|| phase of MeDOPE with respect to temperature (°C) as determined by XRD performed at station 2.1 of the Synchrotron Radiation Source at Daresbury Laboratory, UK. Lα phase: (□) 1st order, (◇) 2nd order. H|| phase: (■) 1st order, (▲) 2nd order, (●) 3rd order, (▼) 4th order, (♦) 5th order. The shaded area represents possible phase coexistence.](image)

**Figure 4.1.** Change in repeat spacings (Å) for the Lα and H|| phase of MeDOPE with respect to temperature (°C) as determined by XRD performed at station 2.1 of the Synchrotron Radiation Source at Daresbury Laboratory, UK. Lα phase; (□) 1st order, (◇) 2nd order. H|| phase; (■) 1st order, (▲) 2nd order, (●) 3rd order, (▼) 4th order, (♦) 5th order. The shaded area represents possible phase coexistence.

Two sharp orders of diffraction were observed at 31°C that indexed onto a lamellar lattice when measured from the position of the zeroth order. The narrow peak widths and low incoherent scattering infers that most of the sample mass was in a well-ordered lattice. The reciprocal space co-ordinates of the lamellar phase had the ratio: 1:2. The d-repeat (d_L) of MeDOPE at 31°C was 62Å that is in agreement with Gruner and coworkers. Figure 4.1 shows the change in repeat spacing of the Lα and H|| phase as a function of

179
temperature. \( d_L \) increases steadily with temperature up to 54°C. The onset of the \( H_{II} \) phase is observed at approximately 60°C in agreement with the literature.

Although both \( Q_{II} \) and \( H_{II} \) phases of MeDOPE can coexist near \( T_{II} \) (Siegel and Banscbach, 1990), no \( Q_{II} \) phase reflections were observed at our rapid experimental temperature scan rate, as predicted. The reciprocal space reflections had the ratio 1:√3:2: √7:3. No other reflections were present. At this temperature the first reflection unique to the \( H_{II} \) phase (√3) was observed. However, the exact \( T_{II} \) of MeDOPE is debatable because the 1\(^{st}\) and 3\(^{rd}\) order \( H_{II} \) phase lattice points index onto the 1\(^{st}\) and 2\(^{nd}\) order lamellar phase lattice points making distinction between the two phases difficult. \( L_\alpha \) and \( H_{II} \) phase coexistence near the \( T_{II} \) has been reported previously (Colotto et al., 1996).

The \( H_{II} \) repeat spacing of MeDOPE at 60°C was 65Å. Multiplying this value by the factor \( 2/√3 \) yields the lattice basis vector length of the \( H_{II} \) phase (\( d_H \)), the distance from the center of one \( H_{II} \) cylinder to the next. The calculated \( d_H \) was 75Å which is comparable with Gruner's value. This small difference in values probably arose because of different heating protocols and batch-to-batch variations in lipids. \( d_{II} \) steadily decreased to 73Å at 71°C. This occurs because the hydrocarbon chain splay is wider at higher temperatures.

Analysis of the \( L_\alpha \) peak areas of MeDOPE as a function of temperature revealed that the 2\(^{nd}\) order of diffraction dropped in amplitude at 60°C. However, during the phase transition there was no significant increase in
incoherent scattering, indicating that an ordered transition was occurring. Figure 4.2 shows the full-width at $1/e$ ($e=2.718$) height (FW$1/e$ H) values for the $1^{st}$ and $2^{nd}$ order reflections. This parameter is related to sample order. The plot shows that the FW$1/e$ H of the $1^{st}$ and $2^{nd}$ orders of diffraction significantly decreased from approximately 50°C indicating that the lipid system becomes progressively more ordered as the phase boundary approaches. This is to be expected as, close to $T_H$, the recorded reflection is the superposition of a wide $L_\alpha$ Gaussian and a narrow $H_{II}$ Gaussian. As the contribution of the wide $L_\alpha$ Gaussian decreases and the contribution of the narrow $H_{II}$ Gaussian increases, the observed peak appears narrow. Lipid phases may coexist even without thermal gradients across the sample. The observation that the pure lipid specimen can undergo an $L_\alpha\rightarrow H_{II}$ phase transition at fast scan rates without entering the $Q_{II}$ phase is in agreement with Hui et al (1983).
Figure 4.2. Variation in sample order as a function of temperature from XRD results. Sample order is expressed as the full width at 1/e height ($e = 2.73$) of a Gaussian distribution that represents the Bragg intensity peak. L$_{\alpha}$ phase; (◇) 1$^{\text{st}}$ order, (□) 2$^{\text{nd}}$ order. H$_{\parallel}$ phase; (♦) 1$^{\text{st}}$ order, (■) 3$^{\text{rd}}$ order. The shaded area represents possible phase coexistence.

4.4.2. MeDOPE and p15EK (1-mol% and 0.5-mol%)

Figure 4.3 shows a 3-dimensional plot of a series of diffraction images obtained for MLVs containing MeDOPE with p15EK (1-mol%) over the entire temperature range. As MeDOPE has an intermediate $R_o$ value, this lipid has a tendency to form structures of intermediate curvature (Gruner, 1985). $d_o$ for both peptide containing samples was similar to that of the pure lipid. The FW$^{1/e}$ H values were slightly less than those of the lipid control indicating more order in the L$_{\alpha}$ phase lattice at lower temperatures.
Figure 4.3. Typical three-dimensional plot of a real-time series of consecutive XRD patterns obtained on heating from 30°C to 70°C for MeDOPE plus p15EK (1-mol%). The heating scan rate was approximately 30K/hr. The reduction in intensity of the 1st order $L_\alpha$ phase is clearly visible as the $Q_{III}$ phase grows. On the left-hand side of the lamellar peaks it is possible to see the shoulder representing the novel lamellar phase.

In the sample that contained 1-mol% p15EK, a shoulder was evident on both the diffraction orders of the $L_\alpha$ phase at 35°C (figure 4.4.). As the temperature steadily increased, the shoulder of both orders of diffraction grew as the original peak area diminished. The 1st order shoulder outlasted the main lamellar diffraction peak. The shoulder peaks had a lamellar phase signature and a d-repeat of 58Å. This was concomitant with the appearance of very low angle Bragg reflections whose reciprocal space coordinates indexed onto a ratio of $1: \sqrt{2}: \sqrt{3}$. Although the coordinates of only four orders of diffraction are known (this includes the unmeasured zeroth order which is superimposed onto the primary beam) the $L_\alpha$ and $H_{III}$ phases can be excluded. The spacing
ratios of the reciprocal space co-ordinates indicate the emergence of a Q_II phase. The areas under the Q_II peaks increased with temperature. The Q_II lattice had a large repeat spacing of 254Å at 44°C and this decreased reciprocally with temperature to 129Å at 72°C, illustrated in figure 4.5.

Figure 4.4. XRD profiles of multilamellar vesicles composed of MeDOPE plus p15EK (1mol%) at specific temperatures. Diagram clearly shows the emergence of a shoulder on the L_α Bragg reflections and the onset of a Q_II phase.
The initial dimensions were approximately 55% greater than that of previously recorded Q\textsubscript{II} phases (Gruner, 1985, Shyamsunder \textit{et al.}, 1988, Colotto \textit{et al.}, 1996, Colotto and Epand, 1997) although a definite lattice assignment on so few orders is not possible. However, the spacings rule out a lamellar or hexagonal lattice. The dimensions at 72°C closely resemble those in previous studies that classed the lipid as possessing Pn3m or Pn3 space group symmetry (unit cell parameter = 125-140Å) (Gruner, 1985). This unit cell parameter of this structure decreases reciprocally with temperature possibly because of tighter curvature at higher temperatures. The H\textsubscript{II} phase emerged at 71°C, at least some 10°C higher than was seen for either the pure lipid sample or the 2-mol% peptide sample (see below). This temperature is in close agreement with the Q\textsubscript{II}\textbackslash H\textsubscript{II} transition temperature, T\textsubscript{QH}, as measured by Siegel and Banschbach, 1990. As the novel peak disappeared at 55°C there was an increase in sample disorder. The areas under the Q\textsubscript{II} peaks were much less than the areas under either the L\textalpha phase or H\textsubscript{II} phase peaks. The Q\textsubscript{II} peaks were much closer to the incoherent background although this baseline did not itself increase.
Figure 4.5. Change in repeat spacing (Å) for the \( L_\alpha \) and \( Q_{II} \) phases of MeDOPE plus 1-mol% p15EK with respect to temperature (°C) as determined by real-time small angle XRD. The reduced \( d_L \) phase can be readily observed. Onset of the \( H_{II} \) phase occurs at the end of the temperature scan. \( L_\alpha \) phase; ( ) 1st order, ( ) 2nd order. \( d_L \) thin phase; (□) 1st order, ( ) 2nd order. \( Q_{II} \) phase; (grey squares) 1st order, (grey circles) 2nd order, (grey triangles) 3rd order. \( H_{II} \) phase; (grey squares) 1st order, (grey circles) 2nd order, (grey triangles) 3rd order, (■) 4th order, (▲) 5th order. The shaded area represents possible phase coexistence.

The 0.5-mol% p15EK sample behaved similarly to the 1-mol% sample. The \( Q_{II} \) phase emerged at 53°C for the 0.5-mol% FeLV sample, nearly 10°C higher than the 1-mol% FeLV sample. The repeat spacing of this cubic phase was 284Å at 53°C, which decreased to 125Å at 72°C. Again, this phase exhibited a shoulder on the 1st and 2nd diffraction orders of the \( L_\alpha \) phase but this was less pronounced than that seen for the 1-mol% FeLV sample. As before, the shoulder appeared prior to the appearance of the cubic phase and had the same
d-repeat of 58Å. Therefore, addition of these concentrations of p15EK to MeDOPE-containing MLVs produces similar intrinsic and extensive changes in its thermotropic mesomorphic behaviour.

4.4.3. MeDOPE and p15EK (2-mol%)

Lipid dispersions that contained 2-mol% peptide gave diffraction patterns that were the most similar to MLVs composed of pure MeDOPE. Well-ordered diffraction was observed throughout the temperature scan. The $d_L$ peak areas (including the reduction in amplitude at 60°C), $T_H$ and the FW$^{1/2}$ $H$ were all very similar to the lipid control.

Strikingly, a small amount of QII phase forms in this sample after the HI phase has formed. This may be accounted for by the existence of discrete membrane patches containing low concentrations of p15EK. The peptide in these domains may be able to interact more intimately with the phospholipid in a similar manner to that seen for samples containing the lower peptide concentrations. The QII phase was observed from 66°C to 70°C and had a repeat spacing of 148Å that did not change significantly during the small temperature range at which it existed. The QII repeat spacing of the 1-mol% sample was 159Å at this temperature and 165Å for the 0.5-mol% sample.
4.5. Discussion

Two important observations are discussed. The p15EK induced both a Q_h structure and a novel lamellar structure. The results clearly show that this peptide had a marked effect on the thermotropic behaviour of MLVs comprised of MeDOPE. This effect was most evident at the two lower peptide concentrations. A previous DSC study has shown that, at high concentrations the P15EK had less effect upon the T_h of DiPoPE than was seen at low peptide concentrations (Davies et al., 1998a). In the same paper, using SAXS, the presence of peptide-induced novel peaks in the H_h phase of DiPoPE was also less pronounced at high peptide concentrations. Similarly, our X-ray data have shown that the sample containing 2-mol% p15EK behaved like the pure lipid control. It is possible that the peptide, at this high concentration, simply lies along the membrane surface in sufficiently large numbers to prevent the bilayer from curling into an inverse geometry.

At concentrations of 0.5-mol% and 1-mol%, the p15EK has the ability to reduce the T_h of both DiPoPE and MeDOPE and to increase the amount of isotropic $^{31}$P-NMR of both MeDOPE and DiPoPE (Davies et al., 1998a). The idea that fusion peptides can induce this isotropic resonance is not new (Epand et al., 1994, Epand and Epand, 1994, Colotto and Epand, 1997). However, our data show that the previously reported isotropic resonance is directly related to Q_h structure formation (Davies et al., 1998a). The connection between fusion and the formation of non-bilayer phases is only valid for systems that show
these structures during the first heating scan, as was observed here. This is the first observation, to our knowledge, of the induction of Q\textsubscript{II} phase formation by a viral fusion peptide. Previously, this peptide-induced phase has only ever been seen with MeDOPE multilamellar systems containing influenza A fusion peptide at temperatures above T\textsubscript{H} or when samples were subjected to cooling from T\textsubscript{H} (Colotto and Epand, 1997). Observation of the L\textsubscript{a}\text{"}Q\textsubscript{II} phase transition normally takes hours, and thus cannot be correlated to the rapid fusion process, but we have shown that p15EK can reduce the time of this transition to minutes. This suggests that the p15EK is an extremely potent tool for the production of highly curved intermediates.

