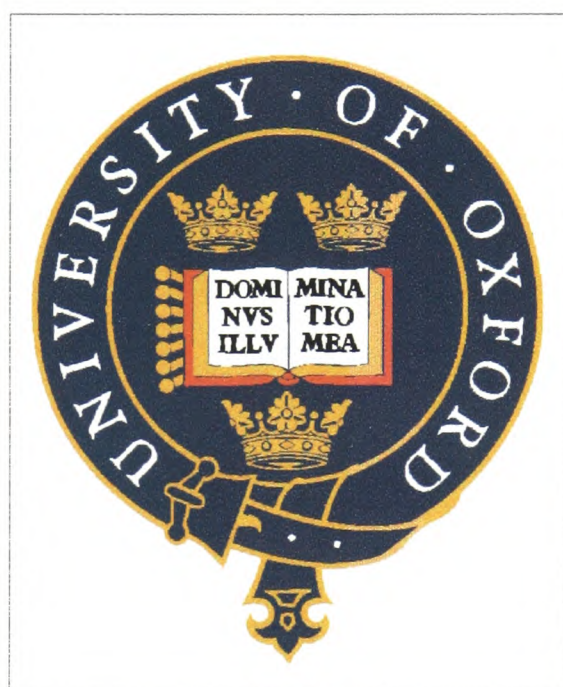


Raman Spectroscopy of Supported Lipid Bilayers and Membrane Proteins

*A thesis submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy in the University of Oxford, by*

Chongsoo Lee



Physical and Theoretical Chemistry Laboratory,
and **University College,**

University of Oxford

Michaelmas 2005



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Off-resonance unenhanced total internal reflection (*TIR*) Raman spectroscopy was explored to investigate supported single lipid bilayers with incorporated membrane peptides/proteins at water/solid interface.

A model membrane was formed on a planar supported lipid layer (*pslb*) by the fusion of the reconstituted small unilamellar vesicles (*SUVs*), and the intensity of bilayer was confirmed by a comparison of Raman spectral intensity in the C-H stretching modes with *C₁₆TAB*. With prominent Raman sensitivity attained, we studied the 2-*D* phase transition of *DMPC* and *DPPC* *pslbs* and the temperature-dependent polarised spectra revealed a broad transition range of ca. 10 °C commencing at the calorimetric phase transition temperature.

We applied polarised *TIR*-Raman spectroscopy to *pslbs* formed by *DMPC SUVs* reconstituted with a model membrane-spanning peptide gramicidin D. A preferential channel structure formed by dissolution of trifluoroethanol could be probed by polarised Raman spectroscopy qualitatively showing an antiparallel β -sheet conformation (different from “standard” one) and our Raman spectra by correlation with *NMR* and *CD* data confirmed single-stranded $\pi^{6.3}$ β -helical channel structure in the single bilayer. We also studied the membrane-penetrating peptide indolicidin in the presence of *DMPC* *pslb* over the chain melting temperature and a β -turn structure was dominantly observed concomitant with membrane perturbation.

Dynamic adsorption of *DPPC* to form *pslb* from a micellar solution of *n*-dodecyl- β -D-maltoside could be examined with high sensitivity of every 1-*min* acquisition. Finally we used polarised *TIR*-Raman scattering to porcine pancreatic phospholipase A₂ hydrolytic activity on *DPPC* *pslbs* and revealed lipid-active conformation different from that of the enzyme alone.

for L. S. P. and J. H. L.

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Table of Contents

CHAPTER 1: INTRODUCTION 1

1.1 Aims and Objectives 1

1.2 Structure of Thesis 2

**CHAPTER 2: INTRODUCTION TO PLANAR SUPPORTED LIPID BILAYERS
..... 4**

**CHAPTER 3: BASICS AND BACKGROUNDS OF TOTAL INTERNAL
REFLECTION (*TIR*)-RAMAN SPECTROSCOPY WITH POLARISATION 11**

3.1 Raman Scattering 11

3.2 Light at Interfaces 15

 3.2.1 Refraction and Total Internal Reflection..... 15

 3.2.2 Fresnel’s Equations 18

3.3 Unenhanced Raman Scattering from Thin Films 23

 3.3.1 Literature Background25

3.4 Comparison with *IR* Absorption Spectroscopy 27

**CHAPTER 4: INTRODUCTION TO MODEL MEMBRANE PEPTIDES AND
PRTEINS 29**

4.1 Gramicidin.....29

 4.1.1 Conformations in Solution30

 4.1.2 Antiparallel $\beta^{6.3}$ helical dimer31

4.2 Indolicidin 38

4.3 Phospholipase A₂ (*PLA*₂)41

CHAPTER 5: MATERIALS AND METHODS50

5.1 Materials50

5.2 Preparation of Small Unilamellar Vesicles (SUVs).....	52
5.3 Incorporation of Gramicidin into Phospholipid SUVs	53
5.4 UV/Vis Absorption, Fluorescence, and Circular Dichroism Spectroscopy	54
5.5 Atomic Force Microscopy (AFM) (For Chapter 7.1)	55
5.6 Cleaning and Assembly Procedures	55
5.7 Formation of Planar Supported Lipid Bilayers	57
5.8 Raman Spectrometer	59
5.9 Measurement of Phase Transitions in Bulk Suspensions (For Chapter 8.5)	65
5.10 Calibration of Laser Intensity	65
5.11 Sample Heating and Damage (For Chapter 8.1)	66
5.12 Preparation of Peptide/Protein Solutions	67
5.13 Procedures for Lipid-Surfactants Mixtures (For Chapter 10 and 11)	67
5.14 Preparation of d_{75}-DPPC Vesicle Solution	68
CHAPTER 6: RECONSTITUTED VESICLES IN SOLUTION	70
6.1 Phospholipid Vesicles	70
6.2 Gramicidin D-incorporated phospholipid vesicles	72
6.2.1 Aspects of the Physical Chemistry	73
6.2.2 Aspects of Biophysics	78
6.3 Discussion	86
6.4 Conclusion	87
CHAPTER 7: PLANAR SUPPORTED LIPID BILAYERS AT WATER/SOLID INTERFACE	88
7.1 Characterisation by Atomic Force Microscopy	88

7.2 Characterisation by <i>TIR</i>-Raman Scattering	93
7.2.1 Difference Spectra	93
7.2.2 Time-dependent <i>TIR</i> -Raman Spectra of Fusion Kinetics	94
7.2.3 Unilamellarity of Supported Phospholipid Bilayers	97
7.2.4 Polarised <i>TIR</i> -Raman Spectra <i>DMPC</i> and <i>POPC pslbs</i>	99
7.3 Conclusion	105
 CHAPTER 8: PHASE TRANSITIONS IN PLANAR SUPPORTED LIPID BILAYERS BY POLARISED <i>TIR</i>-RAMAN MICROSPECTROSCOPY	 106
8.1 Local Heating by Laser	108
8.2 Temp-dependent Polarised <i>TIR</i> -Raman Spectra of <i>DMPC pslb</i>	111
8.3 Temp-dependent Polarised <i>TIR</i> -Raman Spectra of <i>DPPC pslb</i>	117
8.4 Temp-dependent Polarised <i>TIR</i> -Raman Spectra of <i>POPC pslb</i>	120
8.5 Phase Transition Behavior of <i>DMPC</i> , <i>DPPC</i> , and <i>POPC pslbs</i>	122
8.6 Discussion	128
8.7 Conclusion	131
 CHAPTER 9: <i>TIR</i>-RAMAN SPECTROSCOPY OF PEPTIDES INTERACTING WITH PLANAR SUPPORTED LIPID BILAYERS	 133
9.1 Gramicidin D	135
9.1.1 Raman Scattering of Gramicidin D in a Cast Film	135
9.1.2 Gramicidin D with <i>POPC</i> in a Cast Multistack Film	139
9.1.3 Photodamage in Mixed Gramicidin D/Lipid <i>pslbs</i>	148
9.1.4 Gramicidin D in a Supported <i>DMPC</i> Bilayer at Water/Silica Interface	152
9.1.4.1 Manipulation of Spectra	155
9.1.4.2 Curve-fitting of Polarised <i>TIR</i> -Raman Spectra of Gramicidin D in <i>DMPC pslb</i>	163
9.1.5 Discussion of Gramicidin D in <i>DMPC pslb</i>	171
9.2 Interaction of Indolicidin with a <i>pslb</i>	179
9.3 Conclusion	198

9.3.1 Gramicidin D in <i>DMPC pslb</i>	198
9.3.2 Indolicidin-associating <i>DMPC pslb</i>	199
CHAPTER 10: FORMATION OF SUPPORTED LIPID BILAYERS FROM MICELLAR SOLUTION n-DODECYL-β-D-MALTOSE AND <i>DP</i>PC	200
10.1 Co-adsorption of <i>h-DPPC</i> and <i>h-DDM</i>	202
10.2 Co-adsorption of <i>d₇₅-DP</i> PC and <i>h-DDM</i>	206
10.3 Conclusion	219
CHAPTER 11: HYDROLYSIS OF A DPPC PLANAR SUPPORTED LIPID BILAYER BY PHOSPHOLIPASE A₂	221
11.1 Hydrolysis of a <i>DP</i> PC <i>pslb</i> by <i>PLA₂</i> : Lipid Spectra	221
11.2 Hydrolysis of a <i>DP</i> PC <i>pslb</i> by <i>PLA₂</i> : Protein Spectra	229
11.3 Conclusion	236
CHAPTER 12: CONCLUSION	237
REFERENCES	241

List of Abbreviation

AFM	Atomic Force Microscopy
ATR-FTIR	Attenuated Total Reflection Fourier Transform Infrared spectroscopy
CCD	Charge-Coupled Device
CD	Circular Dichroism
C₁₆TAB	Hexadecyltrimethylammonium bromide
<i>d</i>₇₅-DPPC	Deuterated (⁷⁵ C-D's)-1,2-Dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine
DDM	<i>n</i> -Dodecyl- β -D-maltoside
DiOC₁₈	3,3'-Diocadecyloxacarbocyanine perchlorate
DMPC	1,2-Dimyristoyl- <i>sn</i> -glycero-3-phosphocholine
DPPC	1,2-Dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine
DSC	Differential Scanning Calorimetry
FRAP	Fluorescence Recovery After Pattern photobleaching
gD	Gramidicin D
<i>h</i>-DDM	hydrogenated-DDM
<i>i</i>-face	interfacial recognition region of <i>PLA</i> ₂
L_{α}	Liquid crystalline phase
L_{β}'	Gel phase (tilted chains)
LB	Langmuir-Blodgett
NMR	Nuclear Magnetic Resonance
NR	Neutron Reflectivity
P_{β}'	Ripple phase
PLA₂	Phospholipase A ₂
<i>pol</i>	polarised
POPC	1-Palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
pslb	Planar Supported Lipid Bilayer
PTFE	Polytrifluoroethylene
SDS	Sodium Dodecyl Sulfate
SERS	Surface Enhanced Raman Scattering
SUV	Small Unilamellar Vesicle
TFE	Trifluoroethanol
THF	Tetrahydrofuran
TIR	Total Internal Reflection

Trp	Tryptophan
UHQ DW	Ultrahigh Quality Deionised Water
UV/Vis	Ultraviolet/Visible

Chapter 1 Introduction

1.1 Aims and Objectives

The *aim* of this thesis is to develop the technique of total internal reflection (*TIR*)-Raman spectroscopy for the study of planar supported lipid bilayers (*pslbs*) in an aqueous environment.

The specific *objectives* of this work are summarized below.

1. To build a Raman spectrometer for unenhanced (off-resonance) Raman scattering from *pslbs* and to optimise its performance.
2. To use this spectrometer to investigate structural changes in pure lipid bilayers as a function of temperature, making use of the polarisation properties of Raman scattering.
3. To demonstrate that *TIR*-Raman scattering can detect small membrane peptides either embedded in the bilayer (exemplified by the model channel-forming peptide gramicidin) or interacting with the layer (exemplified by the soluble antimicrobial peptide indolicidin).
4. To assess the information that can be obtained on the secondary structure of membrane peptides/proteins by *TIR*-Raman scattering on single *pslbs*.
5. To show that *TIR*-Raman scattering can be used to follow the kinetics of membrane processes, using as exemplars the formation of *DPPC* bilayers from mixed micelles with the surfactant dodecyl maltoside, and the reaction of a *DPPC* bilayer with the enzyme phospholipase A₂.

1.2 Structure of thesis

The aim of my *D.Phil.* project was to develop Raman spectroscopy as a technique for understanding the structure of membranes and membrane peptides/proteins in an aqueous environment. Initially, I investigated the sensitivity of Raman scattering to the structure of single phospholipid bilayers at water/solid interfaces. The next step was to study model membrane peptides/proteins and assess the structural information that can be derived from unenhanced Raman scattering. This thesis focuses on the experimental results, rather than theoretical modelling of spectra. During the data analysis of the Raman spectra of membrane peptides/proteins, it became clear that the poor database of Raman reference data on peptides with different (known) secondary structures was a limitation in the interpretation of the spectra from gramicidin and indolicidin.

This thesis consists of two parts. The first part provides an introduction to planar supported lipid bilayers (*pslbs*) (Chapter 2). I explain why I am interested in a supported single lipid bilayer as a model membrane and discuss previous work on phase transitions in supported lipid bilayers. Chapter 3 introduces the primary experimental technique of this thesis: *TIR*-Raman scattering. The theory part of this chapter is relatively brief since there are numerous textbooks on the theory of the Raman effect. I have limited myself to the theory necessary to understand the Raman data shown in the results sections. Chapter 4 gives background to the structure and function of the membrane peptides and protein described later in the thesis.

The second part of the thesis concerns the results from my experimental studies. Maintaining a strongly developmental approach throughout the project, I typically

optimised techniques with well-defined interfacial systems such as phospholipid bilayers, and then applied these techniques to new, more challenging systems of phospholipid bilayers interacting with the membrane-channel peptide gramicidin and the membrane-penetrating peptide indolicidin. Chapter 6 describes the preparation and characterisation of reconstituted small unilamellar vesicles (with/without the membrane-spanning peptide gramicidin) in solution prior to formation of planar supported lipid bilayers. Chapter 7 describes the development of *TIR*-Raman scattering from planar supported lipid bilayers at the silica/water interface. Chapter 8 contains a study of the temperature dependence of *pslbs* of three lipids: *DMPC*, *DPPC* and *POPC*, which has never been attempted for temperature-dependent conformational/orientational changes of supported lipid bilayers through thermotropic phase transitions using the Raman method. Chapter 9 moves on to the question of the detection of small peptides either incorporated into the lipid bilayer (gramicidin) or interacting with the lipid bilayer from solution (indolicidin). In chapter 10, we use Raman scattering to follow the process of formation of a *pslb* by an alternative route, namely from mixed micelles of a lipid and surfactant in solution. Chapter 11 contains a preliminary study on the interaction of a *DPPC* bilayer with the enzyme *PLA₂*.

To conclude, Chapter 12 assesses the progress made in my D.Phil. and suggests briefly directions for future research.

Chapter 2 Introduction to Planar Supported Lipid Bilayers

Biological membranes play key roles in the life of cells, acting as permeability barriers and privileged sites of communication between the inside and outside of the cellular world. These highly complex and dynamic assemblies, only a few nanometers thick, consist of two main components: a two-dimensional space made up of lipid molecules held together by hydrophobic interactions and self-assembled as a continuous bilayer, and proteins embedded within the membrane or attached to it.

Our current knowledge of the molecular processes occurring at biological membranes is mainly based on studies performed on models of biological membranes, including liposomes and giant vesicles in solution, lipid monolayers at the air water-interface, black lipid films, membrane patches at pipettes, or solid-supported membranes.

Among them, supported lipid bilayers have been widely used as models for cellular membranes since the pioneering studies of Tamm and McConnell (Tamm & McConnell, 1985). There is a growing interest in the development of biofunctionalised surfaces in biotechnological applications such as molecular patterning of receptors for antigen-antibody recognition (Dustin, *et al*, 1998; Groves & Boxer, 2002; Sackmann, 1996) and in biosensors based on electrical and optical detection of embedded ion channels (Anrather, *et al*, 2004). A multitude of surface-sensitive techniques have emerged for the study of bilayer lipid membranes, based on a variety of optical and mechanical sensing principles: fluorescence microscopy, Brewster angle microscopy, atomic force microscopy, surface plasmon resonance, ellipsometry and the quartz crystal microbalance, all of which enable the physico-chemical or structural characterization of bilayers in an aqueous environment.

Single lipid bilayers supported on a solid substrate are produced by two principal methods: Langmuir-Blodgett (*LB*) deposition (in which preformed monolayers of lipid on the surface of water are transferred to the support by dipping) or spontaneous fusion of liposomes. Figure 2.1 shows these two different methods. One issue with supported lipid bilayers is that large integral membrane proteins may be pinned to the substrate by the domains that protrude from the inner leaflet of the bilayer and interact with the hydrophilic substrate, thereby inhibiting their lateral mobility (Hinterdorfer, *et al*, 1994; Poglitsch, *et al*, 1991; Salafsky, *et al*, 1996). Figure 2.1 (C) shows one method of circumventing, or at least limiting, the problem of reduced mobility by first depositing a polymer-cushion before the *LB* transfer of the lipid bilayer (Wagner & Tamm, 2000; Wong, *et al*, 1999). Single lipid bilayers on a solid hydrophilic substrate are believed to be separated from the substrate by a thin film of water (Johnson, *et al*, 1991; Tamm & McConnell, 1985) and can therefore provide a good environmental mimic for the small peptides such as gramicidin and indolicidin studied in my thesis.