Previous differential scanning calorimetry studies (DSC) of MeDOPE-containing MLVs have revealed small shoulders on the low temperature side of the T\textsubscript{H} enthalpic peak presumably caused by intermediates in the L\textsubscript{a}\text{"}H\textsubscript{II} pathway (Siegel \textit{et al.}, 1989, Ellens \textit{et al.}, 1989). Later studies using DSC and X-ray diffraction revealed that the L\textsubscript{a}\text{"}Q\textsubscript{II} phase transition temperature (T\textsubscript{Q}) was approximately 62°C and that the Q\textsubscript{II} structure undergoes a phase transition to the H\textsubscript{II} phase at higher temperatures (72-77°C) (Siegel and Banschbach, 1990). Thus, from figure 4.5, it can be readily observed that p15EK dramatically reduces T\textsubscript{Q} by 18°C, when compared to these values. The H\textsubscript{II} phase that eventually formed had the same lattice parameters as the pure lipid. Importantly, this indicates that the p15EK does not affect R\textsubscript{o} of this lipid species although it does have a destabilising effect upon the L\textsubscript{a} phase.
previous X-ray diffraction study on MeDOPE with the fusion peptide of SIV (SIVwt) revealed that the H$_{II}$ lattice parameters were slightly less than that of the pure lipid control (Colotto et al., 1996). It was reasoned that this fusion peptide destabilises bilayers by increasing the strength of $R_\alpha$. Importantly, the p15EK promotes the facile development of Q$_{II}$ structures rather than H$_{II}$ structures. Precursors involved in the L$_{\alpha}$Q$_{II}$ transition pathway are also thought to be involved in fusion pore formation (Davies et al., 1998a).

The membrane-interactive properties of the p15EK also differ from that of dodecane (C$_{12}$H$_{26}$), which dramatically reduces T$_H$ but still promotes H$_{II}$ formation. Unlike dodecane, most fusion peptides only subtly lower T$_H$ and are not regarded as being of sufficient hydrophobicity to fill hydrocarbon packing interstices in the H$_{II}$ phase. However, p15EK does share one property with dodecane: both massively drop a bilayer\nonbilayer phase transition temperature.

The second important feature of the SAXS images is the appearance of a lamellar structure that had a reduced d-repeat some 25°C below the T$_H$. At first, this structure coexisted with the L$_{\alpha}$ phase. This novel lamellar structure occurred prior to Q$_{II}$ phase formation and throughout its early growth. As the Q$_{II}$ phase peaks grew, the novel peaks diminished. Therefore, the presence of the novel structure overlapped L$_{\alpha}$Q$_{II}$ phase coexistence. This peptide-induced lipid structure seems to represent an intermediary stage in the L$_{\alpha}$Q$_{II}$ phase transition mechanism and possibly in the multi-step fusion event itself. The
available data do not determine whether the novel structure is thermodynamically metastable. Importantly, it is proposed that the ability of the FeLV fusion peptide to produce this transitory structure is crucial to the facile production of the Q II phase observed at greatly reduced temperatures. Obviously, the peptide has caused the coexistence of two lamellar structures. It is believed that this effect is a result of the peptide's local action on the membrane rather than due to bulk phase separation into areas of peptide-rich and peptide-poor concentrations. Such an argument is supported by the fact that no novel peaks were evident at the 2-mol% peptide concentration, the concentration where phase separation would be most obvious.

There are two possible explanations for the observed lamellar structures of reduced d-repeat. The thickness of the water layer between adjacent bilayers may decrease, or the lipid bilayer may thin. Interfacial dehydration of the lipid bilayer will decrease the d-repeat of a membrane (Hui et al., 1983). For fusion to occur, their close approach, from approximately 20Å inwards, is hindered by an exponentially increasing repulsive force (Israelachvili and McGuiggen, 1988, Leiken et al., 1993). Close-approaching membranes require the expulsion of tightly bound water molecules from each membrane interface (Hughson, 1995). Surface dehydration could circumvent repulsive forces and permit close membrane approach. Our SAXS images reveal that the d-repeat of the bilayer decreased by some 8Å in the presence of peptide. This is similar to a previously reported
value where membrane fusion induced by freezing and thawing (Hui et al., 1983). Hui also point out that dehydration does not cause membrane fusion alone but that it increases the opportunity for the event to proceed. The ability of fusion peptides to dehydrate membrane surfaces has already been implicated in modelling studies of a lipid-lined fusion pore (Bentz et al., 1990).

An alternative, or complementary, explanation for the reduced d-repeat induced by p15EK is bilayer compression. This mechanism would also result in a reduced d-repeat. X-ray diffraction studies have shown that low concentrations of alamethicin can adsorb onto bilayer surfaces composed of diphytanoylphosphatidylcholine and cause bilayer thinning (Wu et al., 1995). Wu and co-workers proposed that alamethicin increases the cross-sectional area of the membrane surface by causing lateral expansion of the lipid headgroups. In planar bilayers, the cross-sectional area of the lipid headgroups and hydrocarbon chains must be matched. Therefore, the hydrocarbon chains must increase their cross-sectional area. As the volume occupied by the chains is constant, there is a resultant decrease in chain length. Unlike monolayers solely composed of lipids that have phosphatidylcholine headgroups, monolayers comprised of lipids that have phosphatidylethanolamine headgroups have a natural tendency to curl (Rc). At slightly increased temperatures, the lipid molecules cannot maintain cross-sectional matching and undergo an inverted phase transition. Similar to the HII phase, the QII lattice parameters decrease reciprocally with temperature due to hydrocarbon
chain splay. At $T_{QH}$, the frustration between the free energy of monolayer curvature strain and hydrocarbon chain stretching is so great that the $H_{II}$ phase forms.

The most difficult problem to answer is why does the membrane-thin structure metamorphose to a $Q_{II}$ phase and not $H_{II}$? At a given temperature, the monolayer curvature free energy and the free energy involved in hydrocarbon chain stretching governs the type of lipid phase present (Gruner, 1985). No lipid assembly can fully satisfy the conflicting demands of a simultaneous low curvature free energy and chain packing free energy. This gives rise to frustration within any lipid phase. The amount of frustration in the cubic phase is smaller than in either the $L_{\alpha}$ or the $H_{II}$ phases (Anderson et al., 1988) so that the cubic phase represents a compromise between the $L_{\alpha}$ and $H_{II}$ phase. The free energy of curvature is lower than in the $L_{\alpha}$ phase but higher than the $H_{II}$ phase. Likewise, the free energy of the chains is higher than in the $L_{\alpha}$ phase but lower than the $H_{II}$ phase. Our data show that the p15EK destabilises the $L_{\alpha}$ phase but stabilises lipid transition intermediates. Compared with the $L_{\alpha}$ phase, the lipid intermediates have a reduced amount of frustration. The data show that, in the presence of p15EK, the inverted structure formed is a $Q_{II}$ structure that initially has a large lattice parameter. This indicates that the free energy of curvature of the headgroup region and the hydrocarbon packing free energy are of an intermediary value.
Since fusion peptides affect the exact transition pathway followed, it can be assumed that they must alter the relationship between temperature and the individual shape-dependent free energy of the lipid molecules and/or collective lattice parameters. The p15EK is either providing an alternative route to the $H_{II}$ phase (compared to pure lipid), or it is visualising the same route, since the pathway to the $H_{II}$ phase consists of a number of low energy steps rather than one single transformation.

Conformational studies of various fusion peptides (Martin et al., 1993, 1994) including the FeLV (Davies et al., 1998b), and molecular modelling studies (Callebaut et al., 1994) have shown that these peptides exhibit structural flexibility when exposed to changing physico-chemical conditions. The secondary structure of peptides depends not only on the primary sequence but also on its local and bulk environment (Waterhous and Johnson, 1994). Possession of this physical characteristic may be crucial to the function of fusion peptides. As the dynamic fusion reaction proceeds, the local chemical environment encountered by the fusion peptide will change. In this study, some evidence of this changing lipid environment is observed. Initially, the p15EK appears to causes a reduction in the $L_{a}$ lattice parameter but it may be employed to carry out other tasks until membrane fusion (lipid and aqueous contents mixing between apposed viral and target membranes) is complete. If indeed fusion peptides play not just one, but many roles in the fusion process
because of their structural flexibility they may be regarded as molecular acrobats.

This work with the p15EK supports the link between the mechanism of membrane fusion and inverse cubic phase formation. Furthermore, it also suggests that bilayer thinning may be important in the fusion pathway. This study only deals with simplified peptide lipid interactions. Biologically, the peptide is covalently bound to a larger fusion protein, which is embedded in the viral membrane. During biomembrane fusion the primed fusion peptide will interact, at least initially, with only the outer membrane leaflet in a coordinated fashion. In this experiment, the p15EK, which has the ability to self-aggregate, is free to interact with both sides of multi-lamellar bilayers. Nonetheless, these results may well represent many features of the fundamental peptide membrane interactions but without some of the biological control and specificity.
Chapter 5: Conclusions
5. Conclusions

5.1 Real-time swelling series

Section 3.3 has demonstrated that it can be advantageous to sample a range of points in the reciprocal lattice during neutron diffraction measurements of stacked phospholipid bilayers. This can be achieved in two ways. By recording several static measurements using a number of known relative humidities or, as we have demonstrated here, the sample hydration can be changed during the data collection. Whichever method is used, phase information is gained. If the hydration state is slowly changed without disturbing the sample, it is not necessary to scale the different data sets to each other. Interpolation between the observed points for any order can then be used to determine structure factor sets of any intermediate d-repeat, thereby reducing the errors in difference subtractions. We have demonstrated and quantified the error caused by neglecting to correct the d-repeat differences in subtractions, and also the error introduced by mistakenly using the continuous transform method to adjust the d-repeat of 0% $^2$H$_2$O data.

It is argued that it is valid to interpolate between observed points for any order at 0% $^2$H$_2$O, even though these points do not lie on the continuous transform of the neutron scattering profile (Bradshaw, Darkes & Davies, 1998). However, a more satisfactory approach is to collect the data at 8.06% $^2$H$_2$O. At this isotopic composition the water is effectively invisible to neutrons and neutron
scattering by the water is eliminated. The net result is similar to the so-called 'Minus Fluid Model' proposed by Worthington (Worthington, King & McIntosh, 1973) and developed by White (Hristova & White, 1998), except that in the neutron method, the removal of scattering from water is achieved without mathematical manipulation. Only under these conditions will all observed structure factors lie on the same continuous transform, thereby allowing the same function to be fitted simultaneously to all observed points. This approach is far more rigorous than the fitting of arbitrary functions to individual orders of data collected at any other $^2\text{H}_2\text{O}$ concentration. The accuracy is improved by fitting one curve to all observed points. It also gives phase information and allows the adjustment of d-repeats of data sets for use in difference subtractions.

The use of 8.06% $^2\text{H}_2\text{O}$ has other advantages. The introduction of substances such as peptides into bilayers may affect the distribution of water within and between the stacked bilayers. This will be reflected in difference subtractions. By making the water effectively invisible to neutrons, difference subtractions at 8.06% $^2\text{H}_2\text{O}$ removes this contribution from the difference profile, allowing less ambiguous interpretation of the results.

Taking all these advantages together, the improved accuracy, reduced equilibration time and the ability to calculate structure factor sets at any selected d-repeat (within a given range), the swelling series method has much to recommend its adoption for neutron diffraction studies. This adaptation of
the swelling method may be considered as, not a replacement for the more usual neutron approach of isotopic substitution, but rather as a complimentary technique that can help to improve the accuracy of structure factor determination, whilst also providing an independent ‘second opinion’ for phase determination. Its added advantage of reducing the requirement for sample equilibration time at the equilibration time at the start of an experiment will assist in the optimal utilisation of neutron beam time allocations.

5.2. NKA-phospholipid interaction

The principle biophysical method that has been used in this thesis has been neutron diffraction. For the NKA study in particular, many problems were encountered that led to a disproportionate length of time analysing the data. Although useful biological information has been gained from these experiments, the problems encountered have raised important issues regarding sample preparation. It is important to scrutinise the technique used in order to improve it. This will lead to physiologically more representative results. Section 3.5.4 mentioned the hazards of reconstituting the peptide-containing phospholipid multibilayers from organic solvent. This procedure has been the traditional method of sample preparation for orientated diffraction work because the early studies concentrated only on pure bilayers or the location of cholesterol within them. This method of sample preparation is useful when studying intrinsic membrane components or molecules that are known to
interact intimately with the lipid molecules. Such molecules could also include the hydrophobic anaesthetics and even channel forming peptides. However, modifications are required when investigating the membrane location of interfacial peptides such as NKA. This is because the membrane-embedded deuterium label distribution may not be believable. The label distribution may be kinetically trapped or represent the peptide in a membrane-embedded equilibrium with the interfacial population.

One way to improve on the existing results is to repeat the experiment at 100% rh. The present experiments were carried out at 92% rh. At this rh, there is no free inter-bilayer bulk water, as the phospholipid head group hydration shell is not filled. All the available water is bound to the phospholipid head group. As the rh approaches 100%, there is a non-linear increase in the amount of inter-bilayer water and the samples are more physiological. Presently, there is ongoing work to improve the sample cans at each of the respective neutron diffraction centres. This includes cans that have smaller internal volumes so equilibrium times are reduced.