The mobile lipid molecules in the 2-*D* space of supported lipid bilayers can accommodate any membrane peptide/proteins (either membrane-spanning or membrane-associating) by reconstitution in a liposomal state. The chemical composition of the lipids exerts a profound effect on the physical properties of this 2-*D* membrane. For example, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (*POPC*) has a chain melting temperature, $T_m \sim -2.5^\circ\text{C}$ while for the saturated phospholipid dipalmitoyl-*sn*-glycero-3-phosphocholine (*DPPC*), $T_m \sim 36^\circ\text{C}$ (Koynova & Caffrey, 1998). *POPC/DPPC* mixtures in the liquid crystalline state are intermediate in order between pure *POPC* and *DPPC* at the same temperature in vesicle solution (Curatolo, *et al*, 1985). Since membrane peptides generally function in a fluid membrane environment, the question of how the phase behaviour of a *pslb* mimics that of a free membrane is an important issue that will be addressed later in this thesis.

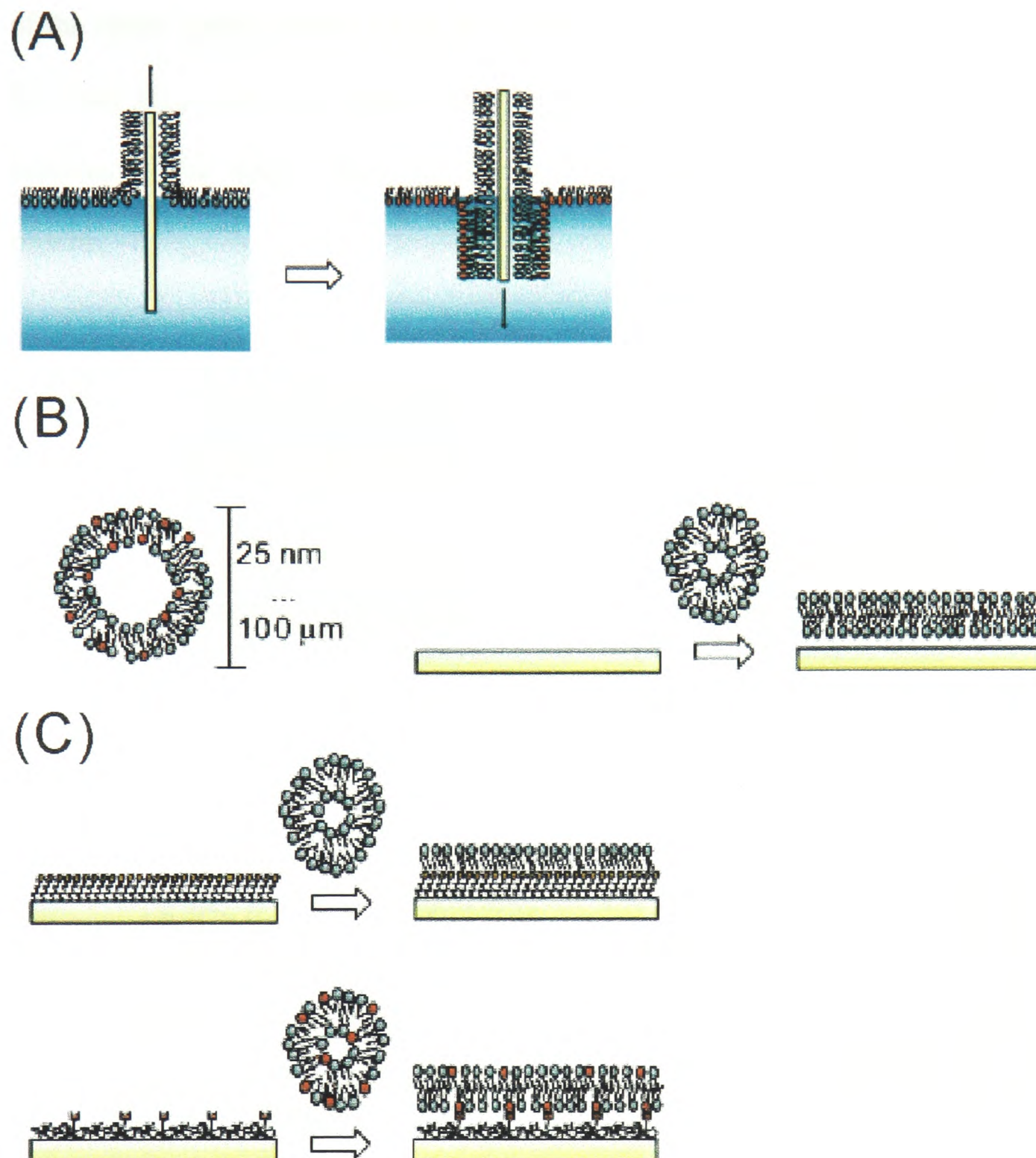


Figure 2.1 Preparation of supported single lipid bilayers: (A) *LB* method, (B) direct fusion of liposomes, (C) spontaneous spreading of liposomes on solid substrate pre-treated with a hydrophobic monolayer such as alkanethiols on Au or alkyl silanes on glass or silica (upper) or by deposition of polymer-cushion with functionalized tethers (lower). (From Richter, *et al*, 2003.).

Figure 2.2 shows schematically the main lamellar phases displayed by bulk

lipids. The so-called ‘main transition’ is a gel/fluid transition attributed to the melting of the lipids' carbon chains (A or B $\rightarrow$ D). This transition is of great interest, since, in the fluid (L_α) state, the supported lipids provide lateral fluidity in the plane of the substrate. The ripple phase (C) is sometimes observed between the gel and fluid phases.

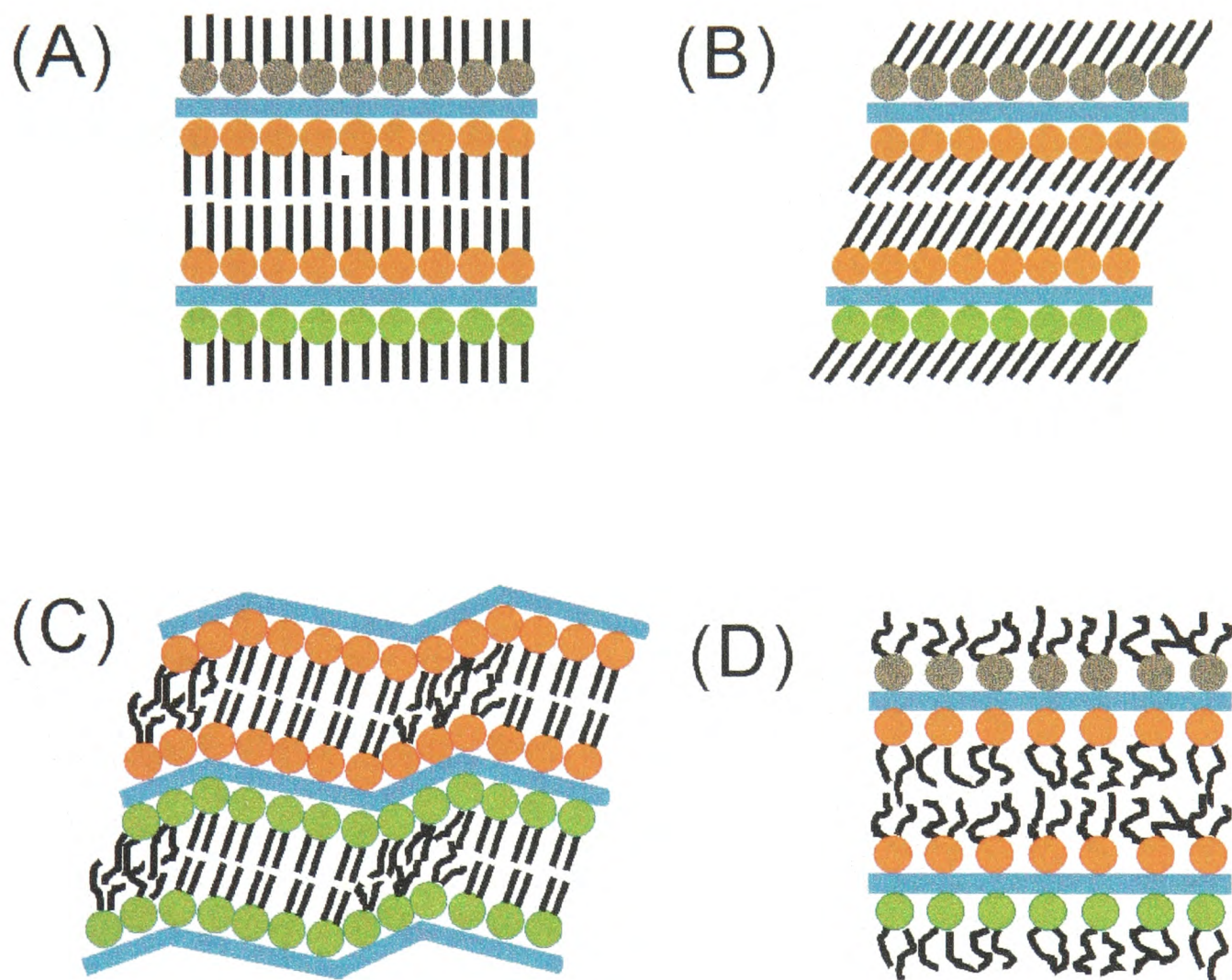


Figure 2.2 Schematic diagrams of various lamellar phases: A. gel phase (untitled chains), L_β ; B. gel phase (tilted chains), L_β ; C. ripple phase, P_β ; D. liquid crystalline phase, L_α .

There have been a few studies on phase transitions in single supported lipid bilayers by differential scanning calorimetry (DSC) (Yang & Appleyard, 2000) and by direct visualization by atomic force microscopy (AFM) (Charrier & Thibaudau, 2005;

Feng, *et al*, 2002; Tokumasu, *et al*, 2003). The supported lipid layer in Yang's work was prepared by chopping fresh mica into microscopic chips (a few microns) and blending and suspending the chips into the vesicle solution. In contrast to the *DSC* results showing one main phase transition at 36 °C for *DPPC* vesicles, their supported *DPPC* layer exhibited two phase transitions separated by 2 °C, the lower of which is 2 °C higher than the $L_{\alpha} \rightarrow L_{\beta'}$ transition temperature of lamellar vesicles in bulk solution. There has been one report of the use of attenuated total reflection Fourier transform infrared spectroscopy (*ATR-FTIR*) (Brumm, *et al*, 1996), but no prior studies by Raman spectroscopy.

The understanding of main phase transition of single-component lipid bilayers supported on mica (such as 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, *DMPC*) has been controversial. Tokumasu *et al* and Xie *et al* have studied the gel/fluid phase transition of single bilayers of *DMPC* on mica by *in-situ AFM* measurement. In contrast to a free-standing bilayer for which the main transition is sharp (the width of the transition is < 1 °C (Heimburg, 1998)) and occurs at 23.7 °C (Needham & Evans, 1988), they show that the transition in the supported bilayer is much broader (8 °C) and shifted to ~28 °C (Tokumasu, 2003). Tokumasu *et al* explain this large transition width through a finite-size-limited first-order transition model in which the diameter of intrinsic domains is 4.2 nm, whereas Xie *et al* interpret this behavior in the framework of a classic van't Hoff transition. Charrier & Thibaudau concluded, however, that the independent melting of each leaflet of the bilayer and the large broadening of the transition could be interpreted through a basic thermodynamic framework in which the surface tension varies during the transitions. In Chapter 7, I present the first temperature-dependent study of the structure of the acyl chains by *TIR-Raman* spectroscopy.

There is a rich variety of different classes of phospholipids, some of which are

shown in Figure 2.3. To permit fusion onto the negatively charged surface of silica, I limited to zwitterionic lipids. I chose to use saturated and unsaturated phosphatidylcholines (Figure 2.3A).

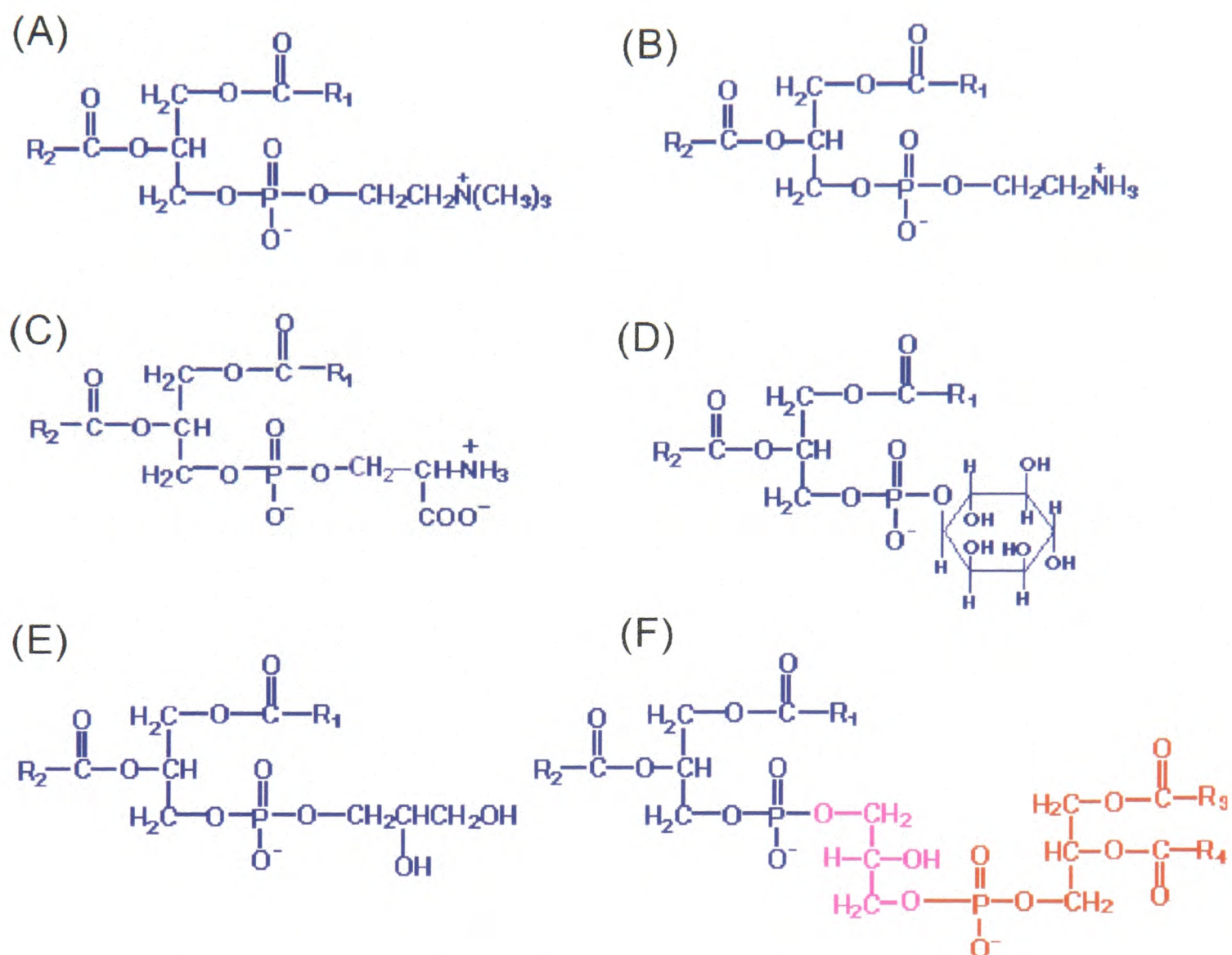


Figure 2.3 Major classifications of phospholipids: (A) phosphatidylcholine, (B) phosphatidylethanolamine, (C) phosphatidylserine, (D) phosphatidylinositol, (E) phosphatidylglycerol, (F) diphosphatidylglycerol.

Phosphatidylcholines (*PC*) have been widely used in model membrane studies. It is the most abundant lipid class in mammalian membranes and a major membrane component in eukaryotic organisms. It is a crucial element in metabolism, such that *PC* is the major delivery form of the essential nutrient choline. *PC*'s role in the maintenance of cell-membrane integrity is vital to all of the basic biological processes. *PC*,

particularly *PC* rich in polyunsaturated fatty acids, has a marked fluidizing effect on cellular membranes. Decreased cell-membrane fluidization and breakdown of cell-membrane integrity, as well as impairment of cell-membrane repair mechanisms, are associated with a number of disorders, including liver disease, neurological diseases, various cancers and cell death. *PC* is absorbed into the mucosal cells of the small intestine, mainly in the duodenum and upper jejunum, following some digestion by the pancreatic enzyme phospholipase A_2 (see Chapter 11), producing lysophosphatidylcholine (lysolecithin). Reacylation of lysolecithin takes place in the intestinal mucosal cells, reforming *PC*, which is then transported by the lymphatics in the form of chylomicrons to the blood. *PC* is transported in the blood in various lipoprotein particles, including very-low-density lipoproteins, low-density lipoproteins, and high-density lipoproteins; it is then distributed to the various tissues of the body. Some *PC* is incorporated into cell membranes.

Chapter 3 Total internal reflection (TIR)-Raman spectroscopy

3.1 Raman scattering

When light is incident on a dust-free, transparent, optically perfect medium most of it is transmitted. However, a very small fraction of the light is scattered - almost all of this scattered light has the same frequency as the incident light, but a tiny portion of it is found to be shifted to higher or to lower frequencies. Furthermore, the frequency difference between the incident and scattered radiation corresponds to the separation of energy levels within the scattering system.

The phenomenon of light scattering with a concomitant change in frequency is known as the Raman effect. It was predicted theoretically in 1923 by Smekal (Smekal, 1923), and first observed in liquids in 1928 by the eponymous Chandrasekhra Venkata Raman & K.S. Krishan (Raman & Krishan, 1928). The first observation of the Raman effect was quickly followed by reports of Raman scattering in many other systems. By 1934, G. Placzek (Placzek, 1934) had predicted almost all of the phenomena now observed.

Simply speaking, Raman spectroscopic experiments require the illumination of a sample with monochromatic light, and the observation of scattered light of altered frequency. The origin of Raman spectra is markedly different from that of infrared (IR) absorption or reflection spectra. The frequencies at which Raman scattering occurs can be described classically as beat frequencies arising from the interaction of induced dipoles with incident radiation.

Classically the electric field strength, E of monochromatic light fluctuates with time, t , thus:

$$E = E_0 \cos(\omega_0 t), \quad (3.1)$$

where E_0 is the amplitude and ω_0 is the frequency of the light. The electric dipole moment, μ , induced in a molecule by irradiation with this light is then

$$\mu = \alpha E = \alpha E_0 \cos(\omega_0 t). \quad (3.2)$$

If the molecule is vibrating harmonically with a frequency ω_m the nuclear displacement, q , is given by

$$q = q_0 \cos(\omega_m t + \phi), \quad (3.3)$$

where q_0 is the vibrational amplitude and ϕ is the phase of the vibration. For small vibrations (small q), α is a linear function of q , and can be expanded as a Taylor series (we invoke the electrical harmonic approximation, so neglect higher powers of q). That is,

$$\alpha = \alpha_0 + \alpha' q + \dots, \quad (3.4)$$

where α_0 is the polarisability at the equilibrium position and $\alpha' = \left(\frac{\partial \alpha}{\partial q} \right)_0$.

Equations (3.2) and (3.4) combine to give

$$\mu = (\alpha_0 + \alpha' q + \dots) E_0 \cos(\omega_0 t) \quad (3.5)$$

$$\mu = \alpha_0 E_0 \cos(\omega_0 t) + \alpha' q E_0 \cos(\omega_0 t) + \dots \quad (3.6)$$

and use of Equation (3.3) to substitute for q then yields

$$\mu = \alpha_0 E_0 \cos(\omega_0 t) + \alpha' q_0 E_0 \cos(\omega_0 t) \cdot \cos(\omega_m t + \phi) + \dots \quad (3.7)$$

and a trigonometric identity tells us that therefore

$$\mu = \alpha_0 E_0 \cos(\omega_0 t) + \frac{1}{2} \alpha' q_0 E_0 \left[\cos\{(\omega_0 + \omega_m)t + \phi\} + \cos\{(\omega_0 - \omega_m)t - \phi\} \right] + \dots \quad (3.8)$$

We can see that the first term in Equation (3.8) represents oscillations of the induced dipole at a frequency of ω_0 and the second term corresponds to induced dipole oscillations at frequencies of $\omega_0 - \omega_m$ and $\omega_0 + \omega_m$. Light is radiated at these frequencies. The essential features of elastic scattering of light (*i.e.* without frequency/energy change) were described in Lord Rayleigh's classical radiation theory of 1871, and the phenomenon bears his name. The inelastically scattered light comprises

two parts; the Stokes shifted Raman scattered light at $\omega_0 - \omega_m$, which occurs with excitation of the molecular vibration mode, and the anti-Stokes shifted Raman scattering band at $\omega_0 + \omega_m$, which reduces the energy of the molecular vibration. Anti-Stokes Raman scattering requires the molecule initially to be in an excited state, and, since the population of vibrational states follows a Boltzmann distribution, anti-Stokes bands are very much weaker than Stokes bands. For this reason, most Raman experiments analyse only the Stokes-shifted light, although there are cases when the anti-Stokes part of the spectrum is useful (*e.g.* to measure temperature).

In general, an assembly of molecules does not vibrate in-phase (*i.e.* ϕ varies between 0 and 2π randomly) and so light scattered by different molecules is incoherent and does not exhibit interference effects. Therefore Raman scattered light does not have a single, well-defined direction. Furthermore, the intensity of scattering is linearly proportional to the number of scatterers.

Looking again at Equation (3.8), we can see that no Raman scattering occurs if $\left(\partial\alpha/\partial q\right)_0 = 0$. Therefore, for a vibration to be Raman-active, the polarisability must change with vibration around the equilibrium nuclear displacements.

Whether or not α varies in time and/or with vibration, it is always non-zero, so we can always expect Rayleigh scattering to occur. Much of the early difficulty in observing Raman scattering arose from the fact that it is always accompanied by Rayleigh scattering, which is typically stronger by about 10^2 to 10^4 times. For this reason Raman scattering is perceived as a weak effect. In fact, it is easy to induce detectable Raman effects from ultrathin films and small sample volumes – the main difficulty involves suppression of other signals (arising from bulk phases, and Rayleigh scattering), which can wholly or partially mask the Raman spectrum.

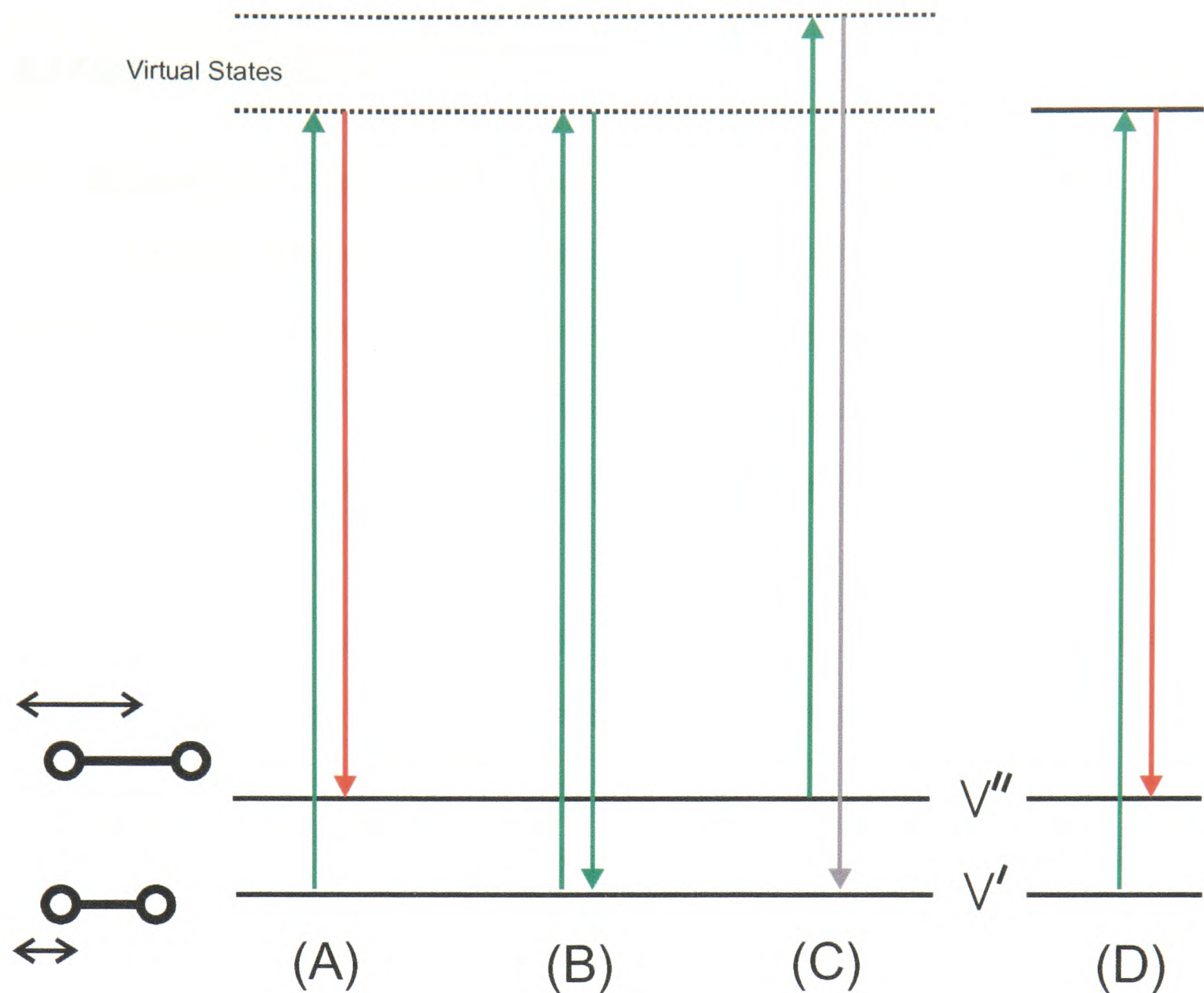


Figure 3.1 Schematic diagram of transitions during Raman spectroscopy: (A) Stokes scattering red-shifts the incident light, (B) Rayleigh scattering occurs elastically and (C) anti-Stokes scattering blue-shifts the incident light. Raman scattering occurs with resonance enhancement (D) when the pump beam is resonant with an electronic excitation. The ground and first excited vibrational states are labelled v' and v'' respectively. Transition to the 'virtual states' is schematic only: quantum mechanical methods relate the scattering to the sum over all possible states in the system, but there is no transition to an intermediate state. See pp 115-116 in the reference (Long, 1977).

3.2 Light at Interfaces

3.2.1 Refraction and total internal reflection

Consider light incident through a medium of refractive index n_i onto an interface with a medium of refractive index n_t , at an angle θ_i to the surface normal (see Figure 3.2). Some of the light will be reflected at an angle θ_r , and some will be transmitted at an angle θ_t in general (*i.e.* neglecting the special case when θ_i exceeds a critical angle).

The Law of Refraction tells us that the incident, reflected and transmitted beams all lie in the same plane. Furthermore, the angles are related by

$$\theta_i = \theta_r, \quad (3.9)$$

$$n_i \sin \theta_i = n_t \sin \theta_t. \quad (3.10)$$

Equation (3.10) was first discovered empirically by Willebrord van Roijen Snell in 1621, and it has come to be known as Snell's Law. The physical basis of Snell's Law is conservation of photon momentum parallel to the surface. Snell's law describes specular reflection; diffuse reflection (when $\theta_i \neq \theta_r$) is relatively weak from smooth surfaces.

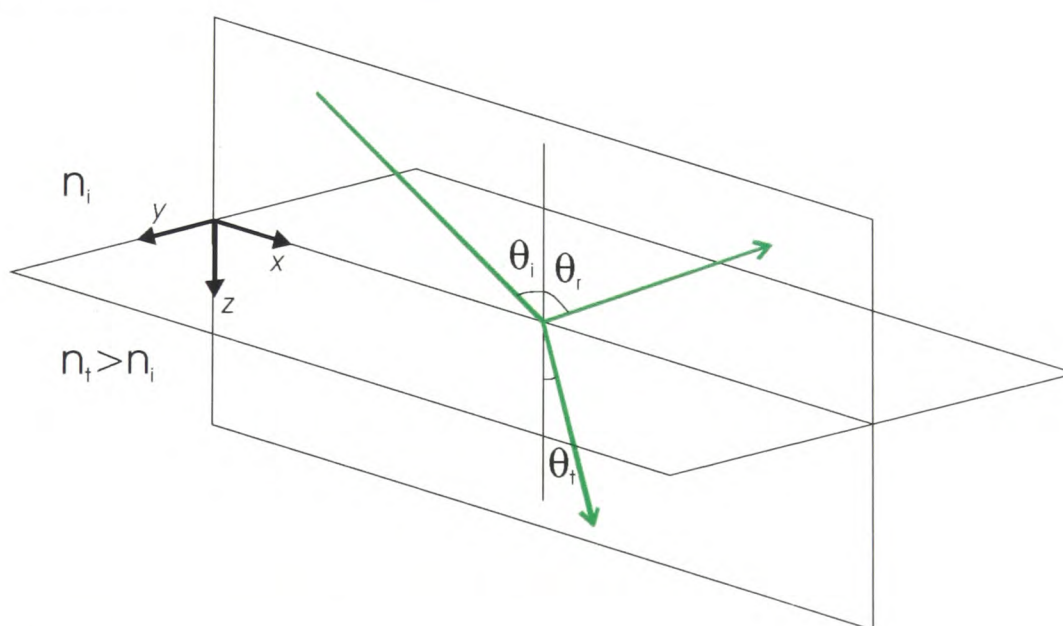


Figure 3.2 Ray tracing diagram illustrating reflection and refraction geometry governed by Snell's Law.

From Equation (3.10), a beam incident on an interface is bent towards the surface normal as it passes from the optically less-dense medium (*i.e.* that having lower refractive index) to the optically more dense. The reflected portion of the beam is said to undergo external reflection. Conversely, a beam passing from the denser medium is bent away from the surface normal, while the remainder of the incident beam is internally reflected. In this case, there exists a critical angle of incidence, θ_c , which produces an angle of transmission of 90° *i.e.* the transmitted beam is tangential to the surface. The critical angle is given by Snell's Law as:

$$\theta_c = \sin^{-1} \left(\frac{n_t}{n_i} \right). \quad (3.11)$$

At incident angles greater than θ_c , all of the light is reflected back into the optically dense medium, *i.e.* there is total internal reflection (*TIR*), and none of the incident light is transmitted into the optically rarer medium. Nevertheless, there must be an electromagnetic field on both sides of the interface in order to satisfy the boundary condition on the continuity of the electric field parallel to the surface. However, the transmitted wave does not, on average, transfer any energy across the interface. In fact, the wave propagates along the surface in the plane of incidence, and its electric field strength exponentially fades with distance away from the interface – it is known as an evanescent ('quickly fading') wave. The penetration depth, d_p , of the electric field into the optically rarer medium is given by (Born, *et al*, 1999)

$$d_p = \frac{1}{\beta} = \frac{\lambda}{2\pi n_i} \left(\frac{\sin^2 \theta_i}{n_{ii}^2} - 1 \right)^{-\frac{1}{2}}, \quad (3.12)$$

where β is the exponential decay length, λ is the wavelength of incident light, and $n_{ii} = n_t/n_i$ is the ratio of refractive indices of the two media. The d_p is typically of the order of λ , and the electric field becomes negligible at a distance of only a few

wavelengths into the second medium. The evanescent wave is inhomogeneous: the wavefronts (surfaces of constant phase) are perpendicular to the surfaces of constant amplitude. Note that the penetration depth for the square of the electric field (the relevant quantity for Raman scattering) is $\frac{1}{2\beta}$, as defined above. The evanescent electric field can be used to investigate material lying close to an interface, by observation of either absorption or scattering phenomena. Since d_p varies with λ , we can reduce the depth of illumination of the second medium by choosing probe light with shorter wavelengths. The dependence of d_p on θ_i tells us that in principle, we can depth profile the sample close to the interface simply by changing the incident angle. Additionally, when $\theta_i = \theta_c$, the penetration depth is infinite. In practice, here ‘infinite’ means macroscopic, and the electric field will be limited by the electric field profile of the incident beam. The value of d_p will determine the strength of the water signal in TIR-Raman spectra of lipid bilayers at the silica-water interface. To minimize d_p , one maximises θ_i , but this has deleterious effects on the strength of the Raman signal from the lipid bilayer, as described in the next section.