A more physiologically representative method of preparing multibilayers that interact with interfacial or surface peptides is described as follows. The method is based on successful attempts to lower the pH of inter-bilayer water. How is it possible to change the pH of this water compartment if its hydration status relies on the environmental humidity? This is a problem because regardless of the pH of the hydrating solvent, the pH of the water in the
atmosphere is always neutral. However it can be achieved by placing prepared multibilayers that are on a slide into a hydration chamber at 100% rh. If this is left for 24 hours the sample will have equilibrated with the environment. It takes this long because the investigator reduces the rh of the hydration chamber when placing the sample in it! After 24 hours, a drop of unbuffered water at an appropriate pH is placed on the lipid at one end of the slide using a long Pasteur pipette. A long pipette is used so that the lid of the hydration chamber is moved only slightly ajar. This ensures that the rh remains very close to 100%. This is most important because if the rh is at 100%, the water droplet on the slide will not evaporate. The water molecules within the droplet and the inter-bilayer compartment will exchange over time. As the volume of water in the droplet is very much larger than the amount of inter-bilayer water, the pH of the inter-bilayer water will equal that of the added water droplet. This usually takes a further 24 hours to achieve. Afterwards, a pipette sucks up the added droplet and the sample is now ready for diffraction.

The same technique can be used to introduce peptides to the multi-bilayers. Instead of altering the pH, the added water contains peptide of a known concentration. With this method, the peptide approaches the membrane from aqueous phase. One problem with this technique could be the equilibrium times required. It may take too long for the peptide to diffuse throughout the sample (Thomas Hauss-personal communication). However, by decreasing the number of bilayer stacks, it may be fruitful to attempt this for NKA as the
results could be compared directly to the other approach. If the difference profiles of NKA gave a similar outcome, then the membrane-embedded label distribution would be readily believed. Conversely, any label distribution at the membrane surface may be qualified by the similar set of arguments that described the membrane-embedded label when using the traditional technique. Therefore, for peptides that have suitable solubility in water, two different approaches are required for a better-controlled experiment. In this way, actively looking for a result will be avoided as much as possible.

If the neutron data for NKA can be interpreted as describing a surface location for this peptide, there would be general agreement with previously observed CD and NMR spectroscopic results. NKA and SP may pack side-by-side when stored in secretory granules. The packing is caused by inter-molecular H-bonds rather than non-specific hydrophobic aggregation (Choo et al., 1994). On release into the synaptic cleft, the peptides dissociate into monomeric entities. This may be caused by H-bond competition for the peptide backbone by solvent water or ions. The structurally flexible tachykinins then associate with patches of anionic membrane surfaces close to the NK receptors. The longer binding times to anionic surfaces maximises the peptide’s two-dimensional diffusion and increases the likelihood of a receptor collision. A process of induced-fit with the receptor and possibly with lipid molecules themselves further alters the tertiary structure of the peptide. At present, neutron diffraction data and $^1$H-NMR studies suggest a similar structure and
location for both tachykinins. This is in agreement with the putative receptor binding binding site(s) being close to the membrane interface. Therefore, it is argued that the lipid-induced structure of NKA and SP is very similar, and receptor selectivity is controlled by non-covalent interactions with specific NK receptors.

5.3. FeLV fusion peptide-phospholipid interaction

A similar XRD experiment was carried out at the ELETTRA synchrotron light source in Trieste, Italy. The purpose of this preliminary experiment was to examine the effect of the fusion inhibitor, LPC, on peptide-induced MeDOPE mesomorphism. One of the controls samples contained MeDOPE with 1-mol% p15EK as described in the section 4.4 giving the same results but this time, X-ray images were recorded of the sample as it cooled. Images were recorded non-continuously due to the possibility of over-exposing the sample to ionising radiation. As the QII phase cooled, more and more orders of diffraction were observed. Strikingly, at 20°C, the cubic phase still existed. This finding fuels speculation that the cubic phase is the most thermodynamically stable phase for MeDOPE containing 1-mol% p15EK at room temperature rather than the Lα phase. The Lα phase may be metastable at ambient temperature. It was also interesting to note that LPC raised the onset of the QII phase by approximately 10°C. Presently, these results represent preliminary data and await further analysis.
The XRD work has shown that the isotropic resonances observed by Davies and co workers were in fact cubic in nature. This strengthens the link between the $L_o/Q_{II}$ pathway and membrane fusion.

The XRD experiments have shown the FeLV fusion peptide to be a potent inducer of non-bilayer structures. Its potency may be investigated in more detail by performing similar experiments on a range of lipid systems. For example, the non-bilayer lipid DiPoPE does not form detectable $Q_{II}$ structures whilst undergoing an $L_o/H_{II}$ phase transition but such structures may be detectable by XRD if the fusion peptide-containing MLVs experience very slow temperature scans. Also, the effect of fusion inhibitors and bilayer-stabilisers will be important in dissecting the transition mechanism further.

One of the problems with real-time XRD experiments is the primitive way that sample heating is achieved. The use of a computer-controlled calorimeter is most desirable for better and more meaningful results.

Knowledge of the location and orientation of the fusion peptide relative to the lipid phase present will complement the real-time X-ray images. The neutron diffraction work on the SIV fusion peptide will give information on its position in a bilayer. This may represent initial peptide-lipid contacts prior to fusion pore formation. However, it is a static picture that cannot be used to predict the future events. But if a series of static images could be collected at various stages of the fusion event then this could be used to test the existing theoretical models. For example, work is ongoing to prepare orientated $H_{II}$ phases using
non-bilayer lipids that would be suitable for neutron diffraction. If the location of the peptide could be deduced in this phase, then both the starting and finishing position would be known. A similar experiment may be performed if a Q_{II} phase could be isolated. Therefore, we are many experiments away from a detailed description of the molecular events of viral fusion peptide-induced biomembrane fusion.
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Appendix: Publications arising from research conducted during the course of this Ph.D.
Appendix

Publications


Communications

Oral presentation: “Biophysical studies on viral fusion peptide-model membrane interactions”.

Summer Seminar, Department of Preclinical Veterinary Sciences, R(D)SVS, University of Edinburgh, Edinburgh, June 1999.

Poster: “Interaction of tachykinins with phospholipid bilayers: a neutron diffraction study”

International Conference on Neutron Scattering, University of Toronto, Toronto, August 1997.

Poster: “Membrane-thinning by the feline leukaemia virus fusion peptide”

Biophysical Conference, Baltimore, February 1999,

Poster: “Revealing the membrane-bound structure of neurokinin A using neutron diffraction”

European Conference on Neutron Scattering, Budapest, August 1999.
Real-time swelling-series method improves the accuracy of lamellar neutron-diffraction data

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Neutron-diffraction data were collected from stacked bilayers of 1,2-dioleoyl-sn-glycero-phosphocholine under conditions of increasing relative humidity at both 0 and 8.06% 2H2O. Over the period of data collection, the d-repeat of both swelling-series samples increased. Each family of structure factors, representing each of the five orders of diffraction, are shown to lie on smooth curves, allowing structure factors of intermediate d-repeat to be determined. In the case of the 8.06% 2H2O data, but not the 0% 2H2O data, all observed structure factors lie on a single continuous transform. 8.06% 2H2O has a net neutron-scattering density of zero; its use in neutron-diffraction experiments presents a novel application of the so-called 'minus fluid' approach, without mathematical manipulation. The data are used to demonstrate the increased accuracy inherent in this real-time swelling-series approach. A quantitative analysis of errors caused by differences in d-repeat in difference subtractions is presented.

1. Introduction

The swelling-series method has been widely utilized in X-ray diffraction measurements of lamellar phospholipid preparations as a technique for the determination of structure-factor phases. The process consists of recording structure-factor amplitudes at a range of points in reciprocal space which, when scaled to each other and plotted, trace out the continuous transform of a single bilayer. In practice, this is achieved by the use of a number of samples, each prepared to a different lamellar spacing by controlling the humidity of the atmosphere or the osmotic pressure of the solution (King & Worthington, 1971).

In an earlier paper (Bradshaw et al., 1998), we described an adaptation of the swelling-series method in which oscillations of the observed diffracted intensity were caused by changing the relative humidity (rh) of a sample. This experiment was performed in situ by swelling dehydrated orientated stacks of phospholipid in the sample can of a neutron diffractometer whilst several consecutive θ–2θ scans were conducted. We demonstrated that this method, when applied to lamellar phospholipids, has the potential to improve both the accuracy of measurement of structure factors and their phase assignment. Such an approach is better suited to neutrons than X-rays because of the lower levels of radiation damage which allow a single sample to be scanned repeatedly. Each programmed scan gives one structure-factor value for each order of diffraction when the Bragg geometry for that order is satisfied. A number of scans are conducted as the bilayers swell by taking up water. In each scan, the position of any structure factor will have shifted slightly from the previous...
scan, thereby sampling a different region of reciprocal space and having a correspondingly different amplitude. The net result is that a family of structure-factor measurements is obtained for each order.

In the previous paper, we presented data from a sample of 1,2-dioleoyl-sn-glycero-phosphocholine (DOPC) collected under real-time conditions of increasing rh of pure water. The term pure water means that the water contained no salts to hold the rh of the sample environment below 100%. The isotopic composition of the pure water was 100% $^1$H$_2$O (i.e. no salts added, 0% $^2$H$_2$O). We demonstrated that at this isotopic composition of water the observed structure factors did not lie on a single continuous transform. This result is to be expected because the scattering density of the unit cell changes as more water is incorporated into the sample. Incorporation of this negatively scattering water affects the bilayer structure in two ways. The mean scattering density is reduced and the neutron-scattering contrast is altered. Therefore, the observed structure factors do not lie on the same continuous transform because the scattering structure is effectively different even if the physical structure of the bilayer remains unchanged. It is common practice, however, to take calculated structure-factor values from the continuous transform and use these values in Fourier subtractions if the d-repeat of the samples differ by approximately 1 Å (Büldt et al., 1979). Our previous paper revealed the potential errors in subtractions by doing this. In this paper, we present a swelling series of structure factors collected from DOPC at 8.06% $^2$H$_2$O and compare them with the earlier set collected at 0% $^2$H$_2$O.

2. Materials and methods

2.1. Sample preparation

DOPC was purchased from Avanti Polar Lipids and used without further purification. 20 mg samples were dissolved in chloroform. An artist's airbrush, using nitrogen as propellant, was used to deposit the lipid onto quartz microscope slides. The slides were placed in a vacuum over the desiccant phosphorous pentoxide (P$_2$O$_5$) for 12 h in order to remove all traces of the solvent. The slides were then hydrated before being placed back in the vacuum desiccator for a further 12 h in order to remove as much water as possible. Since some water molecules tightly bound to the phosphoryl head group may have remained, samples for measurement with 8.06% $^2$H$_2$O were hydrated in an humid atmosphere with water at this isotopic composition before being dried again under vacuum for the second time. The samples were protected from light whenever possible in order to reduce the chance of lipid peroxidation.

2.2. Neutron data collection

Neutron-diffraction measurements were carried out on the D16 membrane diffractometer at the Institut Laue et Langevin, Grenoble, France. The sample environment was a standard aluminium can, in which temperature control is achieved by circulating water through an integral water jacket and humidity control is achieved by changing the solution in a Teflon water bath at the base of the can. Each anhydrous DOPC sample on its quartz slide was quickly transferred straight from its vacuum desiccator into the D16 can together with a bath of pure water at either 0 or 8.06% $^2$H$_2$O. A series of continuous θ=2θ scans was immediately initiated. Each scan (from θ = 1.5 to 150°) took approximately 3 h to complete. The samples were run at 298 K. The mosaic spread of the second order of diffraction was determined for each sample using standard procedures.

2.3. Data analysis

The two-dimensional array of detector counts for each frame of data was corrected for variations in pixel response. The complete set of frames from each scan were then collapsed into a linear spectrum and combined to generate a pseudo θ-2θ scan. All of the analysis to this stage was carried out by the D16 instrument software. The background around each peak was fitted and subtracted using SigmaPlot. Gaussian distributions were then fitted to the Bragg reflections and the angular position, width and area of each peak recorded. Absorption and Lorentz corrections were applied and the square-root of the intensities taken to produce the structure-factor amplitudes.

3. Results and discussion

3.1. 0% $^2$H$_2$O data

Structure factors for the 0% $^2$H$_2$O samples were calculated from the observed intensities and plotted against their spatial frequencies, as shown in Fig. 1. Reference to this figure shows that the 0% $^2$H$_2$O neutron structure factors determined at a range of spatial frequencies do not lie on the same continuous transform. Each order traces out its own curve through the observed points. A quadratic expression was used to join the observed points of each order in turn.

The constantly changing relative humidity within the sample can was reflected in a constantly changing d-repeat throughout the data-collection period. This meant that no two of the observed structure factors, even those within the same scan, indexed onto the same reciprocal lattice. Interpolation between the observed points of each order produced sets of structure factors for a range of d-repeats. These structure-factor sets were used to calculate continuous transforms $C(x)$ using

$$C(x) = \sum_{h=0}^{h_{max}} F(h) \sin(\pi d x - \pi h) / (\pi d x - \pi h),$$

where $F(h)$ are the observed structure factors, $d$ is the Bragg spacing, $x$ is the distance along the bilayer normal to the plane and $h$ is the order number. The resulting continuous transforms show a smooth variation in amplitude, reinforcing faith in the phase assignments (Fig. 1).

The sets of structure factors were also used to calculate a family of trans-bilayer coherent neutron-scattering density profiles, as shown in Fig. 2(a). Within this family of profiles no
scaling was necessary, since each profile was produced by the same sample. However, each family of profiles was put on a per-lipid scale using the ‘relative–absolute’ method (Jacobs & White, 1989; Wiener & White, 1991; Wiener et al., 1991). This was achieved by scaling against previous data collected from the same lipid on the same diffractometer (Duff et al., 1994; Bradshaw et al., 1994; Bradshaw, 1997). This scaling process also yields the value of \( F(0) \), the zeroth order, for each structure factor set, as discussed below.

### 3.2. 8.06% \( \text{H}_2\text{O} \) data

In the case of the data collected at 8.06% \( \text{H}_2\text{O} \), all observed points were simultaneously fitted to a continuous transform using a least-squares minimization procedure. Sets of model structure factors \( F(H) \), each corresponding to a \( d \)-repeat of \( D \), were fitted against all observed data points, including the calculated \( F(0) \), to satisfy

\[
F(h) = \sum_{H=0}^{H_{max}} F(H) \sin(\pi Dh/d - \pi H) \quad (\pi Dh/d - \pi H).
\]

The process was repeated using a number of different values of \( D \), since analytical continuation theory (King & Worthington, 1971) predicts that all the model structure factors should lie on the same continuous transform. The results are shown in Fig. 1(b). Sets of model structure factors resulting from this least-squares fitting procedure are shown in Table 1 and scattering-density profiles calculated from them are shown in Fig. 2 (bottom).

One distinct advantage of the swelling-series method is the potential increase in the accuracy of intensity measurement. Any change in \( d \)-repeat caused by temperature or humidity fluctuations or incomplete equilibration can result in large differences in intensity of any single order. This effect is seen at its most extreme in the first order of the 0% \( \text{H}_2\text{O} \) series, where a change in \( d \)-repeat of 2.5 Å (from 50.8 to 53.2 Å) causes a 280% change in amplitude (which is equivalent to nearly an 800% change in intensity). The indexing of each structure factor to its own spatial frequency, rather than assuming that they all fit the same reciprocal lattice, removes this potential source of error. In the case of data collected at 8.06% \( \text{H}_2\text{O} \), there is the added advantage that all observed structure factors can be used to define the same continuous transform, even when they all index to different reciprocal lattices.

### 3.3. Zeroth order

The trans-bilayer distribution of coherent neutron-scattering density can be described as a one-dimensional bilayer profile which is constructed using Fourier summation. Each order of diffraction contributes a frequency component cosine function that gives a term in the summation. For centro-
symmetric phospholipid bilayer structures, the Fourier equation is

$$\rho(x) = \rho(0) + \sum_{h=1}^{h_{\text{max}}} F(h) \cos(2\pi x h / d),$$

where \(\rho(0)\) is the mean value of \(\rho(x)\). The value \(\rho(0)d\) is equal to the area under the bilayer profile and also equals the unobservable zeroth order, \(F(0)\). Its value can be determined by summing the total coherent scattering length of each atom in the unit cell. If the unit cell is considered to contain one phospholipid molecule, then \(\rho(0)d\) is equivalent to the total coherent scattering length of one phospholipid molecule plus its associated waters. The number of waters present per lipid must therefore be known from other methods.

The Fourier series of the bilayer now becomes

$$\rho(x) = \rho(0) + (2/d) \sum_{h=1}^{h_{\text{max}}} F(h) \cos(2\pi x h / d).$$

Since the mean scattering density of the unit cell is related to the amplitude of \(F(0)\), an alternative form of this equation, which removes the factor of two by including both sides of the diffraction pattern, is

$$\rho(x) = (1/d) \sum_{h_{\text{min}}}^{h_{\text{max}}} F(h) \cos(2\pi x h / d).$$

The mean scattering density of the unit cell can be estimated by summing the coherent scattering lengths of the atoms that comprise the unit cell. Therefore,

$$\rho(0) = (2/d)(n_w b_w + b_{lip}),$$

where \(n_w\) is the number of waters per lipid, \(b_w\) is the coherent scattering length of water and \(b_{lip}\) is the coherent scattering length from a single lipid molecule. The factor of two simply arises from the fact that the bilayer is composed of two monolayers.

\(F(0)\) cannot be determined experimentally, yet an accurate estimate of its amplitude is essential for the construction of continuous transforms from diffraction data. The introduction of extra solvent in the swelling-series method normally also changes the mean scattering density of the unit cell and, therefore, also changes \(F(0)\). This is demonstrated in Table 1. Example values of \(F(0)\) for neutron and X-ray studies are given in Table 2. The change in \(F(0)\) with hydration means that the structure factors from swelling-series measurements of stacked bilayers do not fit on a single continuous transform, as shown in Fig. 1. However, in the special case of neutron structure factors of stacked bilayers hydrated with 8.06% \(^2\text{H}_2\text{O}, F(0)\) does not change with hydration because water of this isotopic composition has a net neutron-scattering density of zero. With this solvent, the value of \(F(0)\) determined at one hydration can be used for all other hydrations, and the fact that \(F(0)\) does not change removes a large source of potential error when fitting the observed points to a single continuous transform (see Fig. 1).

### 3.4. Scaling

Another significant source of error in X-ray swelling series measurements comes from the scaling of the individual data sets to each other. Each point in the swelling series has to be determined from a different sample, since ionizing radiation damage precludes the reuse of samples for more than one measurement. The scaling procedure is classically based upon the formula

$$k = \sum_{h=0}^{\infty} F(h)/d,$$

where \(k\) is a constant. However, this summation must include \(F(0)\) which, as we have already shown, changes with hydration. The scattering-density contrast also changes with humidity (see below). Moreover, the summation should also extend to infinity, which is clearly impractical. The result of carrying out the summation over an incomplete series and with either no zeroth order or at best an approximation is an inaccurate scaling of the data sets to each other. The errors inherent in this process are quantified in Table 1.

None of these limitations applies to the neutron adaptation of the swelling-series method when used with 8.06% \(^2\text{H}_2\text{O}.\) The low levels of radiation damage inherent in the use of neutrons means that several swelling-series points can be obtained from the same sample. In the real-time swelling approach described here, the sample is not even disturbed.
between measurements, so that the requirement for scaling between measurements in the same swelling series disappears.

3.5. Contrast

The introduction of water at any isotopic composition other than 8.06% $^2\text{H}_2\text{O}$ affects not just the value of $F(0)$ but also has an impact on the other structure factors. Fig. 2 compares the bilayer structure at different points on the swelling series. At 0% $^2\text{H}_2\text{O}$, it is clear that water penetration into the headgroup region reduces the height of the phosphate-ester peaks and shifts their centre of mass into the bilayer. This demonstrates that the effect of increasing the water content is to reduce the neutron-scattering contrast of the system. The region to which the negatively scattering water is introduced is immediately adjacent to and, indeed, partially overlaps the region of highest scattering density, namely the phosphates and ester linkages. The change in scattering contrast, the difference between the minimum and maximum scattering density or the maximum deviation from the mean scattering density $\rho(0)$, therefore has an impact on all structure factors. This effect is seen most clearly in Table 1, where $\sum F(h)^2/d$ changes as the $d$-repeat swells, even when $F(0)$ is included in the summation. Moreover, as the level of hydration rises, the $d$-repeat increases and the phosphate peaks appear to move further into the bilayer as the negatively scattering water erodes their hydrated edges. It is not possible, therefore, to use the location of these peaks as a measure of the bilayer thickness, as some authors have chosen to do, since their centre of mass shifts position with changes in hydration.

3.6. Sample disorder

The $d$-repeat of the 0% $^2\text{H}_2\text{O}$ swelling-series sample increased from 50.8 to 53.2 Å and that of the 0% $^2\text{H}_2\text{O}$ sample increased from 50.4 to 51.8 Å over the period of data collection. The mosaic spreads of the swelling-series samples were comparable to those from a similar sample measured under more conventional steady-state conditions. For example, the half-width at 1/e height ($e = 2.71828$) of the second order of the 0% $^2\text{H}_2\text{O}$ swelling-series sample was 0.34, 0.38, 0.38 and 0.36 Å for the four scans, compared with 0.38 Å for a steady-state sample of pure DOPC measured with identical instrument geometry, fully equilibrated to an atmosphere of approximately 100% rh.

These measurements show that there was no noticeable increase or decrease in the sample disorder throughout the swelling-series measurements. The low mosaic spread is typical for measurements of (static) unsaturated phospholipids such as DOPC, but is perhaps not expected in dynamic systems as reported here. In order to minimize disorder in the sample, two complementary factors are important in minimizing the degree of swelling during the measurement of each single order. Firstly, the sample should equilibrate with the atmosphere inside the sample can faster than the can atmosphere equilibrates with the water at the base of the can. In our measurements, this was achieved by using only one water bath, in which the surface area of the water was relatively small (approximately 5 cm²). Secondly, the time spent in scanning each order should be the minimum consistent with good counting statistics. In this respect, the high neutron flux of the D16 instrument was advantageous to the study.

3.7. Adjusting the $d$-repeat

A standard procedure in neutron measurements is that of difference calculation, which is most powerful when both data sets in a subtraction have exactly the same $d$-repeat. However, this is rarely the case; typically, one has to be content with a difference of up to 2% or so. Fig. 2 shows a series of bilayer scattering profiles, calculated from points on the lines interpolated between the swelling-series points. The difference between profiles that differ by only 1.0 Å is apparent. This error is quantified in Fig. 3. The figure shows that a difference in $d$-repeat of 1.0 Å in a difference subtraction with 0% $^2\text{H}_2\text{O}$ data introduces an error of close to 20% in the result (plot a).

### Table 2

<table>
<thead>
<tr>
<th>Lipid (s)</th>
<th>X-rays 5 w/l</th>
<th>Neutrons 0% $^2\text{H}_2\text{O}$ 5 w/l</th>
<th>Neutrons 100% $^2\text{H}_2\text{O}$ 5 w/l</th>
<th>Neutrons 8.06% $^2\text{H}_2\text{O}$ 5 w/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC (18:1)</td>
<td>272.3</td>
<td>6.2</td>
<td>270.0</td>
<td>7.9</td>
</tr>
<tr>
<td>DOPG (18:1)</td>
<td>267.2</td>
<td>8.5</td>
<td>293.1</td>
<td>10.1</td>
</tr>
<tr>
<td>DOPE (18:1)</td>
<td>258.8</td>
<td>6.7</td>
<td>275.5</td>
<td>8.4</td>
</tr>
<tr>
<td>DOS (18:1)</td>
<td>270.6</td>
<td>11.1</td>
<td>31.9</td>
<td>12.8</td>
</tr>
<tr>
<td>DPPC (16:1)</td>
<td>254.3</td>
<td>6.9</td>
<td>27.7</td>
<td>8.5</td>
</tr>
<tr>
<td>DPPG (16:1)</td>
<td>256.5</td>
<td>3.9</td>
<td>24.7</td>
<td>5.5</td>
</tr>
<tr>
<td>DPPE (16:1)</td>
<td>251.4</td>
<td>6.1</td>
<td>27.0</td>
<td>7.8</td>
</tr>
<tr>
<td>DPPS (16:1)</td>
<td>243.0</td>
<td>4.3</td>
<td>25.2</td>
<td>6.0</td>
</tr>
<tr>
<td>DPPE (16:1)</td>
<td>254.8</td>
<td>8.8</td>
<td>29.6</td>
<td>10.4</td>
</tr>
</tbody>
</table>

1 Water of this isotopic composition has a net neutron-scattering length of zero, so the values of $F(0)$ quoted apply to all hydration levels. 2 DOPC: 1,2-dioleoyl-sn-glycero-phosphocholine; DOPG: 1,2-dipalmitoyl-sn-glycero-phosphoglycerol; DPPC: 1,2-dipalmitoyl-sn-glycero-phosphatidylcholine; DPPE: 1,2-dipalmitoyl-sn-glycero-phosphatidylethanolamine; DPPE: 1,2-dipalmitoyl-sn-glycero-phosphoserine. § Waters per lipid.
This error is approximately halved if the data are collected at 8.06% $^2$H$_2$O (plot b).

It has been proposed (Bildt et al., 1979) that when the two sets of structure factors to be used in a subtraction do not have exactly the same $d$-repeat, one of the sets can be recalculated using the continuous-transform method (1). Fig. 1 demonstrates that this is only possible in the unique situation where the data are collected at 8.06% $^2$H$_2$O, since at 0% $^2$H$_2$O the observed structure factors do not actually trace out a single continuous transform, as shown in the figure. Fig. 3(c) quantifies the error inherent in mistakenly using the continuous transform to adjust the $d$-repeat of data collected at 0% $^2$H$_2$O. Up to a difference in $d$-repeats of approximately 0.6 Å the ‘correction’ has little effect upon the error. Above 0.6 Å difference, the ‘correction’ procedure actually increases the magnitude of the error.

However, the swelling-series method described here allows a degree of adjustment of the $d$-repeats of lamellar structure factors. At 8.06% $^2$H$_2$O, the continuous-transform method can be used. Even at 0% $^2$H$_2$O, the swelling method allows interpolation between the measured values of each structure factor in order to determine the bilayer structure at any $d$-repeat within the range covered by the measurements.