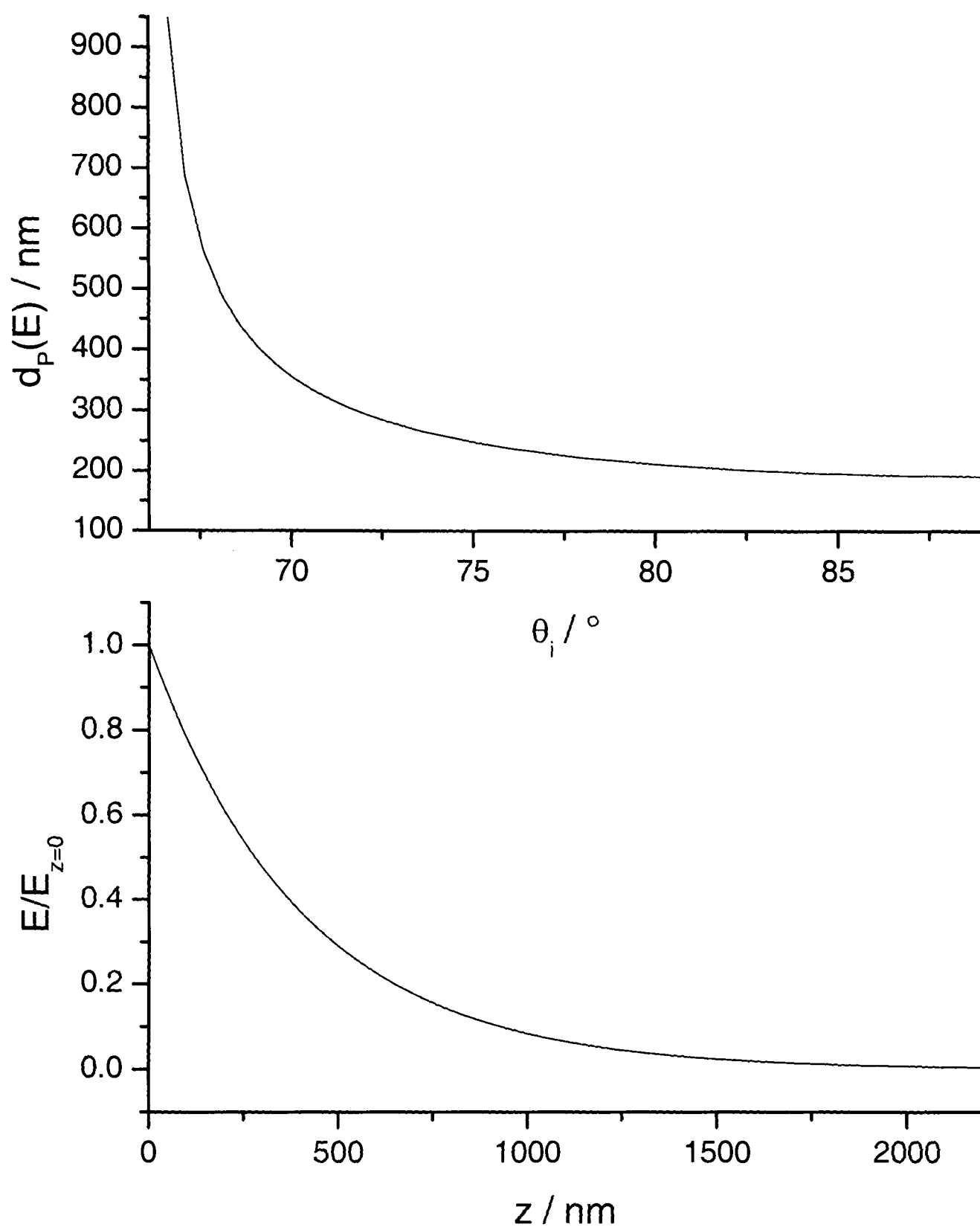


Figure 3.3 Graphs showing (top) the penetration depth of the electric field, $d_p(E)$, against incident angle θ_i , and (bottom) the decay of electric field strength, E , with distance, z , from the interface. The graphs relate to a silica–water interface with 532-nm light and $\theta_c = 66.1^\circ$. For the lower graph, $\theta_i = 69.1^\circ$.

3.2.2 Fresnel's equations

In 1823 Augustine Fresnel considered light incident on the interface between

two homogeneous and isotropic media, and derived equations that relate the amplitudes of the electric vectors of the transmitted and reflected waves to the amplitude of the incident electric field. The proportion of incident energy transmitted and that reflected depends on the incident angle, the ratio of refractive indices across the interface, and the polarisation of the incident light.

Fresnel's equations can be derived from the continuity of E_x , E_y and ϵE_z across a sharp interface between two homogeneous and isotropic bulk media, which have zero conductivity and are therefore perfectly transparent. Light with the electric vector in the plane of incidence (p -polarised light) behaves independently of light with the electric vector perpendicular to the plane of incidence (s -polarised light). The amplitude reflectivity for s -polarised light, r_s , and p -polarised light, r_p , are given by the Fresnel equations (3.13) and (3.14) (Born, *et al*, 1999; Hecht, 1998):

$$r_s = \frac{n_i \cos \theta_i - n_t \cos \theta_t}{n_i \cos \theta_i + n_t \cos \theta_t} = -\frac{\sin(\theta_i - \theta_t)}{\sin(\theta_i + \theta_t)}, \quad (3.13)$$

and

$$r_p = \frac{n_t \cos \theta_i - n_i \cos \theta_t}{n_i \cos \theta_i + n_t \cos \theta_t} = \frac{\tan(\theta_i - \theta_t)}{\tan(\theta_i + \theta_t)}. \quad (3.14)$$

For angles of incidence above the critical angle, θ_t is a complex quantity defined by Snell's law (Equation 3.10). $\cos \theta_t$ is then defined by the identity

$$\cos^2 \theta_t + \sin^2 \theta_t = 1 \quad (3.15)$$

For $\theta_i > \theta_c$, the magnitude of r_p and r_s has to be unity (since there is no transmitted beam), but the phase is not zero and in fact varies with the angle of incidence.

The components of the electric field at the interface are

$$E_x = -(E_p - r_p E_p) \cos \theta_i, \quad (3.16)$$

$$E_y = E_s + r_s E_s \quad (3.17)$$

$$\text{and } E_z = (E_p + r_p E_p), \quad (3.18)$$

where r_p and r_p are the Fresnel amplitude reflection coefficients given above.

Parallel and perpendicular polarisations propagate independently in the absence of any anisotropy in the media, so the incident light will only induce electric field oscillations in its own plane. Therefore, p -polarised incident light will give rise to E_x and E_z oscillations, and s -polarised light will give E_y oscillations.

From Equations (3.13) to (3.18) one can derive coefficients known as Fresnel transmission factors that relate the components of electric field in the transmitted medium to the electric field in the incident medium. For angles of incidence above the critical angle the Fresnel factors are complex quantities, which means that field in the evanescent wave is not in phase with the incident field.

$$K_{sy} = \frac{2 \cos^2 \theta_i - 2i \cos \theta_i (\sin^2 \theta_i - n_{ti}^2)^{1/2}}{1 - n_{ti}^2}. \quad (3.19)$$

$$K_{px} = \frac{2 \cos \theta_i (\sin^2 \theta_i - n_{ti}^2) + 2i n_{ti}^2 \cos \theta_i (\sin^2 \theta_i - n_{ti}^2)^{1/2}}{n_{ti}^4 \cos^2 \theta_i + \sin^2 \theta_i - n_{ti}^2}. \quad (3.20)$$

$$K_{pz} = \frac{2 n_{ti}^2 \cos^2 \theta_i \sin \theta_i - 2i \cos \theta_i \sin \theta_i (\sin^2 \theta_i - n_{ti}^2)^{1/2}}{n_{ti}^4 \cos^2 \theta_i + \sin^2 \theta_i - n_{ti}^2}. \quad (3.21)$$

, where $n_{ti} = n_t/n_i$. These equations give the electric field in water, not in the lipid bilayer. For E_x and E_y , the two (macroscopic) fields are the same, since the parallel component of the electric field is continuous across the interface. For the z -component (3.21), ϵE_z is continuous so the Fresnel coefficient has to be multiplied by $(n'/n_i)^2$ to obtain the field in the bilayer, (n' is a refractive index of the bilayer at the interface).

The dependence of the K -factors on incident angle in the Figure 3.4 has two important aspects from my point of view.

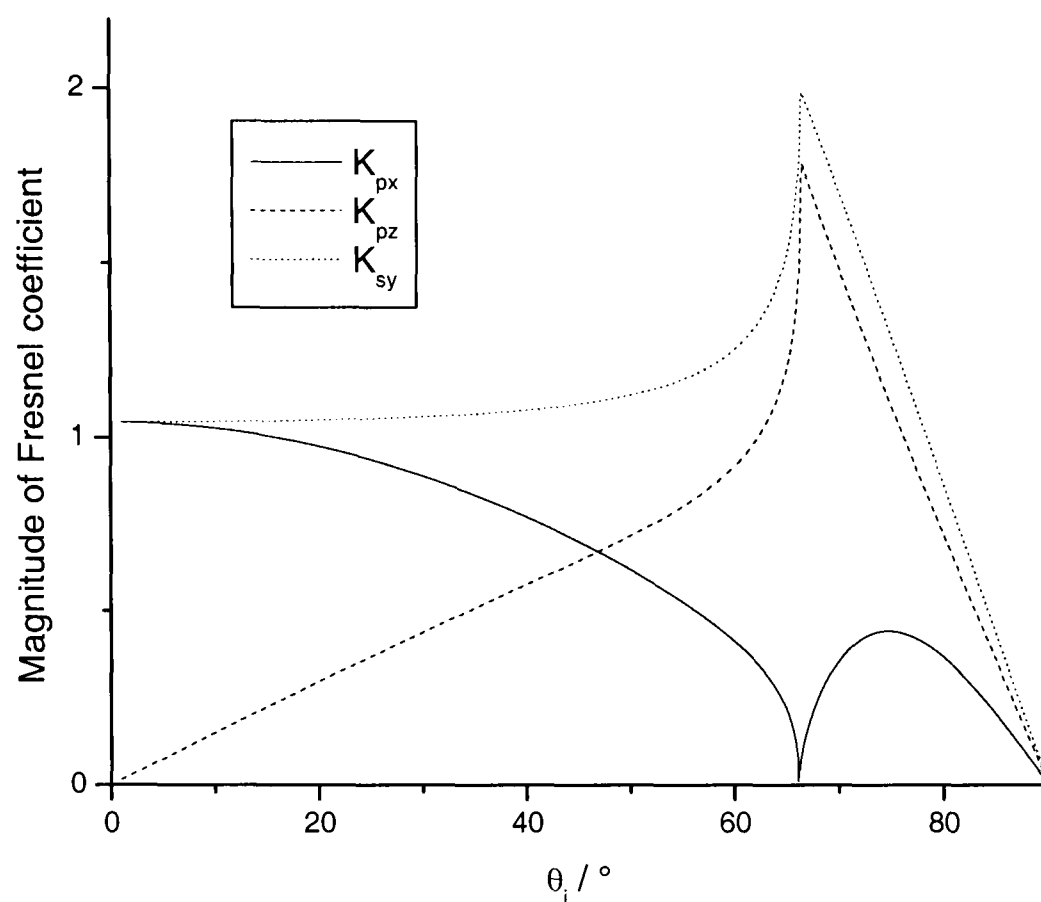


Figure 3.4 Variation of Fresnel K-factors with incident angles for a beam incident from silica ($n = 1.458$) on a film ($n = 1.4$, thickness $\ll \lambda$) at the silica–water interface. The K-factors determine the electric field experienced at an interface for a given macroscopic electric field.

First, the magnitudes of K_{pz} and K_{sy} increase greatly when light is incident at the critical angle. This fact means that to maximise the electric field at the interface, we should employ a *TIR* geometry for beam delivery, at an angle close to θ_c . Spectroscopic signals from the interface can thus be maximised.

The second important point is that K_{px} becomes zero at the critical angle. Consequently, a *p*-polarised beam incident at θ_c produces an interfacial electric field that oscillates purely in the *z*-direction.

Light incident along a surface normal (as in many spectroscopic experiments) can have electric field oscillations in the *x*- or *y*-direction relative to the film, but these

directions are equivalent relative to the surface. There is then no distinction between *s*- and *p*-polarised light. It is impossible to use normal incidence to deliver an electric field vector which oscillates in the *z*-direction. Oblique incidence provides more possibilities for polarised beam delivery. The special case of *TIR* at θ_c is the only way to get an electric field at the interface with purely *z*-oscillations.

For structural studies it is desirable to deliver electric fields with different polarizations. In general the optical response in a material, *e.g.* its induced polarisation or its Raman tensor depends on the direction of the applied field. By delivering light which oscillates in different directions, we can learn about the orientational properties or degree of order of a thin film at an interface. Consider the components of the polarisability tensor, α :

$$\alpha = \begin{bmatrix} \alpha_{xx} & \alpha_{xy} & \alpha_{xz} \\ \alpha_{yx} & \alpha_{yy} & \alpha_{yz} \\ \alpha_{zx} & \alpha_{zy} & \alpha_{zz} \end{bmatrix}.$$

The subscripts *ij* of α_{ij} components denote the direction of the polarisability response and the direction of the applied field. For a film with uniaxial symmetry there are four independent components of α : $\alpha_{xx} = \alpha_{yy}$, $\alpha_{zx} = \alpha_{xz} = \alpha_{yz} = \alpha_{zy}$, $\alpha_{xy} = \alpha_{yx}$, and α_{zz} .

All of these α components, except α_{zz} , can be probed by delivering light to an interface at the critical angle for *TIR* and observing the material's response from the *z*-direction. We need then simply select the correct polarisation of the incident light, and correspondingly discriminate our observations by their direction of polarisation. The component α_{zz} cannot be directly observed along the *z*-axis in the Figure 3.2 although some signal may be collected if the numerical aperture is large.

From the perspective of the study of lipid bilayers at the silica-water interface,

delivering light exactly at θ_c will give the largest interfacial electric field (and hence largest signal) and pure polarisation components. However, at θ_c , d_p is infinite so the lipid signal will be swamped by Raman scattering from the water. Furthermore, the signal strength will be exceedingly sensitive to alignment, making quantitative comparisons difficult. At angles of incident a couple of degrees above θ_c , there will be some mixing of polarisations, but also a smaller penetration depth (and hence weaker bulk signal). The interfacial electric fields are still quite strong, and they are much less sensitive to alignment. As a final point, it is worth noting that d_p is always much greater than the thickness of the lipid bilayer, so the field experienced by the lipid is negligibly different from the interfacial field calculated from Fresnel's equations.

3.3 Unenhanced Raman scattering from thin films

The development of surface enhanced Raman scattering (*SERS*) in the 1970's eclipsed unenhanced and resonance Raman scattering as the preferred technique for studying thin films by Raman spectroscopy. The need for roughened metallic substrates in *SERS* and the difficulties in quantification do not make *SERS* an attractive technique for studying biophysical systems under conditions mimicking their native environment. Recent technological advances in lasers, charge-coupled device (*CCD*) detectors, holographic notch filters (*HNFs*) and confocal microscopes have led to great improvements in unenhanced signal levels. Interest in the use of unenhanced Raman as a broad-based analytical tool for the study of monolayers and thin films has thus been rekindled.

The majority of Raman experiments now use commercial confocal Raman microspectrometers (see the Figure 3.5). In this configuration, the excitation laser is delivered to the sample through the same microscope objective that collects the Raman scattered light. Raman spectroscopy employed in this way has become a routine

laboratory technique, providing spectra and images non-invasively from small (but not ultrathin) samples with speed, ease, and little or no sample preparation.

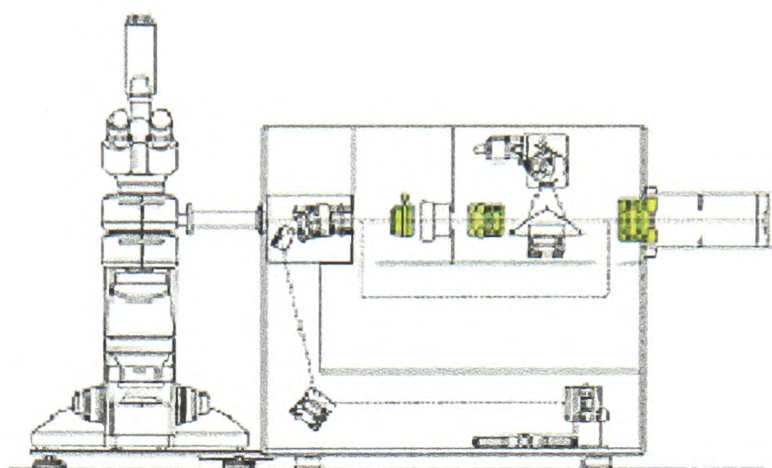


Figure 3.5 Typical optical arrangement for a commercial confocal Raman microscope (adapted from Renishaw website). Samples are placed under the microscope (on the left). Light is delivered to and collected from the sample through the microscope.

There are various strategies for increasing the intensity of Raman scattering, including resonance enhancement and use of higher incident intensity, higher frequency of scattered light, and/or larger scattering volume. Resonance enhancement is highly effective, increasing the signal by 10^6 , but is not generally applicable to all molecules and suffers from the production of the increased fluorescence and sample damage. While the incident beam intensity is limited by damage to optics or to the sample, the intensity of the incident laser beam does not itself determine the strength of the Raman signal. It is the surface electric field that is important for the spectroscopy of thin films, and this electric field strength can be enhanced in an internal reflection geometry. At the critical angle, the *s*-polarised incident field is enhanced by a factor of 2 at the interface (compared to the field in the incident medium), while the *p*-polarised incident electric field is magnified by a factor of $2 \times (n_i/n_t)$. Taking into account other benefits

of delivering the pump beam externally to the collection optics (such as reduced fluorescence background) Greene (Greene, 2004a) has shown that the signal-to-noise ratio in a *TIR*-Raman experiment can be two orders of magnitude better than in a conventional confocal microscopy experiment.

3.3.1 Literature background

A general summary of literature concerned with the optical aspects of *TIR*-Raman scattering may be found in the *D.Phil.* thesis of Phillip Greene and is not repeated here. I restrict this review to experimental studies. The review below is based on a comprehensive literature survey by Phillip Greene.