The above discussion appears to have neglected the suggestion that the bilayer structure might change during the hydration process. It has been suggested that the conformation of phosphate-containing head group of dimyrystoyl-phosphatidylecholine in the L$_o$ phase is dependent upon hydration level (Worcester, 1976). It is possible that the 0% $^2$H$_2$O data are affected by this phenomenon, though it is not certain that the current resolution ($h = 5$) would be sufficient to show this. X-ray diffraction measurements have also shown that there can be structural rearrangements of the fatty-acyl chains as a function of hydration (Hristova & White, 1998). It is unlikely that neutron diffraction would be sensitive to these structural changes in undeuterated lipids. Moreover, unless any structural rearrangement occurs over a very small change in $d$-repeat (the smooth curves through each 0% order in Fig. 1 do not show this to be the case), then calculating an intermediate set of structure factors between the observed points will simply result in an intermediate structure. This view is further reinforced by the observation that the 8.06% $^2$H$_2$O data appear to fit very closely to a single continuous transform, thereby indicating that neutron diffraction is insensitive to any lyotropic structural changes of the lipids that may have occurred during these measurements.

4. Conclusions

We have demonstrated that it can be advantageous to sample a range of points in the reciprocal lattice during neutron-diffraction measurements of stacked phospholipid bilayers. This can be achieved in two ways: by recording several static measurements using a number of known relative humidities or, as we have demonstrated here, by changing the sample hydration during the data collection. Whenever method is used, phase information is gained. If the hydration state is slowly changed without disturbing the sample, it is not necessary to scale the different data sets to each other. Interpolation between the observed points for any order can then be used to determine structure-factor sets of any intermediate $d$-repeat, thereby reducing the errors in difference subtractions. We have demonstrated and quantified the error caused by neglecting to correct the $d$-repeat differences in subtractions and also the error introduced by mistakenly using the continuous-transform method to adjust the $d$-repeat of 0% $^2$H$_2$O data.

We have argued (Brashaw et al., 1998) that it is valid to interpolate between observed points for any order at 0% $^2$H$_2$O, even though these points do not lie on the continuous transform of the neutron-scattering profile. However, a more satisfactory approach is to collect the data at 8.06% $^2$H$_2$O. At this isotopic composition the water is effectively invisible to neutrons and neutron scattering by the water is eliminated. The net result is similar to the so-called ‘minus fluid model’ proposed by Worthington et al. (1973) and developed by Hristova & White (1998), except that in the neutron method the removal of scattering from water is achieved without mathematical manipulation. Only under these conditions will all observed structure factors lie on the same continuous transform, thereby allowing the same function to be fitted simultaneously to all observed points. This approach is far more rigorous than the fitting of arbitrary functions to indi-

![Figure 3](image-url)

Figure 3 Determination of errors introduced into difference subtraction data by subtracting structure-factor sets that do not have the same $d$-repeat. Experimentally determined structure factors (five orders) for DOPC at various $d$-repeats were subtracted from each other and the difference expressed as a percentage. The points shown are the mean of five independent calculations with the error bars showing the maximum spread. (a) 0% $^2$H$_2$O data; each data point represents the mean of five calculations of the form percentage error = 100\$\sum_{i=1}^{\infty} |F_i - F_{\infty}|/100\sum_{i=1}^{\infty} |F_i|$, (b) 8.06% $^2$H$_2$O data; errors calculated as in (a), (c) 0% $^2$H$_2$O data; errors calculated as in (a) except that the continuous-transform method (1) was used to correct one of the data sets before the subtraction. The magnitude of the error increases as the coherent neutron scattering density of water deviates from zero. In other words, the errors are lowest at 8.06% $^2$H$_2$O, greater at 0% $^2$H$_2$O and are therefore predicted to be greatest at 100% $^2$H$_2$O.
vidual orders of data collected at any other $^2$H$_2$O concentration. The accuracy is improved by fitting one curve to all observed points. It also gives phase information and allows the adjustment of $d$-repeats of data sets for use in difference subtractions.

The use of 8.06% $^2$H$_2$O has other advantages. The introduction of substances such as peptides into bilayers may affect the distribution of water within and between the stacked bilayers. This will be reflected in difference subtractions. By making the water effectively invisible to neutrons, difference subtractions at 8.06% $^2$H$_2$O removes this contribution from the difference profile, allowing less ambiguous interpretation of the results.

Taking all these advantages together, the improved accuracy, reduced equilibration time and the ability to calculate structure-factor sets at any selected $d$-repeat (within a given range), the swelling-series method has much to recommend its adoption for neutron-diffraction studies. We therefore propose this adaptation of the swelling method, not as a replacement for the more usual neutron approach of isotopic substitution, but rather as a complimentary technique which can help to improve the accuracy of structure-factor determination whilst also providing an independent ‘second opinion’ for phase determination. Its added advantage of reducing the requirement for sample-equilibration time at the start of an experiment will assist in the optimal utilization of neutron beam-time allocations.

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References
X-ray diffraction study of feline leukemia virus fusion peptide and lipid polymorphism

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Abstract The structural effects of the fusion peptide of feline leukemia virus (FeLV) on the lipid polymorphism of N-methylated dioleoylphosphatidylethanolamine were studied using a temperature ramp with sequential X-ray diffraction. This peptide, the hydrophobic amino-terminus of p15E, has been proven to be fusogenic and to promote the formation of highly curved, intermediate structures on the lamellar liquid-crystal to inverse hexagonal phase transition pathway. The FeLV peptide produces marked effects on the thermotropic mesomorphic behaviour of MeDOPE, a phospholipid with an intermediate spontaneous radius of curvature. The peptide is shown to reduce the lamellar repeat distance of the membrane prior to the onset of an inverted cubic phase. This suggests that membrane thinning may play a role in peptide-induced membrane fusion and strengthens the link between the fusion pathway and inverted cubic phase formation. The results of this study are interpreted in relation to models of the membrane fusion mechanism.

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Key words: Membrane thinning; Cubic phase; Hexagonal phase

1. Introduction

Biomembrane fusion is a ubiquitous process that plays a crucial role in such fundamental events as spermatozoid-egg fusion and mitosis. Despite this fact, the molecular rearrangements of the lipids and the precise kinetic events involved are still unclear. This is largely because the fusion event is transient and involves only local, isolated patches of lipid. Biomembrane fusion is a protein-regulated event [1,2]. Enveloped virus particles use membrane fusion in order to introduce their infective nuclear material into a host cell. The most widely studied membrane 'fusion protein' is the hemagglutinin of the influenza A virus [2]. This virus, like all animal enveloped viruses, utilizes a specialised, extra-membranous glycoprotein 'spike' as a fusion catalyst.

Although the initial triggers of the fusion event show great diversity, the actual macromolecular rearrangements of the membrane are thought to be similar for many fusing systems [3]. The multi-step fusion process involves the merging of two distinct, planar bilayers to form highly curved fusion intermediates [4] and there has been much evidence to support this hypothesis [5-10]. The formation of highly curved lipid mesomorphs also occurs during the lamellar liquid-crystal/inverted cubic (Lc/Qh) phase transition and the lamellar liquid-crystal/inverse hexagonal (Lc/Hx) phase transition [11]. The ability of a number of agents to promote fusion appears to be correlated to their ability to lower the Lc/Hx transition temperature (Tc) [12–14]. Similarly, some fusion inhibitors raise Th [15]. Although the Qh and the Hx phases, which are kinetically stable, are unlikely to exist at the site of a developing fusion pore, knowledge about the topology of the interface as these phases begin to form may have implications in biological fusion pathways.

The exact structures of the intermediates involved in the Lc/Qh and Lc/Hx phase transitions have not been described uniquely [16–19]. Experimental data [20] and theoretical free energy calculations of intermediate lipid structures [21] suggest that the most likely pathway is via the formation of a low energy stabi-like structure [22], then 'hemifusion' intermediates (also known as transmonolayer contacts, TMCs). A recent study showed that intermembrane connections could appear some 22°C below the Th [23]. The intermembrane connections evolve to form isolated TMCs, then aggregated TMCs, which are a basis for Hx phase growth via the formation of quasi-hexagonal phase domains [23]. The integrity of the TMC structure is the committing step in membrane fusion. Ruptured TMCs form intermembrane attachments (ILAs) or fusion pores. Many ILAs in close proximity may nucleate to form Qh phases. There is evidence to suggest that specialised fusion proteins also catalyse the formation of similar lipid intermediates [3,24–26]. Therefore, studying the structural effects of fusion peptides in the proximity of inverted phase boundaries provides instructive insight into the fusion mechanism.

In this paper, we report the effect of the fusion peptide from feline leukemia virus (FeLV) on the thermotropic lipid phase behaviour of multi-lamellar vesicles (MLVs) composed of N-methylated dioleoylphosphatidylethanolamine (MeDOPE). The fusion peptide of FeLV is the hydrophobic N-terminus of p15E, itself part of the viral envelope glycoprotein spike gp85. Sequential small angle X-ray scattering (SAXS) images were recorded from pure lipid dispersions and lipid dispersions containing different amounts of FeLV peptide as the temperature was progressively increased. We interpret the findings in relation to biomembrane fusion.

2. Materials and methods

FeLV fusion peptide was synthesised by Albachem (Edinburgh, UK) to the sequence EPISLTVALMLGLTGGIAAGVGTGKT, as used in our previous studies (e.g. [27]). MeDOPE was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. A stock solution of FeLV peptide (1.0 mg/ml) in chloroform:methanol (2:1) was prepared by thorough vortexing and ultrasonication. An ice/water mixture ensured that sonication

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did not heat the solution above room temperature. The appropriate amount of stock solution was dispensed to a pre-weighed amount of MeDOPE in a Pyrex test-tube. The dissolved lipid and peptide were then dried under a stream of oxygen-free nitrogen. The resultant lipid films were placed under vacuum overnight in the presence of phosphorous pentoxide (P2O5) to remove trace amounts of solvent. Each film was constituted in buffer (20 mM PIPES, 1 mM EDTA, 150 mM sodium chloride and 0.002% sodium azide at pH 7.4) and vortexed thoroughly for 10 min at room temperature. All samples had a low lipid concentration of 100 mM, equivalent to 7.57% (w/v), thereby ensuring an excess water condition. The lipid dispersions were then subjected to five freeze-thaw cycles as previously described [27] to ensure that the lipid was in the fully hydrated Lα phase regardless of the thermal history of the lipid.

X-ray diffraction measurements were performed at station 2.1 of the Synchrotron Radiation Source at Daresbury Laboratory, UK. The X-ray wavelength was 1.51 Å, the specimen to detector distance was 1.5 m. A teflon-lined brass chamber with mica windows and thermocouple was used as a sample holder and a circulating water bath controlled the sample temperature. The temperature increased linearly at a rate of 30 K/h, while data were collected continuously, at a rate of two frames per minute. Geometric and detector response corrections were applied using the XOTOKO program. Angular calibration was achieved using rat-tail collagen as a standard [28] immediately prior to the exposure of each new liposomal sample. The exact location and intensity of Bragg reflection were determined by Gaussian fitting, using PeakFit v4 software (Jandel Scientific, Chicago, IL, USA).

3. Results

3.1. MeDOPE control

This type of experiment may be run as a series of temperature jumps, each one followed by a pause whilst the sample equilibrates to the new temperature before a single frame of data is obtained. Whilst this approach ensures equilibrium across the sample, information is lost during heating. Therefore, the approach chosen for these measurements was a continuous temperature scan, with consecutive frames of x-ray data recording the whole transition process. In this way, it is unlikely that the sample ever reaches equilibrium, but the chance of observing intermediate structures is maximised. This is only possible with bright synchrotron X-ray sources. MeDOPE was chosen for this study because its T1/2 of approximately 60°C is experimentally convenient, allowing for a long temperature ramp from ambient conditions. MeDOPE is classed as a ‘non-bilayer lipid’ [29]. Such lipids are thought to play a role in biological membrane fusion. In addition, MeDOPE has an intermediate spontaneous radius of curvature (Rc) value [8] and has been shown to form Qθ phases when treated to specific thermal protocols [8, 30, 31]. MeDOPE has an intermediate Rc value, so this lipid has a tendency to form structures of intermediate curvature [8].

In the X-ray data, two sharp orders of diffraction were observed at 31°C, which indexed onto a lamellar lattice (reciprocal lattice ratio 0:1:2) when measured from the position of the zeroth order. The narrow peak widths and low incoherent scattering inferred that most of the sample mass was in a well-ordered lattice. The lamellar repeat distance (dL) of MeDOPE at 31°C was 62 Å, which is in agreement with Gruner et al. [8]. Fig. 1 shows the lattice basis vector lengths of the Lα and HII phases as a function of temperature. dL increases steadily with temperature up to 54°C. HII phase onset is observed at approximately 60°C in agreement with the literature. Although both Qθ and HII phases of MeDOPE can co-exist near T1/2 [31], no Qθ phase reflections were observed at our relatively rapid temperature scan rate, as predicted. The exact T1/2 is difficult to ascertain because the dL and dL/2 reflections of both HII and Lα overlapped each other with MeDOPE, making differentiation between the two phases difficult. The first reflection unique to HII phase (dL/3) was observed at approximately 60°C. The co-existence of Lα and HII phases near the T1/2 has been reported previously [32]. Above 62°C, some five HII reflections were observed, with the reciprocal ratio of 0:1:3:5:7:9.

The HII lattice basis vector length of MeDOPE at 60°C was 65 Å. Multiplying this value by the factor 2/3 yields dH, the distance from the centre of one HII cylinder to the next. The calculated dH was 75 Å, which is comparable with Gruner’s value. dH steadily decreased to 73 Å at 71°C. This occurs because the hydrocarbon chain splay is wider at higher temperatures.

Fig. 2 shows the full-width at 1/e (σ = 2.718) height (FW1/eH) values for the first and second order reflections. This parameter is related to the sample order. The width of the
3.2. MeDOPE and FeLV fusion peptide (1.0 and 0.5 mol%)

Bragg reflections significantly decreases from those around 50°C, indicating that the lipid system becomes progressively more ordered as the phase boundary approaches. This is because, close to \( T_{H_1} \), the recorded reflection is the superposition of a wide \( L_a \) Gaussian and a narrow \( H_{II} \) Gaussian. As the contribution of the wide \( L_a \) Gaussian decreases and the contribution of the narrow \( H_{II} \) Gaussian increases, the observed peak appears narrow. Lipid phases may co-exist even without thermal gradients across the sample. The observation that the pure lipid specimen can undergo a \( L_a/H_{II} \) phase transition at fast scan rates without entering the \( Q_{II} \) phase is in agreement with Hui et al. [7].

3.3. MeDOPE and FeLV fusion peptide (2.0 mol%)

The highest peptide concentration gave diffraction patterns that were the most similar to those from MLVs composed of pure MeDOPE. Well-ordered diffraction was observed throughout the temperature scan. The \( d_L \) peak areas (including the reduction in amplitude at 60°C), \( T_{H_1} \) and the FWL/eH were all very similar to the lipid control. Only a small amount of \( Q_{II} \) phase was observed and then only after the \( H_{II} \) phase

Fig. 3. X-ray diffraction profiles of MLVs composed of MeDOPE plus FeLV fusion peptide (1.0 mol%) at specific temperatures. The diagram clearly shows the emergence of a shoulder on the \( L_a \) Bragg reflections and the onset of a \( Q_{II} \) phase.

Fig. 4. Lattice basis vector length versus temperature for the \( L_a \) and \( Q_{II} \) phases of MeDOPE plus FeLV fusion peptide (1.0 mol%) as determined by time-resolved small angle X-ray diffraction. The novel lamellar phase with reduced \( d_L \) can be readily observed (crossed symbols). Onset of the \( H_{II} \) phase occurs at the end of the temperature scan. The shaded areas represent transition temperature ranges, during which more than one phase is observed simultaneously. Symbols as in Fig. 1; \( Q_{II} \) phase symbols in grey.
was established. This may have been accounted for by the existence of discrete membrane patches containing low concentrations of peptide. The peptide in these domains may be able to interact more intimately with the phospholipid in a similar manner to that seen for samples containing the lower peptide concentrations. The Q1H phase was observed from 66 to 70°C and had a basis length of 148 Å that did not change significantly during the limited temperature range at which it existed. At this temperature, the Q1H basis length was 159 Å for the 1.0 mol% sample and 165 Å for the 0.5 mol% sample.

4. Discussion

These results clearly show that FeLV fusion peptide has a marked effect on the thermotropic behaviour of MLVs comprised of MeDOPE. This effect was most evident at the two lower peptide concentrations, when the peptide induced both a Q1H structure and a novel lamellar structure.

A previous differential scanning calorimetry (DSC) study has shown that, at high concentrations, the FeLV peptide had less effect upon the Tm of DPoPE than was seen at low peptide concentrations [27]. In the same paper, using SAXS, the presence of peptide-induced novel peaks in the H1 phase of DPoPE was also less pronounced at high peptide concentrations. Similarly, our X-ray data have shown that the sample containing 2.0 mol% peptide behaved like the pure lipid control. It is possible that, at high concentration, peptide is simply lying along the membrane surface in sufficiently large numbers to prevent the bilayer from curling into an inverse geometry. Alternatively, peptide aggregation in these higher concentration samples may prevent the peptide from interacting with the phospholipid molecules in the same way. We have already reported [34] that FeLV fusion peptide is able to form amyloid-like cross-β fibrils.

At concentrations of 0.5 and 1.0 mol%, the FeLV peptide reduces the Tm of MeDOPE and increases the amount of isotropic 31P nuclear magnetic resonance [27]. The idea that fusion peptides can induce this isotropic resonance is not new [9,10,33]. However, our data show that the previously reported isotropic resonance [27] is directly related to Q1H structure formation. The connection between membrane fusion and the formation of non-bilayer phases is only valid for systems that show these structures during the first heating scan, as was observed here. This is the first observation, to our knowledge, of Q1H phase formation by a viral fusion peptide. Previously, this peptide-induced phase has only ever been seen with MeDOPE systems containing influenza A fusion peptide at temperatures above Tm or in samples cooled from Tm [33]. Development of the Q1H phase normally takes hours and thus cannot be correlated to the rapid fusion process, but we have shown that FeLV peptide can reduce the time of this transition to minutes. This suggests that the FeLV peptide is an extremely potent trigger of highly curved intermediates.

Previous DSC measurements of MeDOPE-containing MLVs have revealed small shoulders on the low temperature side of the Tm enthalpic peak [13,34], presumably caused by intermediates in the Ld/H1 pathway. Later studies using DSC and X-ray diffraction revealed that the Ld/H1 phase transition temperature (Tg) was approximately 62°C and that the Q1H structure undergoes a phase transition to the H1 phase at higher temperatures (72–77°C) [31]. Thus, from Fig. 3, it can be readily observed that the FeLV peptide dramatically reduces Tg by 18°C, when compared to these values. The H1 phase that eventually formed had the same lattice parameters as the pure lipid. Importantly, this indicates that the peptide does not affect R, of this lipid species although it does have a destabilising effect upon the Ld phase. A previous X-ray diffraction study on MeDOPE with the fusion peptide of simian immunodeficiency virus revealed that the H1 lattice parameters were slightly less than that of the pure lipid control [32]. It was reasoned that this fusion peptide destabilises bilayers by increasing the strength of R, greatly. Importantly, the FeLV peptide promotes the facile development of Q1H structures rather than H1 structures. Precursors involved in the Ld/Q1H transition pathway are also thought to be involved in fusion pore formation [35].

An important feature of the SAXS data is the appearance of a lamellar structure with reduced dL some 25°C below the Tm. This structure occurs at the Ld/Q1H transition, co-existing with first the Ld and then the Q1H phases. This peptide-induced structure seems to represent a transitory stage in the Ld/Q1H phase transition mechanism and possibly in the multi-step fusion event itself. The correlation of the appearance of this novel structure with the greatly reduced Tg suggests that the two are causally connected. We believe that this effect is a result of the peptide’s local action on the membrane rather than due to bulk phase separation into areas of peptide-rich and peptide-poor concentrations. This argument is supported by the fact that no novel peaks were evident at the 2.0 mol% peptide concentration, the concentration where phase separation would be most obvious. The available data do not determine whether the novel structure is thermodynamically metastable.

There are two possible explanations for these lamellar structures of reduced dL. The thickness of the water layer between adjacent bilayers may decrease or the lipid bilayer may thin.

Interfacial dehydration of the lipid bilayer would decrease the dL [36]. The close approach of two membranes is hindered, from approximately 20 Å inwards, by an exponentially increasing repulsive force [37,38]. It is necessary to displace tightly bound water molecules from each membrane interface [39]. Surface dehydration, by a fusion peptide, could circumvent repulsive forces and permit close membrane approach. Our SAXS images reveal that the dL-repeat of the bilayer decrease by some 8 Å in the presence of peptide. This value is similar to that reported by Hui et al. [36] who studied membrane fusion induced by freezing and thawing. They make the point that dehydration does not cause membrane fusion alone but that it increases the opportunity for the event to proceed. The ability of fusion peptides to dehydrate membrane surfaces has already been implicated in modelling studies of a lipid-lined fusion pore [40].

An alternative, or complementary, explanation for the observed reduction in dL is bilayer compression. X-ray diffraction studies have shown that low concentrations of alamethicin adsorbed onto phospholipid bilayer surfaces can cause bilayer thinning [41]. Wu et al. proposed that alamethicin increases the cross-sectional area of the membrane surface by causing lateral expansion of the lipid headgroups. The cross-sectional areas of the lipid headgroups and hydrocarbon chains must be matched in planar bilayers, so the hydrocarbon chains must increase their cross-sectional area to compen-
sate for the peptide at the surface. As the volume occupied by the chains is constant, there is a resultant decrease in the bilayer thickness. Since monolayers of phosphatidylethanolamine lipids have a natural tendency to curl, at elevated temperatures, the lipid molecules are not able to maintain cross-sectional matching and undergo an inverted phase transition. Like the $H_2$ phase, the $Q_1$ lattice parameters decrease reciprocally with temperature due to hydrocarbon chain splay. At $T_{QH}$, the frustration between the free energy of monolayer curvature strain and hydrocarbon chain stretching must be so great that the $H_2$ phase forms.

Why does the membrane-thin structure metamorphose to a $Q_1$ phase and not $H_2$? At a given temperature, the monolayer curvature free energy and the free energy involved in hydrocarbon chain stretching governs the type of lipid phase present [29]. No lipid assembly can fully satisfy the conflicting demands of a simultaneous low curvature free energy and chain packing free energy, giving rise to frustration. Cubic phases have a lower free energy of curvature than the $L_0$ phase but higher than the $H_2$ phase. Conversely, the free energy of the chains is higher than in the $L_0$ phase but lower than the $H_2$ phase. This means that the amount of frustration in the cubic phase is smaller than in either the $L_0$ or the $H_2$ phases [42], so that the cubic phase represents a compromise between the two. Our data show that the peptide destabilises the $L_0$ phase but stabilises lipid transition intermediates. Compared with the $L_0$ phase, the lipid intermediates have a reduced amount of frustration. The data show that, in the presence of fusion peptide, the inverted structure formed is a $Q_1$ structure. The initially large lattice parameter of this phase indicates that the free energy of curvature and the hydrocarbon packing free energy are of an intermediary value.

This work with FeLV fusion peptide supports the proposed link between the mechanism of inverse cubic phase formation and membrane fusion. It also indicates that bilayer thinning may be important in the fusion pathway. This study only deals with simplified peptide lipid interactions. In vivo, the peptide is covalently bound to a larger fusion protein, which is embedded in the viral membrane. During fusion, the primed fusion peptide will interact, at least initially, with only the outer membrane leaflet. In this experiment, the fusion peptide is free to interact with both sides of multi-lamellar bilayers. Nonetheless, these results may well represent many features of the fundamental peptide membrane interactions but without the same control and specificity.

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References

Improved accuracy and phasing of lamellar neutron diffraction data by real-time swelling series method

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Improved accuracy and phasing of lamellar neutron diffraction data by real-time swelling series method

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Abstract

The analytic continuation method has been widely used to phase Bragg reflections from lamellar samples of phospholipids. Here we describe its application to neutron diffraction data, collected from liquid-crystalline (L\textsubscript{\alpha} phase) samples of 1,2-dioleoyl-sn-glycero-phosphocholine (DOPC) under a state of shifting lamellar spacing. To provide a comparison with the more normal neutron approach of isotopic substitution using \textsuperscript{2}H\textsubscript{2}O, data were collected from similar samples, using the same experimental and optical conditions. We compare the two methods in terms of accuracy of structure factor determination, unambiguity of phase assignment and ease of use. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Neutron diffraction; Phospholipid; Phase assignment

1. Introduction

When performing neutron diffraction experiments on lamellar phospholipid samples, it is necessary to ensure that each sample has reached equilibrium with the humid atmosphere of the sample can, before starting the data collection. It is normal practice, upon loading a new multi-bilayer lipid sample, to monitor the position of a strong lamellar diffraction peak. As the bilayers take up water from the atmosphere the lamellar distance (D-repeat) increases, a process which is reflected in a decreasing angle of diffraction. This is often preceded by a period of reducing lamellar spacing as the sample continues to dehydrate from its exposure to the, usually, dryer outside atmosphere. The whole process may take several hours, depending upon the size of the can, the surface area of the water baths and the previous state of the sample. Obviously, it helps if the disturbance to the sample's hydration state is kept to a minimum by transferring it as quickly as possible from its sealed container to the experimental vessel.

The movements of diffraction peaks during sample equilibration are accompanied by a rise or fall...
in diffracted intensity as the Bragg angles sample different spatial frequencies on the molecular (bilayer) transform. This property has been used to determine the structure factor phases in X-ray experiments, when a pseudo-continuous transform is mapped out using a number of samples, each prepared to a different lamellar spacing by controlling the humidity of the atmosphere or the osmotic pressure of the solution [1]. Here we show that the oscillations of a Bragg peak in a sample seeking equilibrium may be used both as an aid to phasing the peak and to improve the accuracy of its measurement. The method is presented in a side-to-side comparison with the more widely used $^2\text{H}_2\text{O}/\text{H}_2\text{O}$ exchange approach to phasing, in which the isotopic substitution of hydrogen by deuterium is used in an adaptation of the crystallographic method of isomorphous derivatives.

2. Materials and methods

2.1. Sample preparation

1,2-dioleoyl-sn-glycero-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. 20 mg of the sample was deposited on quartz microscope slides (75 mm x 25 mm) using an artist’s airbrush. The spraying solvent was chloroform. The slides were placed in a vacuum dessicator for 12 h to remove all traces of the solvent.

2.2. Neutron data collection

Neutron diffraction measurements were carried out on the D16 membrane diffractometer at the Institut Laue Langevin, Grenoble, France. The sample environment was a standard aluminium can, in which temperature control is achieved by circulating water through an integral water jacket, and humidity control by changing the solution in two Teflon water baths at the base of the can. All samples were run at 25°C. The water baths contained pure water, at one of three isotopic compositions: 0%, 50% or 100% $^2\text{H}_2\text{O}$.