The first introduction of *TIR* geometry as a spectroscopy was made by *IR* absorption spectroscopy in Harrick's work to the impurity and polyethylene-adsorbates onto the optical cell in 3-6 μm water absorption bands with penetration depth of 0.15 μm (Harrick, 1960). His efforts were also applied to monolayer adsorbates of protein plasma albumin using multi internal reflection fluorescence spectroscopy (Harrick & Loeb, 1973). Although the necessary theoretical work had been done in 1933 (Taylor & Glover, 1933), the early demonstrations of *TIR*-Raman scattering emerged in 1980 (Iwamoto, *et al*, 1980, 1981) with the introduction of an internal reflection element (*IRE*). In principle the interface needs only to be accessible to light from one side – the scattered light can then be collected through the *IRE*. Their *TIR*-Raman spectroscopy was performed at the critical angle for 700-*nm* thick polymer films on *IREs* (Iwamoto, *et al*, 1981), where the signal was enhanced by 40 times compared to spectra recorded under conventional illumination. Bovine serum albumin (*BSA*) films were also investigated (Iwamoto, *et al*, 1981). Iwamoto *et al.* investigated how well the penetration depth (and therefore the sampled layer) can be tuned with incident angle, using polymer coatings of various thicknesses down to 6 *nm* (Iwamoto, *et al*, 1980).

Patents exist from as far back as 1985 for apparatus to collect *TIR*-Raman spectra from transparent samples (Iwamoto, *et al*, 1985), and yet the technique remains quite poorly-known. As long ago as 1976, Raman spectra were obtained from layers adsorbed at the CCl_4 –water interface with a *TIR* technique (Takenaka & Nakanaga, 1976). Other early examples of resonant-enhanced *TIR*-Raman spectra include species adsorbed at the silica–water (Takenaka & Yamasaki, 1980) and SnO_2 electrode–aqueous solution interfaces (Fujihira & Osa, 1976). In 1990, the most relevant work to our Raman setup in early *TIR*-Raman spectroscopy was performed by Hoelzer *et al* (Hoelzer, *et al*, 1990).

As Raman apparatus has been improved, *TIR*-Raman spectroscopy has been recently shown to be a viable method and attention moved to more demanding investigations, involving, molecular structure and orientation. Works by Nicklov *et al* demonstrated that H_2O structure was enhanced at interface of hydrophobic *LB* film of ω -tricosenoic acid coated on sapphire prism by using single-pase polarised *TIR*-Raman spectroscopy (Nicklov, *et al*, 1993). Kawai *et al* performed non-resonance Raman studies of *LB* monolayers of stearic acid- d_{35} and cadmium stearate- d_{35} on water surfaces (Kawai, *et al*, 1989). Umemura and co-workers analysed the unenhanced *TIR*-Raman spectra obtained from multilayer *LB* films of dipalmitoyl diphosphatidylcholine (*DPPC*) to correlate the order of molecules with surface-pressure during deposition (Okamura, *et al*, 1991). They report that at high surface pressures, the *LB* films were well-ordered, while disordered films deposited at lower pressures gradually reoriented with time to form stable, highly ordered aggregates.

Beyond successful primitive attempts, the inherent weakness of the Raman scattering process is not a fundamental obstacle to acquisition of monolayer Raman spectra any more, as has been demonstrated with spectra from Newton black films (Lecourt, *et al*, 1998), *LB* films at the solid–solid interface (Beattie, *et al*, 2000), surfactants adsorbed at the solid/liquid interface (Bain & Greene, 2001) and cuticular

wax layers at the air-solid interface (Greene & Bain, 2005). There are, however, no previous studies of lipid bilayers at aqueous interfaces by Raman scattering.

3.4 Comparison with IR absorption spectroscopy

Raman scattering should be regarded as complementary to *IR* absorption as a vibrational spectroscopy. Quite apart from the different selection rules for *IR*, there are a number of strengths associated with Raman spectroscopy. (i) Raman experiments offer better spatial resolution than *IR* spectroscopy, due to the shorter incident wavelengths typically used for Raman scattering. (ii) Raman spectroscopy can provide information for a wider frequency range than *IR* spectroscopy in a single measurement. Particularly of note is the facility with which Raman scattering can probe low-frequency vibrations. (iii) Bulk water is less of a problem in Raman spectroscopy of aqueous systems than in *IR* spectroscopy due to the low Raman cross-section of water. (iv) The penetration depth of the pump laser in *TIR*-Raman scattering is less than that of the *IR* beam in attenuated total internal reflection *IR* (*ATR-IR*) spectroscopy (since d_p is wavelength-dependent), increasing the surface sensitivity of Raman scattering. (v) Raman spectra contain more orientational information than *IR* spectra (four independent tensor components compared to two). (vi) The delivery of light to an aqueous interface is easier in Raman scattering, since many materials absorb *IR* radiation (notably water).

Raman spectroscopy also has several drawbacks. Intrinsic low sensitivity is the first problem, but this has become more a historical detail rather than a fundamental experimental limitation. One of the principal difficulties is background fluorescence – several strategies exist to reduce fluorescence, including a change of the laser wavelength (either to longer wavelength where the sample does not absorb, or to shorter wavelengths where the fluorescence is red-shifted from the Stokes scattering) and use of time-gated signal collection – the Raman signal is simultaneous with the pump laser,

while the fluorescence is delayed in time. *FT*-Raman systems are effective for reducing fluorescence, but their sensitivity is insufficient for observation of unenhanced Raman scattering from surface species. The low sensitivity arises from the use of near-*IR* lasers: Raman intensities vary with the fourth power of the scattered light frequency, and detectors are relatively low in sensitivity for $\lambda > 1 \mu\text{m}$. One-photon fluorescence is not an issue in *FT-IR* spectroscopy.

ATR-IR spectroscopy is now quite a well-established technique for studying proteins at solid-water interfaces (Martin, *et al*, 2003), usually with D₂O rather than H₂O to reduce overlap between the O-H bending mode and the Amide I region of the peptide backbone. Use of D₂O is not necessary in *TIR*-Raman scattering due to the much weaker water background.

Chapter 4 Introduction to model membrane peptides and proteins

This chapter provides background information on the structure and function of the membrane peptides and proteins employed in the work described in the thesis: gramicidin D (*gD*), indolicidin and phospholipase A₂ (*PLA*₂). Gramicidin is a channel-forming membrane-bound peptide that is widely used for model studies on membrane peptides. Until recently, there was controversy over the secondary structure of the active channel form of gramicidin in lipid bilayers. The generally accepted structure is now a single-stranded $\pi^{6.3}$ β -helix dimer (left in Figure 4.1, where the superscript is the number of residues per turn in the helix) rather than the alternative proposition of an intertwined double-stranded $\pi\pi^{5.6}$ -helix (Figure 4.2). Indolicidin is a water-soluble antimicrobial peptide whose mode of action in membranes has been the subject of much debate. Various structures have been proposed for indolicidin interacting with a membrane including a β -turn or poly-L-proline type II helix. One reason for the choice of gramicidin and indolicidin for Raman studies is the high tryptophan content of both peptides, which provides for useful markers in the Raman spectrum. *PLA*₂ is a difficult protein to characterize by x-ray or *NMR* spectroscopy since it is only active when interacting with a lipid bilayer. Our primary interest is not to derive information about the protein structure, but to show that Raman scattering can be used to follow changes in the lipid structure when the *pslb* interacts with the phospholipase and is subsequently hydrolysed by the enzyme.

4.1 Gramicidin

Gramicidin A (*gA*) is a linear pentadecapeptide (15 residues) synthesized by *Bacillus brevis*. It has a very unusual structure composed of alternating D- and L-amino

acid residues with the primary sequence of (C-terminal) $\text{HCO-L-Val}^1\text{-Gly}^2\text{-L-Ala}^3\text{-D-Leu}^4\text{-L-Ala}^5\text{-D-Val}^6\text{-L-Val}^7\text{-D-Val}^8\text{-L-Trp}^9\text{-D-Leu}^{10}\text{-L-Trp}^{11}\text{-D-Leu}^{12}\text{-L-Trp}^{13}\text{-D-Leu}^{14}\text{-L-Trp}^{15}\text{-NHCH}_2\text{CH}_2\text{OH}$ (N-terminal) (Sarges & Witkop, 1965a). It acts as an antibiotic, particularly against gram-positive bacteria (hence the name), and finds commercial applications as a topical bacteriostatic agent. Natural sequence variants occur at position 11 with substitution of *Trp* by *Phe* (gramicidin B, *gB*) or *Tyr* (gramicidin C, *gC*); the *Val* in the first position may also be substituted by *Ile* (Sarges & Witkop, 1965b, c). Often experiments have been performed with the natural mixture (termed gramicidin D, *gD*) which is predominantly *gA* (typically 80 % *gA*, 5 % *gB* and 15 % *gC*) (Weinstein, *et al*, 1980). Gramicidin forms channels in lipid membranes that are selective for monovalent cations (Finkelstein & Andersen, 1981). Water and protons also pass through the channel, but divalent cations and anions are essentially impermeant (Myers & Haydon, 1972). Single gramicidin channels behave in an ohmic manner with a conductance of about 5.8 pS in 100 mM NaCl at 20 °C (Hladky & Haydon, 1972).

4.1.1 Conformations in solution

The presence of alternating *D*- and *L*-amino acid residues in gramicidin produces some secondary structures not generally encountered with peptides and proteins that contain *L*-amino acids exclusively. Additionally, gramicidin has polymorphism depending on the solvent type, temperature and peptide concentration. This polymorphism is manifest in solution, in the solid state, and even in membranes (Wallace, 1991). The amino-acid sequence of gramicidin is very hydrophobic and results in the peptide being virtually insoluble in water. In a polar organic solvent, such as a 1:1 mixture of dimethylsulphoxide/acetone, in the absence of lipid, gramicidin is monomeric (Roux, *et al*, 1990), whereas in nonpolar solvents like dioxane, and in various alcohols such as ethanol and methanol, it has been shown to exist as a mixture

of dimeric forms in equilibrium with monomers (Veatch & Blout, 1974). The rate of conversion between polymorphic forms is also solvent and temperature dependent.

The conformation of gramicidin was originally investigated in organic solution due the difficulty of obtaining high resolution x-ray crystal structures of gramicidin in a membrane. There were thoughts that organic solvents might mimic the hydrocarbon interior of lipid membranes and the early structures obtained from crystals grown in these solvent appear quite reasonable as ion channels. However, there were various compelling reasons for believing that these did not correspond to the structure of the predominant active membrane-bound channel. The circular dichroism (*CD*) spectrum of gramicidin in lipid vesicles was found to be significantly different from the spectrum in solution and could not be represented by any combination of the spectra of the different double helical dimers or monomeric solution forms (Masotti, *et al*, 1980; Wallace, *et al*, 1981). Nevertheless, the early studies in solution have been indispensable for the current understanding of the structure and function of gramicidin in membranes.

4.1.2 Antiparallel β^3 helical dimer

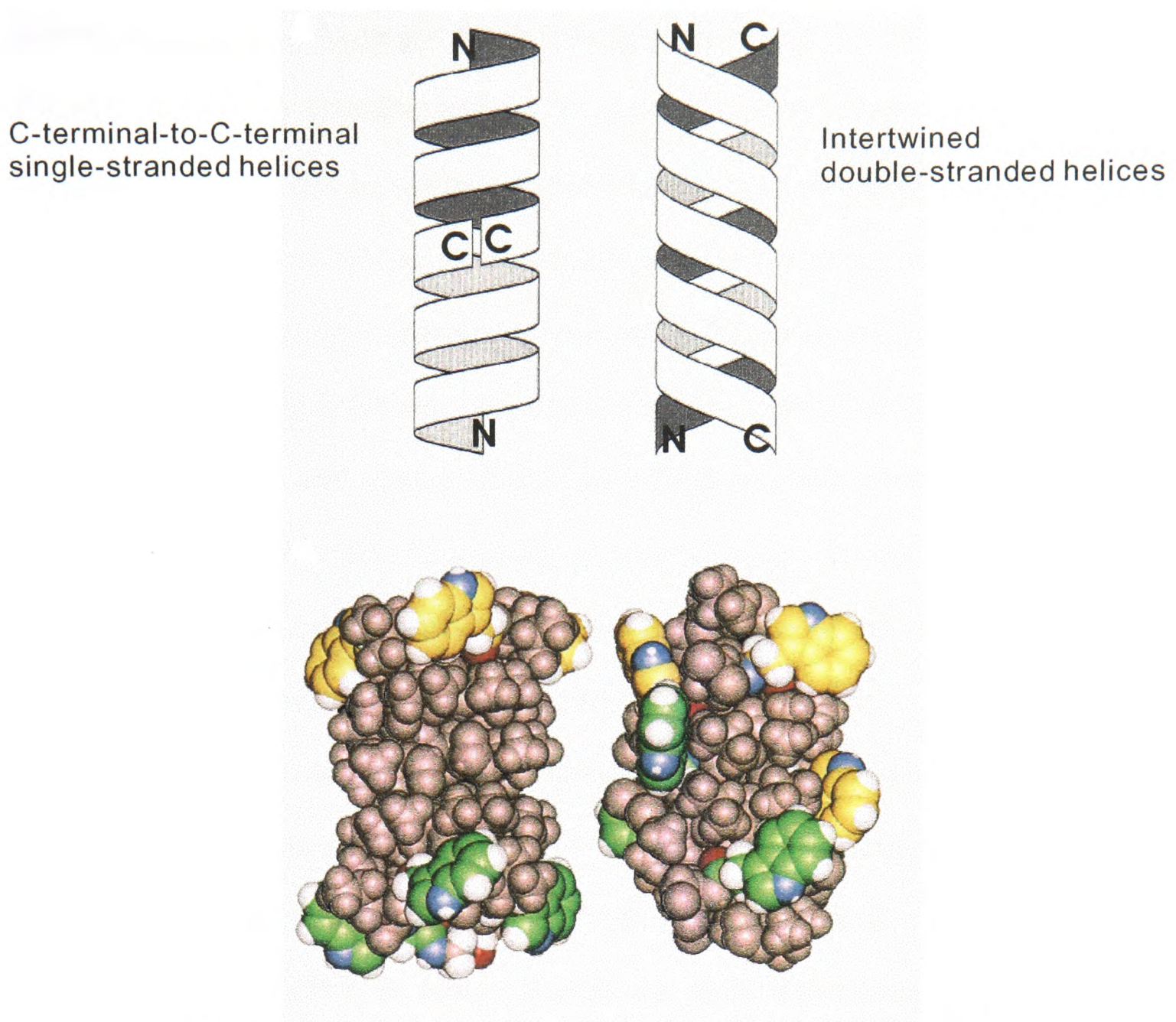
The different gramicidin structures found in organic solutions result in a significant dependence of the structure of the membrane-bound form on the solvent from which the lipid membrane was reconstituted, as found by *CD* and *NMR* measurements (Bano, *et al*, 1989, 1991; LoGrasso, *et al*, 1988; Killian, *et al*, 1988). The nature of the lipid is also important. Sychev found that the double-stranded/single-stranded dimer equilibrium is shifted toward the double-stranded form in bilayers formed by polyunsaturated phospholipids (Sychev, *et al*, 1993). Furthermore, phospholipid bilayers with unsaturated acyl chains produce different conformation depending on the degree of unsaturation of the acyl chains. As the degree of unsaturation is increased from 1-palmitoyl-2-oleoylphosphatidyl-choline/*n*-decane to

1,2-dilinoleoylphosphatidylcholine/*n*-decane bilayers, the average channel duration and channel-forming potency of gA are increased (Girshman, J., *et al*, 1997).)

Greathouse found that the conformational preference of gA in micelles formed by short-chain phosphatidylcholines varies as a function of the acyl chain length: the single-stranded dimeric channel form dominates at acyl chain lengths above eight; while the double-stranded form predominates when the acyl chain length is less than eight. (Greathouse, *et al*, 1994).

Early model-building studies (Urry, 1971; Urry, *et al*, 1971) and energy calculations (Ramachandran & Chandrasekaran, 1972) suggested that the channel-active form adopts a single-stranded $\pi^{6.3}$ β -helix dimer conformation as shown in left structures of the Figure 4.1.

For non-biologist, briefly I note the properties of π -helix here. It is an extremely rare secondary structural element in proteins. Hydrogen bonds within a π -helix display a repeating pattern in which the backbone C=O of residue *i* hydrogen bonds to the backbone H-N of residue *i*+5. Like the 3.10 helix, one turn of π -helix is sometimes found at the ends of regular alpha helices but π -helices longer than a few *i*, *i*+5 hydrogen bonds are not found. One of examples is depicted in the Figure 4.2). Subsequent studies revealed that gramicidin adopts two major folding motifs: the head-to-head single-stranded $\beta^{6.3}$ π -helix dimer (SS in the Figure 4.1) suggested by Urry and intertwined double-stranded helix forms, typically $\pi\pi^{5.6}$ or $\pi\pi^{6.4}$ (which I will call intertwined double-stranded $\beta^{5.6}$ or $\beta^{6.4}$ -helix, DS in the Figure 4.1).



Courtesy of Comment, 1999, *Nature Structural Biology* 6, 609

Figure 4.1 (a) Primary sequence, (b) schematic diagrams of gramicidin, and (c) space-filling models: head-to-head single-stranded β -helical dimer (SS) (left) and intertwined double-stranded helices (DS) (right)

The DS structures have two polypeptide chains forming a series of intermolecular hydrogen bonds along their entire length and then rolled up to form a π helix. The antiparallel DS structures have been designated the “pore” form. In the SS structure each of the two polypeptide chains forms six hydrogen bonds along most of its length (one helix in each leaflet of the bilayer). In the SS structure most of hydrogen bonds are intramolecular, with the residues at the N-termini of each chain forming

several intermolecular hydrogen bonds joining together the two helices of the dimer. This SS structure is typically referred to as the “channel” form. Both motifs are composed of β -sheet-like secondary structures with intra-chain hydrogen bonding, when rolled up to produce π helices, form tube-like structures with the lumens of the tubes composed of the relatively hydrophilic polypeptide backbones and the surfaces of the tubes coated by the hydrophobic side chains. Figure 4.2 shows the different helices of right-handed α -helix and single-stranded and double-stranded π -helix). Both types of structures are suitable for functioning as ion channels in membranes, as their hydrophilic interiors can function as a water-filled passage for ion translocation, while their hydrophobic surfaces permit them to embed in the membrane.