In the $^2\text{H}_2\text{O}/\text{H}_2\text{O}$ exchange experiments, each sample was run at all three $^2\text{H}_2\text{O}$ concentrations. Each sample was rehydrated at 25°C in an atmosphere of 100% relative humidity for at least 24 h before being transferred to the D16 sample can. At each subsequent change of solvent, the sample was first dried out, then rehydrated in an atmosphere saturated with water at the new isotopic composition for at least 24 h. The scanning protocol consisted of sequential $\theta$ (sample angle) scans around the predicted Bragg angle for each order. Each scan covered the angle $-0.6^\circ$ to $+0.6^\circ$.

The mosaic spread of the second order of diffraction was determined for each sample, using standard procedures.

For the swelling series measurements, the dry DOPC sample, on its quartz slide, was quickly transferred straight from its vacuum desiccator into the D16 can together with two water troughs, containing water. A series of continuous $\theta-2\theta$ scans was immediately initiated. Each scan (from $\theta = 1.5^\circ$ to $15.0^\circ$) took approximately 3 h to complete.

2.3. Data analysis ($1^\circ$ $^2\text{H}_2\text{O}/\text{H}_2\text{O}$ exchange

The two-dimensional array of detector counts for each frame of data was corrected for variations in pixel response by division by a corresponding array of data recorded from water (H$_2$O). The complete set of frames from each scan were then collapsed into a linear spectrum and combined to generate a pseudo-(\(\theta - 2\theta\)) scan. Up to this stage, all of the analysis was carried out by the D16 instrument software.

The background around each peak was fitted and subtracted using SigmaPlot (Jandel Scientific Software GmbH), a commercial spreadsheet and graphing package. Gaussian curves were then fitted to the Bragg reflections and the angular position, width and area of each peak recorded. Absorption and Lorentz corrections were applied and the intensities square-rooted to produce structure factor amplitudes. The relative scaling of the different data sets and the phases of each of their orders were determined by least-squares fitting to straight line functions, as shown in
Fig. 1. The whole procedure has been described previously [2].

The D-repeat (lamellar repeat distance) was calculated by least-squares fitting of the observed angles of diffraction (2θ) to the Bragg equation: \( h^2 = 2D \sin(2\theta) \) where \( \lambda \) is the wavelength of neutrons, \( h \) is the order of diffraction and \( x \) is the angular offset (misalignment) of the detector.

The data were placed on a ‘relative absolute’ [3–5] scale using the known neutron scattering densities of \( ^2\text{H}_2\text{O} \) and \( \text{H}_2\text{O} \) to scale the (DOPC + \( ^2\text{H}_2\text{O} \)) – (DOPC + \( \text{H}_2\text{O} \)) difference. This method requires knowledge of the molar percentage of water in the samples, which was determined as described below.

2.4. Determination of water content

The neutron diffraction sample preparation was repeated using \( ^{14}\text{C} \)-labelled DOPC and tritiated water so that scintillation counting could be used to determine the water : lipid molar ratio. Microscope coverslips (22 × 22 mm) were cut into half to provide a suitable substrate which would fit into a standard scintillation counter tube. In order to give the same sample thickness, approximately 5.0 mg of lipid was spread on each half-coverslip. After vacuum dehydration and rehydration for 24 h, in small batches, at 25°C, the coverslips were quickly transferred to tubes of scintillant. \( ^{14}\text{C} \) and \( ^{3}\text{H} \) activity were measured using a Packard 1900CA Liquid Scintillation Analyzer. Calibration samples containing tritiated water (1.0 µl) or \( ^{14}\text{C} \)-phospholipid equivalent to 5.0 mg of DOPC were used to calibrate the scintillation counter channel and cross-channel response. Clean coverslips served as controls for the experiment, to ensure that the amount of water condensing on the reverse side was negligible.

2.5. Data analysis (2) swelling series

Background subtraction and Gaussian-fitting were carried out as for the \( ^2\text{H}_2\text{O}/\text{H}_2\text{O} \) exchange data. After correction with absorption and Lorentz factors the structure factors were calculated from the intensities and plotted against their spatial frequencies, as shown in Fig. 2. Interpolation between the points produced sets of structure factors, which were then used to calculate [1] the continuous transforms, also shown in the figure.

3. Results and discussion

Figs. 1 and 2 show the structure factors determined by each method. Neutron scattering density profiles, calculated by Fourier transformation of the \( ^2\text{H}_2\text{O}/\text{H}_2\text{O} \) exchange data are shown in Fig. 3. Fig. 4 is a swelling series of bilayer profiles calculated from sets of structure factors determined by interpolation between the observed points, as shown in Fig. 2.
Fig. 2. Plot of structure factor amplitude versus spatial frequency of data points collected by real-time swelling series from stacked bilayers of DOPC at 25°C. The dry DOPC sample was placed in a D16 sample can along with water troughs containing 100% H2O. Four consecutive 0-2θ scans were run over a total period of 12 h. The three inset graphs are enlargements of the regions corresponding to the third, fourth and fifth orders of diffraction. Also shown are continuous transforms calculated using the method of King and Worthington [1].

The D-repeats of the 2H2O/H2O exchange samples were 53.5 ± 0.5, 53.1 ± 0.5 and 53.1 ± 0.5 Å for the 0%, 50% and 100% 2H2O samples, respectively. The D-repeat of the swelling series sample increased from 50.8 to 53.2 Å over the 12 h of data collection. The mosaic spreads (half-width at 1/e height) for the 2H2O/H2O exchange samples were 0.38°. The mosaic spreads of the swelling series sample was 0.34°, 0.38°, 0.38° and 0.36° for the four scans. The water content of pure DOPC bilayers at 25°C was determined to be 26.8 ± 0.5 waters per lipid. The tritium signal from the control coverslips was less than 1/200th of that from the sample coverslips.

There was no noticeable increase or decrease in the sample disorder throughout the swelling series measurements, as determined by mosaic spread. The low mosaic spread is typical for measurements of (static) unsaturated phospholipids, such as DOPC, but is perhaps not expected in dynamic systems as reported here. We explain our findings in terms of the sample quickly achieving equilibrium with the atmosphere inside the sample can compared to the very much slower equilibration of the can atmosphere with the water at the base of the can. The design of the water baths was such that the surface area was relatively small (approximately 5 cm²) and positioned some 1.5 cm below the rim.

Both of the methods used in this study give phase information. In the case of the 2H2O/H2O exchange approach, the phases are determined as those which give the best fit to straight lines when the structure factors of each order are plotted against the percentage of 2H2O (see Fig. 1). Since
the distribution of $^2\text{H}_2\text{O}$ approximates to a Gaussian peak centred at the origin, the phases are predicted to alternate, with odd-numbered orders negative and even ones positive. However, this Gaussian approximation breaks down as the order number increases because the width of the $^2\text{H}_2\text{O}$ peak is wide, and is actually better represented as a pair of Gaussian distributions [2, 4]. In the case of the swelling series approach, all possible phase combinations are tried, the correct one being identified as that which gives best agreement between the observed points and the continuous transform calculated from them [1]. This is shown in Fig. 2. Reference to the figure shows that the neutron structure factors, determined at a range of spatial frequencies, do not lie on the same continuous transform. The explanation for this is discussed below. However, it is clear from the figure that each order traces out its own curve through the observed points. This is even true for the first order reflections, which show the largest change in spatial frequency. The point at a spatial frequency of $0.0197 \text{Å}^{-1}$ was the first point measured, from an effectively dry sample; even though it lies well outside the continuous transforms shown, it still fits the curve plotted through the other first-order points. This curve may be interpolated to calculate sets of structure factors of any intermediate $D$-

repeat (since the hydration of the sample increased steadily throughout the experiment, no two measured points index to the same $D$-repeat). Continuous transforms, calculated from these points, using the method of King and Worthington show a smooth variation in amplitude, reinforcing faith in the phase assignments.

Interpolation between the measured values of each structure factor allows determination of the bilayer structure at any $D$-repeat within the range covered by the measurements. This feature complements the standard neutron procedure of difference calculation, which is most powerful when both data sets in a subtraction have exactly the same $D$-repeat. However, this is rarely the case; typically one has to be content with a difference up to 2\% or so. Fig. 4 shows a series of bilayer scattering profiles, calculated from points on the lines interpolated between the swelling series points. The difference between profiles which differ by only 1.0 Å is apparent. Using the swelling series method, each set of structure factors in a difference subtraction can be calculated for the same $D$-repeat, thereby removing ambiguity from the subtraction result.

It is apparent from Fig. 2 that the observed points do not trace out a continuous transform. One possible explanation is that the bilayer structure changes during the hydraton process. This point has been raised by Worcester [6], who has suggested that the conformation of phosphate-containing head group of dimyristoylphosphatidylcholine in the $L_\beta$ phase is dependent upon hydration level. It is possible that our data demonstrate the same phenomenon, though it is not certain that the current resolution ($h = 5$) would be sufficient to show this. However, unless any structural rearrangement occurred over a very small change in $D$-repeat (the smooth curves through each order in Fig. 2 do not show this to be the case) then calculating an intermediate set of structure factors between the observed points will simply result in an intermediate structure.

An alternative (or complementary) explanation is that increasing amounts of (negatively scattering) water modify the unit-cell structure in such a way that it is not valid to fit all of the swelling series to the same continuous transform. This suggestion is supported by Fig. 4 which compares the bilayer
structure at different points on the swelling series. It is clear that water penetration into the head group region reduces the height of the phosphate-ester peaks, and shifts their centre of mass into the bilayer.

Fig. 4 shows that the effect of increasing the water content is to reduce the neutron-scattering contrast of the system. The reason for this is that the region to which the negatively scattering water is introduced is immediately adjacent to and, indeed, totally overlaps (see Fig. 3), the region of highest scattering density, namely the phosphates and ester linkages. In order to avoid this effect, and more-accurately mimic the X-ray technique, it would be necessary to use 8.06% $^2$H$_2$O; at this isotopic composition, water has a net neutron scattering density of zero.

Despite the fact that the individual observed points cannot be fitted to a single continuous transform, for the reasons explained above, it is our assertion that it is still quite legitimate to interpolate between the observed points in order to derive sets of structure factors.

Table 1 compares the structure factors obtained by the two methods. One distinct advantage of the swelling series method is the potential increase in the accuracy of intensity measurement. Any change in $D$-repeat, caused by temperature or humidity fluctuations, or incomplete equilibration, can result in large differences in intensity of any given order. This effect is seen at its most extreme in the first order, where a change in $D$-repeat of 2.5 Å (from 50.8 to 53.2 Å) causes a 280% change in amplitude (which is equivalent to nearly 800% change in intensity). These variations may occur within the duration of the scanning of one sample (typically 12 h or so), or the same sample may equilibrate to a slightly different $D$-repeat in any of the set of measurements in a $^2$H$_2$O/$^4$H$_2$O exchange series. Instead of simply averaging out these differences, the swelling series method, proposed here, provides a much more systematic treatment of the data. Each reflection is indexed individually to its spatial frequency. Moreover, with more than one point for each order, it is possible to determine the precise structure factor amplitude at any intermediate point on the spatial frequency scale. When neutron beam time is limited, the advantage of not needing to wait for hours to be certain of complete equilibration of a new sample is considerable.

Another weakness in the $^2$H$_2$O/$^4$H$_2$O exchange method which is overcome by the swelling series approach is the requirement to scale the different data sets to each other in the former. This requirement is alleviated in the latter, since the entire series of measurements is made on the same sample, without disturbing its alignment in the beam. No correction is therefore necessary.

In practice, we do not see the swelling method being used exactly in the way we have described here. DOPC is a well-understood phospholipid, so it was a reasonable choice for this study. However,

Table 1
Relative absolute neutron structure factors $F^s(h)$ for oriented bilayer stacks of DOPC at 25°C. The $D$-repeat of each set is 53.2 Å

<table>
<thead>
<tr>
<th></th>
<th>$F^s(1)$</th>
<th>$F^s(2)$</th>
<th>$F^s(3)$</th>
<th>$F^s(4)$</th>
<th>$F^s(5)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC ($^2$H$_2$O/$^4$H$_2$O exchange method)$^a$</td>
<td>-7.68</td>
<td>-20.10</td>
<td>9.96</td>
<td>-3.00</td>
<td>-2.09</td>
</tr>
<tr>
<td></td>
<td>±0.30$^a$</td>
<td>±0.50$^b$</td>
<td>±0.34$^b$</td>
<td>±1.72$^b$</td>
<td>±0.57$^b$</td>
</tr>
<tr>
<td>DOPC (swelling series method)$^c$</td>
<td>-8.34</td>
<td>-18.89</td>
<td>9.57</td>
<td>-1.57</td>
<td>-4.13</td>
</tr>
</tbody>
</table>

$^a$These data were calculated by least-squares fitting to measured structure factors at 0%, 50% and 100% $^2$H$_2$O.