The CD-spectrum of gramicidin in lipid vesicles is often used as a signature for the presence of active single-stranded $\beta^{6.3}$ π -helix dimer. (Killian, *et al*, 1988). The characteristic CD spectrum is referred to in Figure 6.5 in Chapter 6.

In 1991, Wallace & Janes obtained the three dimensional structure of the membrane-bound form of gramicidin at atomic resolution by x-ray diffraction of single crystals of gramicidin-lipid complexes showing $\beta^{6.3}$ SS structure (Wallace & Janes, 1991). Gramicidin in oriented multibilayers has been used to evaluate the overall dimensions and the location of ion-binding sites in the channel by x-ray diffraction (Olah, *et al*, 1991).

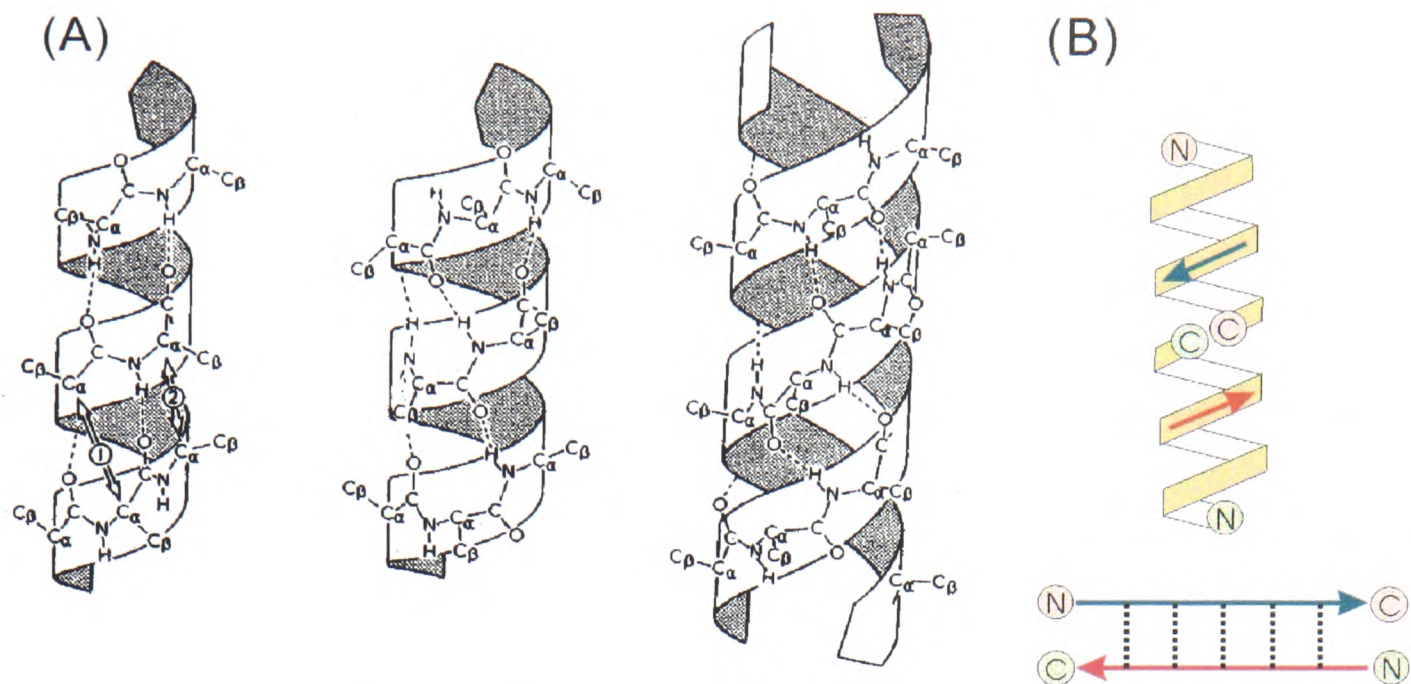


Figure 4.2 (A) Three different types of helical structures: right-handed single-stranded α -LD-helix (left), π -LD-helix (middle), antiparallel double-stranded $\pi\pi$ -LD-helix (right): here the π -LD- and $\pi\pi$ -LD- shown are the tightest possible ones, i.e. π -LD^{4.4} and $\pi\pi$ -LD^{5.6}, respectively), and (B) antiparallel single-stranded π -LD-helix with the accepted conformation of gramicidin as channel form in lipid bilayer, the arrows presents the backbone of upper (in blue) and lower (in red) helices and dotted lines between two helices show hydrogen bonding of β -sheet; the two arrows indicate the N- to C-terminal directionality of the chain conventionally. The opposite direction of two arrows indicate antiparallel β -sheet, in the 3-D model by *NMR* data (from protein data bank, 1MAG, solid-state *NMR* data of *gA* in *DMPC* as shown in the Figure 9.25) each helix has its own intra-hydrogen bonding actually. Figure (A) is published as the Figure 1 in the article by Colonna-Cesari's work (Colonna-Cesari, *et al*, 1977).

Solid-state *NMR* studies in multibilayers with isotopically labeled (¹⁵N, ²H, ¹³C) gramicidin have confirmed single-stranded $\beta^{6.3}$ -helix, oriented with the helix axis perpendicular to the membrane surface (Hing, *et al*, 1990a, b; Nicholson, *et al*, 1991; Smith, *et al*, 1989). The helix was found to be right-handed in dimyristoyl phosphatidylcholine multibilayers (Ketchum, *et al*, 1993; Nicholson & Cross, 1989;

Nicholson, *et al*, 1991; Prosser, *et al*, 1991; Teng, *et al*, 1991). An interpretation of the ion-induced ^{13}C -carbonyl shift changes in the lyso-phosphatidylcholine system by Bystrov & Arseniv revealed a consistent right-handed helical structure (Bystrov & Arseniv, 1988).

The controversy over the channel-active conformation arose with a “new” right-handed intertwined double-stranded *gD* structure (as shown in the Figure 4.3) with 7.2 residues/turn. Burkhardt *et al* proposed that the x-ray crystal structure of the Cs^+ and H^+ complex, showing a right-handed double-stranded helical structure, corresponded to the channel structure in lipid bilayers by ^{15}N -NMR and CD data (Burkhardt, *et al*, 1998).

The membrane-bound gramicidin channel structure has been investigated by IR spectroscopy, which confirmed the β -type hydrogen bonding pattern (Naik & Krimm, 1986). There have been Raman studies of *gA* crystals with/without Cs^+ or K^+ (Naik & Krimm, 1984), *gA* in various organic solutions (Iqbal & Weidekamm, 1980; Rothschild & Stanley, 1974), and *gA* in a DMPC dispersion (1:1 to 1:25 mixture) cosolubilised in a chloroform/methanol solution (95:5) (Short, *et al*, 1987).

None of these studies are directly relevant to membrane studies of gramicidin since a membrane environment is a prerequisite for formation of the channel-active form. Raman spectra of *gA* in $\text{L-}\alpha$ -lysophosphatidylcholine micelles above the main phase transition temperature have been reported (Aslanian, *et al*, 1986). Ward and Sanderson have obtained Raman spectra of *gD* in a phospholipid vesicle trapped in optical tweezers (Ward & Sanderson, unpublished results).

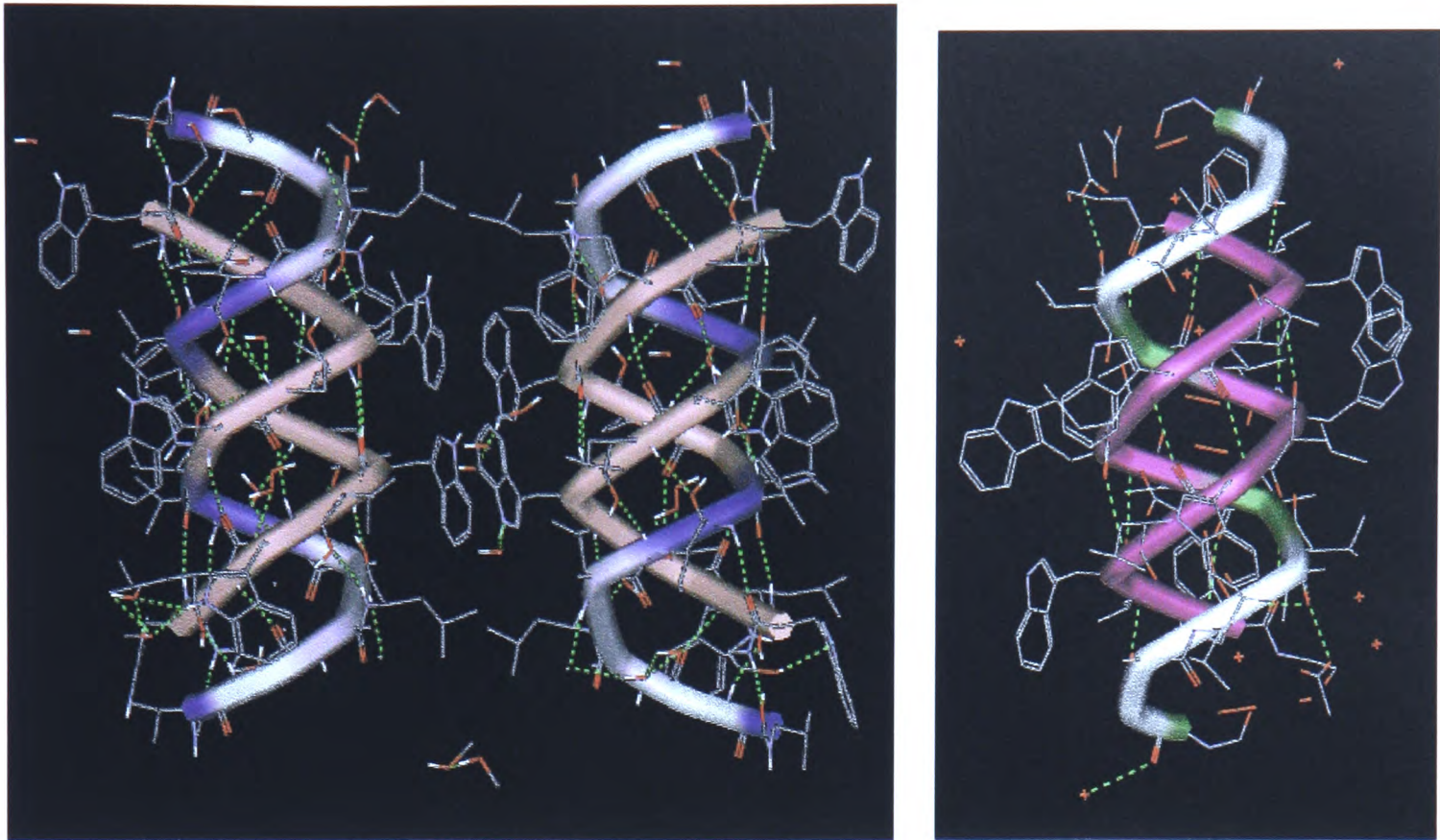


Figure 4.3 Controversial model of *gD* (Cs^+ ion complex in left and H^+ complex in right) proposed by x-ray and ^{15}N -NMR studies (Burkhart, *et al*, 1998) (redrawn from *pdb* 1AV2, x-ray crystallographic data of *gA* in oriented lipid bilayers)

4.2 Indolicidin

Indolicidin is an antimicrobial cationic peptide isolated from the cytoplasmic granules of bovine neutrophils. It is an amidated tridecapeptide with the sequence *Ile*¹-*Leu*²-*Pro*³-*Trp*⁴-*Lys*⁵-*Trp*⁶-*Pro*⁷-*Trp*⁸-*Trp*⁹-*Pro*¹⁰-*Trp*¹¹-*Arg*¹²-*Arg*¹³-NH₂. (Selsted, *et al*, 1992). It is unusually rich in tryptophan (39 % *Trp*) and proline (23 % *Pro*). Indolicidin exhibits antimicrobial activity against a variety of Gram-positive and Gram-negative bacteria (Selsted, *et al*, 1992), protozoa (Aley, *et al*, 1994), fungi (Ahmad, *et al*, 1995), and the enveloped virus *HIV-1* (Robinson, *et al*, 1998). Cationic antimicrobial peptides play a key role in the host defense system of many higher organisms such as plants, insects, amphibians, and mammals (Boman, 1995). The emergence of infections caused by multidrug-resistant micro-organisms has resulted in intense research into cationic antibiotics as a new class of antimicrobial drugs. Four structural classes of cationic antimicrobial peptides have been reported: the amphipathic α -helical peptides, such as the cecropins and melittins; the disulfide-bonded β -sheet peptides, including the defensins; the loop-structured peptides like bactenecin; and extended peptides that are rich in certain amino acids, into which category indolicidin falls (Ahmad, *et al*, 1995; Brogden, 2005). The cytotoxic mechanism of these antimicrobial peptides is still under investigation. Most bactericidal peptides attack the outer and cytoplasmic membranes, leading to target cell lysis (Ahmad, *et al*, 1995; Ladokhin, *et al*, 1997; Schibli, *et al*, 2002). Indolicidin behaviour was initially focused on membrane permeability and partitioning into lipid bilayers, but recently it was reported that membrane permeability did not lead to cell wall lysis (Falla, *et al*, 1996). It has been reported that indolicidin mainly reduces synthesis of DNA, rather than RNA and protein, accompanying *E. Coli* filamentation (Hsu, *et al*, 2005; Subbalakshmi & Sitaram, 1998). Thus, the activity of indolicidin in a membrane is still unconfirmed, and there is speculation that there is

more than one mechanism of antimicrobial action (Hsu, *et al*, 2005).

The structure of indolicidin also needs further investigation. Analysis of *CD*, *NMR* and fluorescence spectra (Hsu, *et al*, 2005; Ladokhin, *et al*, 1999; Rozek, *et al*, 2000) shows that indolicidin forms random coil structures in aqueous solution (Rozek, *et al*, 2000). As long as the concentration of indolicidin is lower than the critical concentration required for cell lysis, it is assumed that indolicidin crosses the membrane, accompanied by the transient perturbation, and transfer to the cytoplasm. It then inhibits cytoplasmic activities of biosynthesis and signal transduction, such as DNA synthesis (Hsu, *et al*, 2005). Above the lytic concentration, it behaves as typical antimicrobial peptides capable of cell lysis (Hsu, *et al*, 2005). Either a poly-L-proline type II helix (PP II helix) (Falla, *et. al.*, 1996) or a type VIa β -turn conformation of Trp^6 - $cis-Pro^7$ - Trp^8 (the *cis*-proline isomer) (Ladokhin, *et al*, 1999) have been proposed for the lipid-bound molecule. More recent works by Rozek (Rozek, *et al*, 2000) and Hsu (Hsu, *et al*, 2005) argue against a type VIa turn in indolicidin bound to either dodecylphosphocholine (*DPC*) or anionic sodium dodecyl sulfate (*SDS*) micelles. The proposed models are shown in the Figure 3.4: (A) rendered model from solution *NMR* data of 16 structures of complex form with *DPC* micelles, (B) computer-modeled membrane-bound conformation by Ladokhin showing a type VIa β -turn conformation of Trp^6 - $cis-Pro^7$ - Trp^8 .