$^b$The errors quoted are the maximum deviation of each order from the least-squares fitted straight line (as shown in Fig. 1) and are, therefore, only an approximation. The two major contributions to the errors are likely to come from combining data from different $D$-repeats and inaccuracies inherent in the measuring process itself. It is not easy to calculate meaningful error estimates for the swelling series data, but they are expected to be no larger than those quoted for the $^2$H$_2$O/$^4$H$_2$O exchange data. Indeed, they are likely to be much improved since the former of the two major contributors to the errors does not apply to these data.

$^c$These data were calculated from curves fitted to structure factors measured from a sample as its $D$-repeat increased from 50.8 to 53.2 Å, over a period of 12 h.
when applied to less well-studied lipids, unexpected lyotropic phase transitions or phase separations may well manifest themselves which, though easy to identify in the neutron data, would detract from the value of the technique. How we do see the swelling method being applied is a way of breaking down each data collection run into several consecutive scans. Each scan would be analysed independently. A comparison of the position and amplitude of each order from the consecutive scans would then allow a more accurate estimation of the amplitude, as well as reveal useful phase information.

Taking all these advantages together, the improved accuracy, reduced equilibration time and the ability to calculate structure factor sets at any selected $D$-repeat (within a given range), the swelling series method has much to recommend its adoption for neutron diffraction studies. We therefore recommend the swelling method, not as a replacement for the more usual neutron approach of isotopic substitution, but rather as a complementary technique which can help to improve the accuracy of structure factor determination, whilst also providing an independent 'second opinion' for phase determination. Its added advantage of reducing the requirement for sample equilibration time at the start of an experiment will assist in the optimal utilisation of neutron beam time allocations. For maximum benefit, and closest parallel to the X-ray method from which it is derived, the samples should be run at 8.07\% $^{2}\text{H}_2\text{O}$, though it will yield increased information content at any $^{2}\text{H}_2\text{O}$ concentration.

**Acknowledgements**

We thank Dr. Valentin Gordelii of the Institut Laue Langevin for expert assistance with the data collection. This work was supported by the Institut Laue Langevin, Grenoble, France.

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A neutron study of the feline leukaemia virus fusion peptide:
Implications for biological fusion?

Sarah M.A. Davies\textsuperscript{a,*}, Malcolm J.M. Darkes\textsuperscript{b}, Jeremy P. Bradshaw\textsuperscript{a,b}

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\textsuperscript{b}Department of Biochemistry, School of Medicine, University of Edinburgh, George Square, Edinburgh EH8 9XD, UK
2. Materials and methods

2.1. Materials

The FeLV peptide sequence, obtained from the Swiss Protein Data Bank, was EPISLTVAL-MLGGLTVGGIAAGVGTGTK. Lysine was included on the carboxy terminus to improve solubility. It is the next naturally occurring residue. The 28-amino acid peptide was synthesised by Albachem (University of Edinburgh, Scotland, UK) using solid-phase synthesis. Its purity was >95%, as determined by analytical HPLC, mass spectrometry and amino-acid analysis. Dioleoylphosphatidylcholine

Fig. 1. (a) Neutron scattering density profiles of pure DOPC/DOPG bilayers (solid line) and of DOPC/DOPG with 1% (mol) FeLV peptide (broken line), and difference profile calculated by subtracting structure factors for DOPC/DOPG bilayers from structure factors for DOPC/DOPG with 1% (mol) FeLV peptide (dotted line). Phospholipid molecules are shown above the profiles to orientate the scattering densities. (b) $^{2}$H$_2$O distribution within and between bilayers of DOPC/DOPG (solid line) and DOPC/DOPG with 1% (mol) FeLV peptide (broken line), and difference profile of $^{2}$H$_2$O distribution due to the presence of FeLV peptide (dotted line).
(DOPC) and dioleoylphosphatidylglycerol (DOPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. Neutron diffraction

Neutron diffraction measurements were performed on the D16 instrument at the Institut Laue Langevin (Grenoble, France). 20 mg of total lipid, or lipid plus 1% (mol) peptide, were vortexed thoroughly in chloroform. The peptide-containing samples were then sonicated until the peptide dissolved. Thereafter, sample preparation, method details and data processing were essentially as described previously [7]. Samples were applied to quartz slides using an airbrush spraying technique. A minimum of 12 h was allowed for sample drying time in vacuum, and also for rehydration with H2O, 50% 2H2O O or 100% 2H2O. All samples were run at 25°C. The data were phased using the information from H2O/2H2O exchange, and put on a ‘relative absolute scale’ using the method in Ref. [8]. 14C-phospholipids and 3H-water were used to determine the water content of each sample. Six orders of diffraction were recorded and analysed. The calculated d-repeats were 55.25 Å for DOPC/DOPG and 55.60 Å for DOPC/DOPG plus FeLV peptide.

3. Results

Fig. 1a shows the neutron scattering density profiles at 8.1% 2H2O of bilayers of 50% (mol) DOPC/DOPG alone and in the presence of 1% (mol) FeLV peptide, and also the difference profile, calculated by subtracting the scattering density due to lipid alone from the profile for lipid with peptide present. Phospholipid molecules are shown above the profile to orientate the relative densities seen. The profile produced with peptide present shows decreased scattering density in the lipid headgroup region, and an increase in scattering density in the lipid tail region. Fig. 1b shows the 2H2O distribution within and between bilayers of pure DOPC/DOPG and DOPC/DOPG plus peptide, and the difference profile for 2H2O distribution due to the presence of the FeLV peptide. Results obtained for samples of pure DOPC with and without FeLV peptide showed negligible effect for the fusion peptide on bilayer structure (data not shown).

4. Discussion

The difference profile for the bilayer neutron scattering density (dotted line, Fig. 1a) shows that the addition of FeLV peptide to the bilayers causes an increase in scattering density close to the bilayer centre, a reduction at the surface and an increase in the water region. The scale of these perturbations is much too high for them to represent simply the scattering density of the peptide: the amount of peptide present is tiny, only 1% (mol). They must therefore be caused by alterations in the phospholipid packing (changes in water distribution have been eliminated from the figure by showing the 8.1% 2H2O profiles). We interpret the difference profile, therefore, as an increase in the packing density of the phospholipid fatty acids, and a decrease in the packing density of their headgroups. This interpretation is totally consistent with the emerging model for the mode of action of a fusion peptide: this suggests that the peptide inserts obliquely into lipid bilayers and precesses, i.e. it traces out an inverted cone-shaped motion. Thus, it will increase the negative curvature of the target lipid monolayers. As our lipids are confined to a planar bilayer configuration, changes in elastic monolayer curvature forces will be expressed as effects on molecular packing. Fig. 2 shows a model of this concept. The profiles in Fig. 1b show that the

Fig. 2. Model of the FeLV peptide inserting into a lipid bilayer and precessing. This favours the bending of the lipid molecules into a negatively curved, non-bilayer conformation.
\(^2\)H\(_2\)O distribution also changes, possibly as a result of bilayer thinning. Our results add support to the hypothesis that fusion peptides act by increasing the negative curvature of the target cell’s outer monolayer, thus causing this to bend towards the opposing bilayer, and allowing closer approach of the two initially distinct membranes.

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Interaction of tachykinins with phospholipid membranes: A neutron diffraction study

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Interaction of tachykinins with phospholipid membranes: A neutron diffraction study

Malcolm J.M. Darkes\(^a\)*, Sarah M.A. Davies\(^b\), Jeremy P. Bradshaw\(^a\),\(^b\)

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**Abstract**

Tachykinins are a group of peptides which bind to G-protein-coupled receptors. Receptor affinity appears to depend on different secondary structures of tachykinin which share the same hydrophobic carboxy-terminal sequence, FXGLM. Receptor activation is thought to be due to the carboxy-terminal submerging into the bilayer and the amino-terminal binding on the surface. Binding of tachykinins to phospholipid bilayers may take place both on the aqueous membrane surface and in the hydrophobic region. The two-state equilibrium appears to depend on the surface charge of the membrane. Deuterating substance P and neurokinin A at their carboxy-terminals, our results show two populations of label for each peptide. One is very close to the water–hydrocarbon interface, the other some 13 Å deeper. We report that the bilayer location of the two tachykinins is remarkably similar, thereby inferring that receptor specificity must be controlled by finer levels of structure. © 1998 Elsevier Science B.V. All rights reserved.

**Keywords:** Tachykinins; Substance P; Neurokinin A; Neutron diffraction

1. Introduction

Substance P (SP) and neurokinin A (NKA) belong to the tachykinin family, a group of six small amphipathic peptides which bind to G-protein-coupled receptors. They are found in a wide range of tissues including the central and peripheral nervous systems and gastrointestinal tract. Tachykinin receptor binding sites appear to involve both the extracellular loops and the transmembrane domains [1]. An address–message model [2] has been proposed where the flexible cationic N-terminal region, or “address domain”, is responsible for receptor selectivity and the structurally extended and conserved hydrophobic C-terminal delivers the message.

The binding of tachykinins to phospholipid bilayers appears to take place both on the aqueous membrane surface and in the hydrophobic region. This two-state equilibrium may depend on the surface charge of the membrane [3]. Nuclear magnetic resonance (NMR), circular dichroism (CD) and molecular modelling studies [4–6] have shown that...
SP has longer binding times and a more complex structure in negatively charged lipid-based systems than zwitterionic ones. In contrast, there is an argument against a membrane-mediated receptor mechanism, based on the observation that a SP analogue with a charged C-terminus, may not insert into the hydrophobic core of membranes, still shows SP agonist activity [4].

Our measurements test the hypothesis that different members of the tachykinin family position their C-terminal message at different depths within the membrane. Neutron diffraction was used to define the location of the specifically deuterated C-terminus of each peptide in synthetic phospholipid bilayers.

2. Materials and methods

Neutron diffraction measurements were carried out on the V1 membrane diffractometer at the Berlin Neutron Scattering Centre, Germany. Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG) were purchased from Avanti Polar Lipids (Alabama, USA) and used without further purification. Peptide synthesis was carried out by Albachem (Edinburgh, UK) to the following sequences: SP (RPKFQFFGLM-NH₂); NKA (HKTDSFVGLM-NFL). Both peptides were synthesised in an undeuterated form and with leucine replaced by a deuterated analogue which contained a total of ten deuterons. Sample preparation, neutron data collection and data analysis were as previously described [7]. The experimental temperature was 25°C. Each tachykinin was added singly at 10 mol% with respect to the lipid and the water baths contained pure water, at one of three isotopic compositions: 0%, 50% or 100% ²H₂O to assist with phase assignment.

The data were placed on a “relative absolute” scale [8] using the known neutron scattering
Fig. 2. Difference neutron scattering density profiles calculated using six orders of diffraction for SP and five for NKA. The profiles show the distribution of $^2$H$_{10}$-leucine in 10% (mol) SP (b) or NKA (c) in bilayers containing DOPC : DOPG 50 : 50. The exact location, width and peak of each label was calculated by fitting Gaussians to the difference structure factor amplitudes in diffraction space, thereby avoiding the termination error observed in Fourier subtractions. The Fourier subtractions (solid line) fit well with the Gaussians (broken line); the poorer NKA fit is probably due to the lower resolution of diffraction data.
Table 1
Location of deuterium label on (2H_{10}-Leu 10)-substance P and (2H_{10}-Leu 9)-neurokinin A. The centre, position and full-width at half-height (FWHH) of Gaussian distributions, fitted by least-squares refinement in reciprocal space, are given

<table>
<thead>
<tr>
<th>Population</th>
<th>Parameter</th>
<th>Substance P</th>
<th>Distribution</th>
<th>Neurokinin A</th>
<th>Distribution</th>
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<td></td>
<td>9.60 ± 0.97 Å</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Position (distance from centre of bilayer)</td>
<td>22.34 ± 0.83 Å</td>
<td>34.3 ± 5.5%</td>
<td>22.17 ± 0.65 Å</td>
<td>30.5 ± 7.5%</td>
</tr>
<tr>
<td></td>
<td>Width (FWHH)</td>
<td>7.54 ± 0.66 Å</td>
<td></td>
<td>6.73 ± 1.13 Å</td>
<td></td>
</tr>
</tbody>
</table>

densities of 2H_{10}-Leu and 2H_{2}O as previously described [7]. This method requires knowledge of the molar percentage of water in the samples, which was determined as described in Ref. [9].

3. Results and discussion

Fig. 1 displays the neutron scattering density profiles across a DOPC : DOPG bilayer. The interbilayer water compartment is at the outer region of the graph. A pair of phospholipid molecules are also shown, to assist with interpretation of the profile.

Fig. 2 shows difference profiles representing the distribution of deuterium label in bilayers containing SP (b) and in bilayers containing NKA (c). The profiles were calculated by the difference method. There are two distributions of label in each sample, one located close to the water–hydrocarbon interface and another located approximately 8 Å from the bilayer centre. This is summarised in Table 1. These findings are consistent with the two-state model. Membrane insertion of SP and NKA allows water to penetrate deeper into the bilayer (data not shown).

Consistent with the monolayer area measurements of Seelig [3] these neutron data clearly demonstrate bilayer penetration of both tachykinins into anionic lipid bilayers. However, the depth of penetration of the two tachykinins studied is remarkably similar. Schwyzer [10] has proposed that receptor binding and specificity of the tachykinins is a property of the membrane-bound conformation of the peptides. Our evidence suggests that, in terms of membrane location, the membrane bound forms of these two peptides is rather similar, implying that receptor specificity is controlled by finer levels of structural detail.

Acknowledgements

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References

PHYSICA B

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