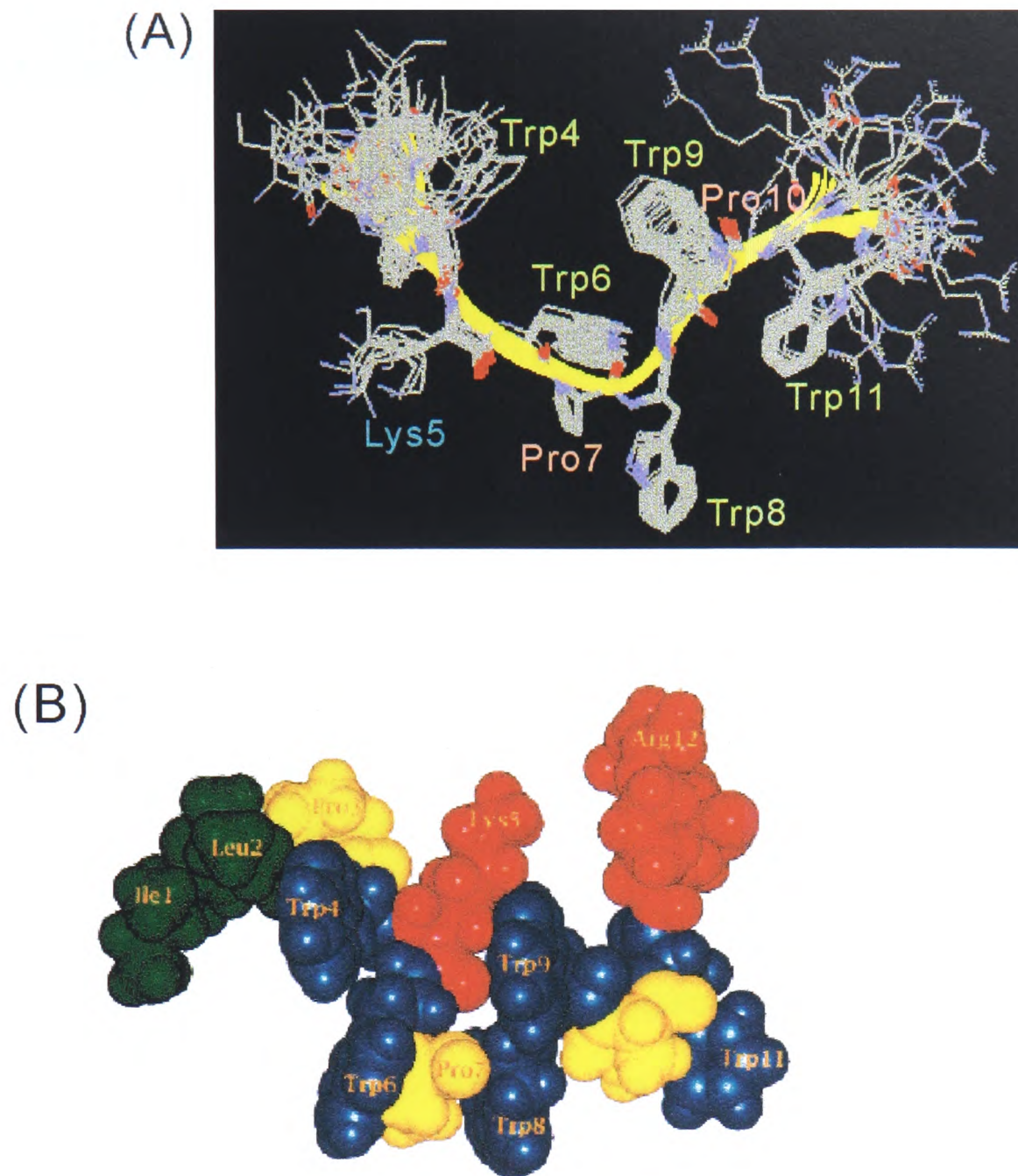


Figure 4.4 Proposed models of indolicidin of (A) complex with lipid-mimic surfactant of *DPC* micelles or (B) computer-modeled membrane-bound conformation showing type VIa β -turn: (A) from *pdb* 1G89, solution *NMR* data of 16 structures (B) from the published article by Ladokhin (Ladokhin, *et al*, 1999). The residues of indolicidin are colored according to the interfacial hydrophobicity scale of Wimley and White (Wimley & White, 1996) in the following manner: highly unfavorable partitioning, red; neutral, yellow; somewhat favorable, green; highly favorable, blue.

4.3 Phospholipase A₂

PLA₂ belongs to the superfamily of lipolytic enzymes that catalyse the hydrolysis of the *sn*-2 ester bond of glycerophospholipids as shown in Figure 4.5 (Six & Dennis, 2000). The active site contains a specific catalytic *His* residue, assisted by an *Asp* residue, which polarises a water molecule that then undergoes a nucleophilic attack on the carbonyl group of the phospholipid substrate. Ca^{2+} plays the role of cofactor to stabilise the transition state and is bound within the highly evolutionarily conserved “calcium binding loop” observed in all ~14 *kDa PLA₂s* (Dennis, 1994; Tischfield, 1997; Wery, *et al*, 1991). This reaction liberates lysophospholipid and free fatty acids. Two products from phospholipid hydrolysis by *PLA₂s* are involved in a number of physiologically important cellular processes: lysophospholipid may be metabolized either to platelet-activating factor which is known as a potent inflammatory and allergic mediator (Balsinde, *et al*, 2002; Dennis, 1987; Irvine, 1982; Snyder 1985) or to lysophosphatic acid, a signaling molecule with mitogenic activities (Fourcade *et al*, 1998; Gennaro *et al*, 1999). Eicosanoids, derived from arachidonic acid, are potent mediators of inflammation, allergy, apoptosis, and tumorigenesis (Bezzine *et al*, 2000; Heller, *et al*, 1998; Touqui & Alaoui-El-Azher, 2001; Valentin, *et al*, 1999).

These water-soluble, heat-stable enzymes have been divided into several groups, depending on their amino acid sequences, structure, and tissue distribution (Dennis, 1997). *PLA₂s* are classified into two structurally and biochemically distinct classes, cytosolic and secretory. The cytosolic types (80 to 110 *kDa*) are involved in phospholipid metabolism and transmembrane signaling. The group IV (85 *kDa*) remains unique in its roles in normal fertility, generation of eicosanoids from inflammatory cells, brain injuries, allergic responses, collagen-induced arthritis, and prostaglandin E₂-mediated bone resorption associated with inflammation (Bonventre, 2004; Sapirstein &

Bonventre, 2000).

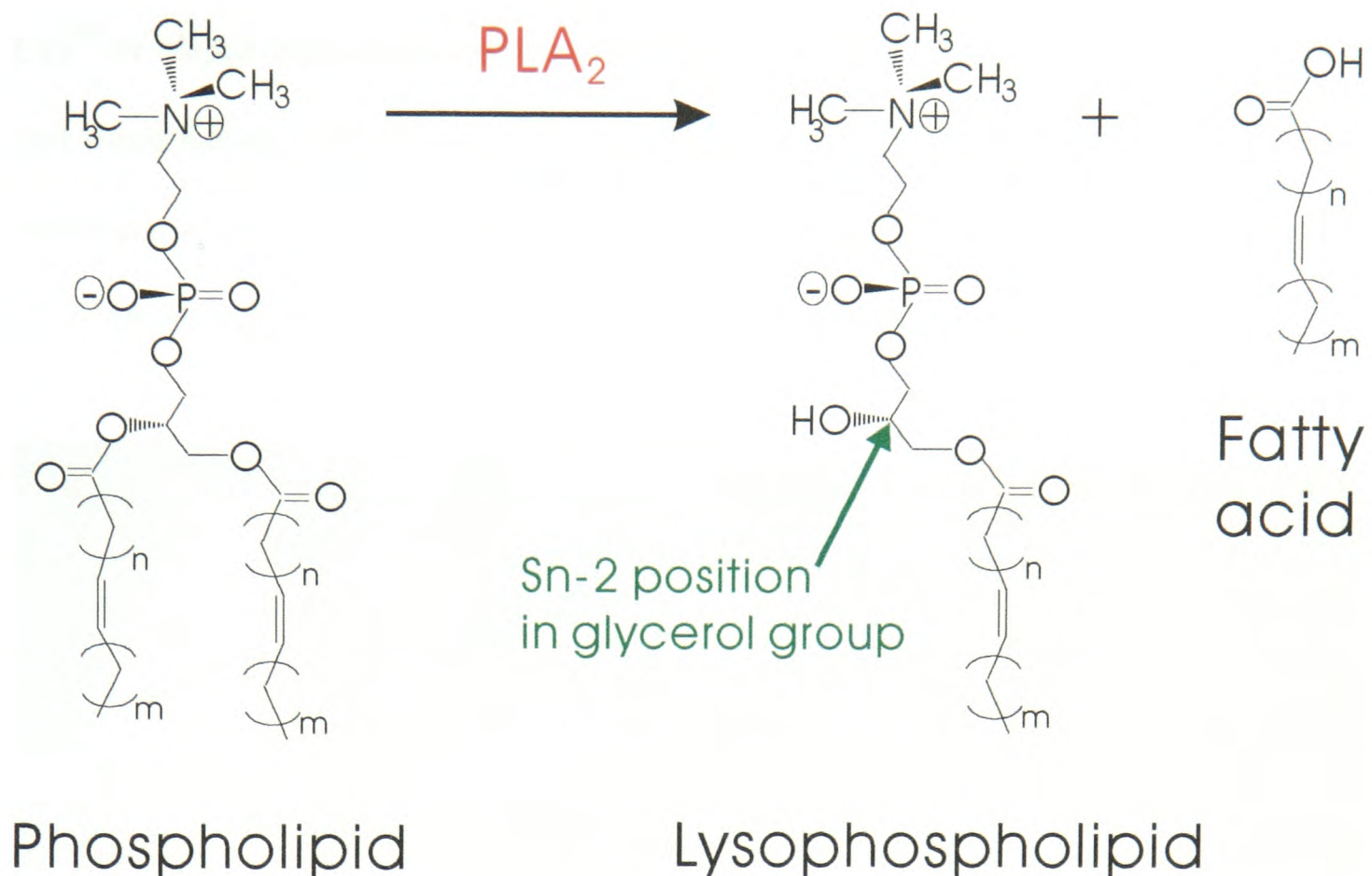


Figure 4.5 Hydrolysis of phospholipid by PLA_2 .

The secretory PLA_2 s (13 to 14 *kDa*) are abundant in mammalian pancreas and in reptile and insect venoms. Two groups of ~14 *kDa* snake venom PLA_2 s, group I from cobras and kraits and group II from rattlesnakes and vipers, are well known. The group I is divided into group IA which is found only in snakes and group IB which appears ubiquitously in mammals. They all have a disulfide bridge connecting Cys-11 to Cys-77 and characteristic three-amino acid “elapid loop” composed of residues 54-56 (Tischfield, 1997). The mammalian group IB PLA_2 has 14 Cys residues and is secreted predominantly by the pancreas to function extracellularly in digestion (Dennis, 1994; Scott & Sigler, 1994). Group II is subdivided into IIA to IIC: Group IIA and IIB are characterised by 14 Cys residues and in common with lack of disulfide bridge between Cys-11 and Cys-77. Group IIB has only been observed in the Gabon viper, but group

IIA has been described for many mammals. Group IIC gene has been characterised in rat and mouse, and it contains 16 Cys residues with neither disulfide bridge of Cys¹¹ to Cys⁷⁷ or elapid loop characteristic of group I. Group III *PLA*₂s are found only in bees and some lizards, which have 10 Cys residues. Group V is distinguished with different molecular mass of 13.6 *kDa* and their characteristics (Tischfield, 1997). Several types of *PLA*₂ are shown in the Figure 4.6.

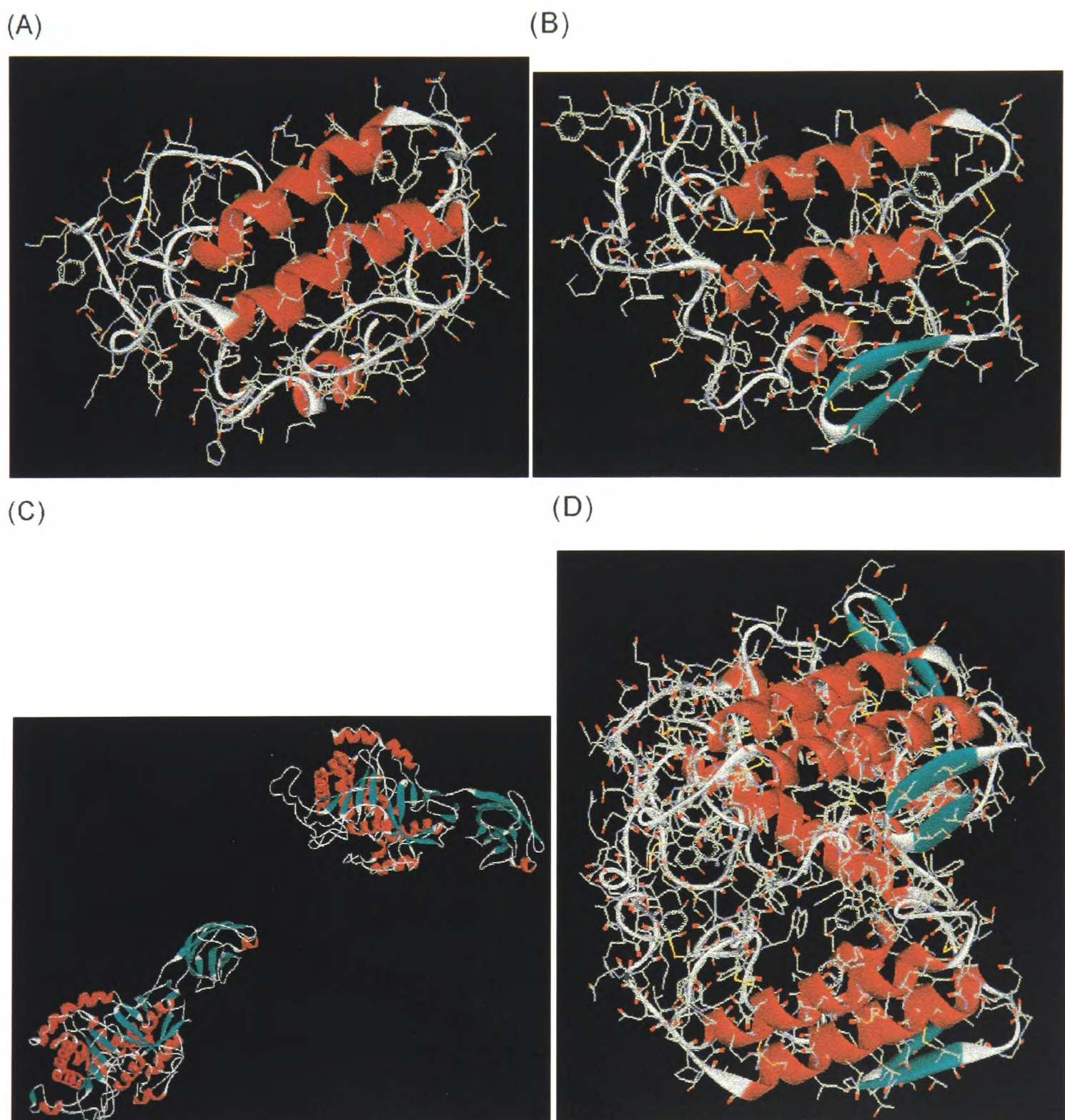


Figure 4.6 Various *PLA*₂ structures (E.C.3.1.1.4) in solution or crystal state (with no lipid) from: (A) porcine pancreas (Type IB, 1PIR in protein data bank (*pdb* code), solution structure by solution *NMR*), (B) porcine pancreas (Type IB, 1P2P, by *x-ray*), (C) human cytosol (1CJY, by *x-ray*), and (D) Naja Naja venom (Type IA, 1A3F, by *x-ray*).

PLA_2 used in my study was porcine pancreatic type IB. It has much the same structure as the PLA_2 from cobra venom, and exactly the same catalytic site, but it is known to exhibit a dormant lag phase in neutral phosphatidylcholine membranes before rapid hydrolysis begins, and is also activated by negatively charged lipids.

Secretory PLA_2 s undergo a substantial increase in activity upon binding to the surface of phospholipid micelles or membranes, an effect known as interfacial activation (Arni & Ward, 1996; Berg, *et al*, 2001; Gelb *et al*, 1995, 1999; Jain & Berg, 1989; Pieterse *et al*, 1974; Scott, *et al*, 1990; Scott & Sigler, 1994). There have been two main issues regarding interfacial activation of PLA_2 : first, the effect of physical properties of the lipid bilayer, including membrane fluidity, curvature and surface charge; second, membrane-induced conformational change of PLA_2 . More negative surface potentials of the membrane increase PLA_2 activity (Jain *et al*, 1986, 1989; Tatulian, 2001; Volwerk, *et al*, 1986; Yu, *et al*, 2000). It has been proposed that increased membrane curvature, defects, and the fluctuations inferred to accompany defects all enhance PLA_2 activity (Sen, *et al*, 1991; Zidovetzki, *et al*, 1992). All these membrane properties vary if profound morphologic rearrangements accompany phase separation of reaction products (both lysophospholipid and fatty acid (Burack, *et al*, 1997)).

While the lysophospholipid is removed from the membrane to a significantly larger extent than the fatty acid (Tatulian, 2001) due to its greater aqueous solubility, the fatty acid has been believed to be the important factor of the control into the PLA_2 activity. *ATR-FTIR* data show predominant, but not complete, removal of the lysophospholipid from the membrane following lipid hydrolysis. Formation of 2:1 fatty acid/phosphatidylcholine complexes has been observed by several studies (Cevc, *et al*, 1988). Such complexes, which are characterised by local negative curvature and increased anionic charge, may serve as PLA_2 binding sites. Very high fractions of the fatty acid

may inhibit PLA_2 activity by laterally segregating into negatively charged patches and sequestering PLA_2 from its substrate (Tatulian, 2001).

Vacklin and coworkers carried out a kinetic study on PLA_2 hydrolysis of phosphatidylcholine bilayers by neutron reflection and ellipsometry (Vacklin, H., *et al*, 2005c). The pattern of hydrolysis of the three different phosphatidylcholines, dioleoyl- (*DOPC*), 1-palmitoyl-2-oleoyl- (*POPC*), and dipalmitoyl- (*DPPC*) by cobra venom PLA_2 was very similar in both neutron reflection and ellipsometry studies, but the rate of hydrolysis and extent of reaction decreased with increasing lipid saturation. A significant amount of surfactant (lyso-phosphatidylcholine) was found to be present in the final *DOPC* bilayers (28 % of surfactant on *DOPC* surface concentration), and it appeared to lead to an increased reaction rate, which suggests that it disturbed the lipid packing so that the ester bonds were more readily accessible to PLA_2 compared to a pure *DOPC* bilayer. However, the increased penetration depth of the enzyme into *DPPC* seemed to serve as an inhibiting effect, which would be consistent with significant accumulation and phase separation of palmitic acid, which is known to increase the packing density of *DPPC* (Inoue, *et al*, 2001). Saturated fatty acids are known to make condensed phospholipid bilayers more rigid, whereas unsaturated fatty acids have a negligible effect. Aggregation of *DPPC* reaction products and phase separation of the enzyme have been directly observed by fluorescence microscopy (Maloney, *et al*, 1995), which is consistent with the idea that, as the enzyme becomes more and more strongly bound to the fatty-acid-rich membrane, it eventually becomes trapped and can no longer release the products or access the next substrate molecule.

While the neutron reflection and ellipsometry use structural parameters such as thickness, surface coverage and refractive index to infer interfacial changes, vibrational techniques can provide a direct measurement of time-dependent structural changes during the hydrolysis reaction. Tatulian (Tatulian, 2001, 2003a) studied a supported

bilayer of 1-palmitoyl(d_{31})-2-palmitoylphosphatidylcholine, in which the sn-1 chain is fully deuterated while the sn-2 chain is not, by attenuated total reflection Fourier transform infrared spectroscopy (*ATR-FTIR*). From the integrated absorbances of the CH_2 and CD_2 symmetric stretching bands after a certain period of hydrolysis, he was able to demonstrate the accumulation of the free fatty acid in the membrane at all different concentrations of PLA_2 . At moderate fractions of PLA_2 -generated fatty acid, complexes between intact phospholipid and fatty acid may form and serve as PLA_2 binding sites, although very high fractions of the fatty acid may inhibit PLA_2 activity by laterally segregating into negatively charged patches and electrostatically sequestering PLA_2 from its substrate.

The effect of the anionic lipid in supported membranes on the activity of PLA_2 was studied by using bilayers composed of a mixture of *POPC* (neutral) and *POPG* (negatively charged lipid) in which the fraction of *POPG* was increased from 0 to 0.5. The methylene stretching intensities as a function of PLA_2 concentration indicated that PLA_2 exhibited higher activity toward membranes with higher fractions of the anionic lipid *POPG*. Higher PLA_2 activity toward negatively charged membranes is shown to result from stronger membrane-enzyme electrostatic interactions (Tatulian, 2001). The interplay between the hydrolytic product (fatty acid) and negatively charged surface of membrane would modulate membrane binding and activity of PLA_2 through morphological and/or electrostatic effects. (Tatulian, 2001)

The enzyme kinetics of PLA_2 has been characterised by an allosteric mechanism expressed in a kinetic model of the Michaelis scheme as shown in the Figure 4.7 (Roger, *et al*, 1998; Yu, *et al*, 1999): K_s^* (which is dissociation constant for surface-bound enzyme and enzyme-activated substrate of $(\text{ES})^*$, which equals k_{-1} / k_1 , the ratio of the dissociation and association rate constants) is influenced by the allosteric effect of the interface, in which the N-terminal segment of i-face is stabilised by the

headgroup interactions and so prevents the bound substrate from leaving the active site, whereas k_{cat}^* (which is the rate constant of decomposition of $(ES)^*$) is activated by interfacial anionic charge. Here '*i*-face' denotes the interfacial recognition region of PLA_2 that comes in contact with the substrate interface.

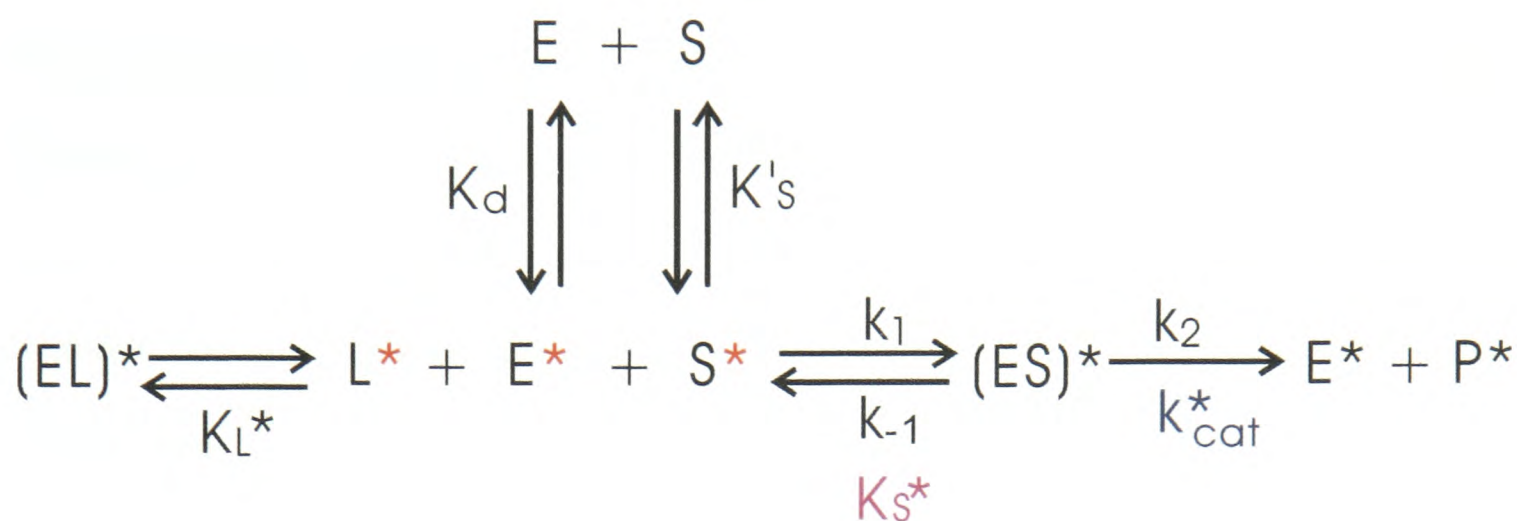


Figure 4.7 Michaelis model for interfacial catalysis that shows relationships between the primary rate and equilibrium parameters (Yu, *et al*, 1999): The species in the interface are marked with an asterisk. E, S, P is enzyme, substrate, and product respectively. L is an active site-directed substrate mimic such as a substrate or product.

If the concentrations in the bilayer are expressed as mole fractions and the bulk concentration in mM, then the equilibrium parameters are as follows. K_d is the dissociation constant at which half the enzyme is in the interface in the E^* form (mM). K'_s is the partition constant at which half the substrate is bound to the interface (mM). K_L^* is the dissociation constant for dissociation of L from $(EL)^*$ (dimensionless). K_s^* is the dissociation constant for $(ES)^*$ (dimensionless), which is the ratio of the rate constants for dissociation and association of the enzyme – substrate complex.

Primary rate constants: k_1 , k_{-1} , k_2 , and k_{cat}^* (all in s^{-1}); k_{cat}^* is the rate constant for the rate-limiting chemical step. The interfacial Michaelis constant, $K_M^* = (k_{-1} + k_2) /$

k_1 .

X-ray studies have failed to identify any conformational change in PLA_2 s in the presence of the lipid-water interface. In the crystal structure of porcine pancreatic PLA_2 (type IB) the N-terminal residues pack in a well-defined helix enabling the α -amino group to form functionally important hydrogen bonds with the active center residues His⁴⁸ and Asp⁹⁹ (Thunnissen *et al*, 1990). The x-ray studies on type I (Scott *et al*, 1990b; Thunnissen *et al*, 1990; White *et al*, 1990), type II (Brunie, *et al*, 1985), and type III (Scott *et al*, 1990a, b) PLA_2 revealed similar structures of these enzymes with and without bound inhibitory substrate analogues. However, small structural differences were found between the free and complexed forms of a type II PLA_2 ; the N-terminal helix and the Ca^{2+} binding loop were shifted towards each other in the uncomplexed enzyme by 1.2 Å (Scott *et al*, 1991). These results were considered as evidence against the enzyme model of PLA_2 activation. The enzyme model hypothesize that conformational changes in PLA_2 are primarily responsible for the interfacial activation of the enzyme (Tatulian, 2001).

NMR data revealed that in the free enzyme the N-terminus and residues His⁴⁸ and Asp⁹⁹ are flexible; they adopt a fixed conformation only upon formation of a ternary complex between the enzyme, substrate micelles, and an inhibitory substrate analog (Peters *et al*, 1992; Van den Berg, *et al*, 1995). These results provide evidence for the enzyme model of the interfacial activation of PLA_2 .

Although the crystal and solution *NMR* structures of PLA_2 are critical for understanding the catalytic mechanism on interfacial activation, the structure of the enzyme in a crystal environment, or even bound to substrate micelles in a suspension, may significantly differ from that of PLA_2 bound to lipid membranes.

The *ATR-FTIR* results uncovered markedly different structures of PLA_2 (of type II) free in solution and bound to lipid bilayers (Tatulian, 1997). He reported the

conversion of a large fraction of α -helical residues into more flexible helices (called dynamic helical structure) induced by membrane binding. Upon interaction with the membrane, this peak splits into two components at ~ 1650 and $\sim 1658\text{ cm}^{-1}$. Less stable α -helices are characterised by stronger carbonyl stretching force constants because of weaker helical hydrogen bonding and, consequently, their amide I vibrational mode occurs at higher frequencies (Dwivedi & Krimm, 1984, Tatulian, 2001). Hydrogen/deuterium ($\text{H}/^2\text{H}$) exchange experiments under various conditions demonstrated that destabilisation of the helices of PLA_2 upon membrane binding might be due to enzyme-substrate hydrogen bonding, which is likely to contribute to enzyme activation (Tatulian, 1997).

Chapter 5. Materials and Methods

This chapter describes the details of the procedures and techniques used in the experiments presented in subsequent chapters.

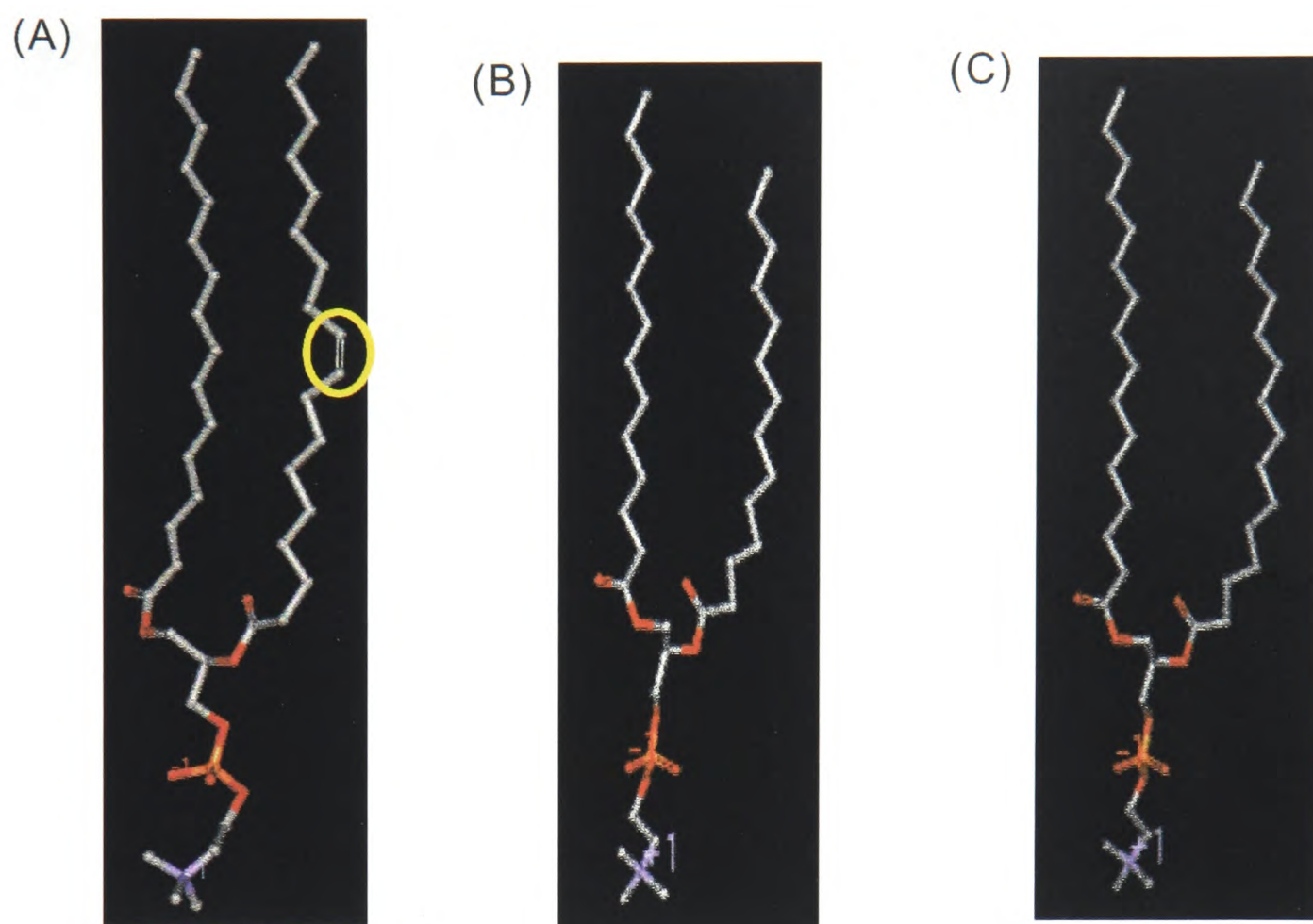
5.1 Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (*POPC*), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (*DMPC*), and dipalmitoyl-*sn*-glycero-3-phosphocholine (*DPPC*) were purchased from Sigma-Aldrich (Gillingham, Dorset, UK) and used without further purification. Solvents of methanol, chloroform, tetrahydrofuran (*THF*), benzene, and trifluoroethanol (*TFE*) were obtained from Sigma-Aldrich (all reagent grade). The fluorescent lipid 3, 3'-dioctadecyloxacarbocyanine perchlorate (*DiOC₁₈*, 99 %) was purchased from Fluka (Gillingham, Dorset, UK). See Figure 5.1 for structures.

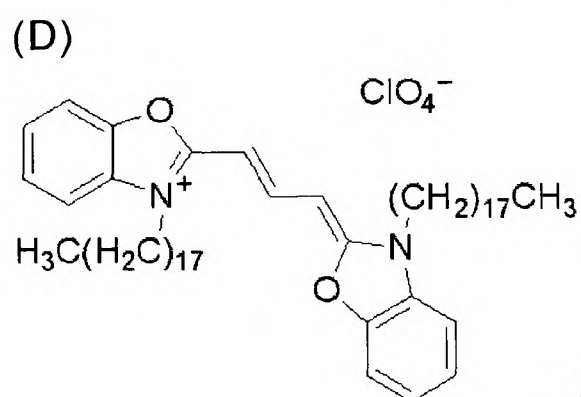
All experiments used ultrahigh quality deionised water (*UHQ DW* – Millipore, resistivity > 18 M Ω ·cm).

Deuterated *DPPC* (*d₇₅-DPPC*) was purchased from Avanti Polar Lipid (Alabaster, AL), β -D-dodecyl maltoside (*DDM*) from Fluka (lyophilised powder, ≥ 99.0 (TLC grade)). See Figure 5.2.

Gramicidin D (*gD*) from *Bacillus brevis* was purchased from Fluka (lyophilised powder) and stored at 4 °C. Indolicidin was custom synthesized by Sigma-Genosys (Cambridgeshire, UK), following an original synthesis paper by Selsted (Selsted, *et al*, 1992), and stored at -20 °C. Phospholipase A₂ (*PLA₂*) from porcine pancreas was purchased from Fluka (lyophilised powder and in ammonium sulfate suspension) and stored at 4 °C. The peptides were all used without further purification.



<i>sn</i> -1: saturated 16 CH ₂ <i>sn</i> -2: unsaturated 18 CH ₂ with <i>cis</i> C=C at 9 th position	<i>sn</i> -1 and <i>sn</i> -2: both saturated 14 CH ₂	<i>sn</i> -1 and <i>sn</i> -2: both saturated 16 CH ₂
<i>POPC</i>	<i>DMPC</i>	<i>DPPC</i>
(C ₄₂ H ₈₂ NO ₈ P, MW: 760.08)	(C ₃₆ H ₇₂ NO ₈ P, MW: 677.93)	(C ₄₀ H ₈₀ NO ₈ P, MW: 734.04)

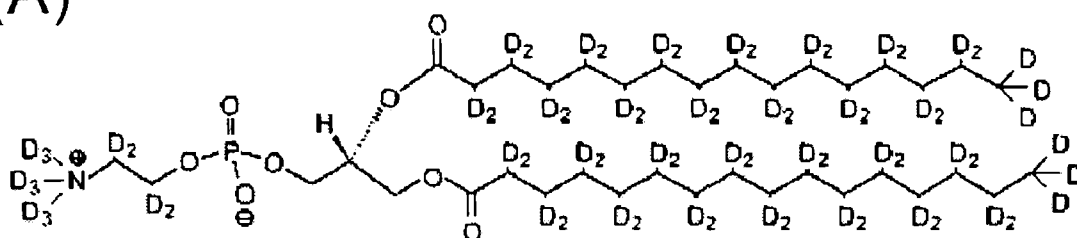


*DiOC*₁₈ (C₅₃H₈₅ClN₂O₆, MW: 881.70)

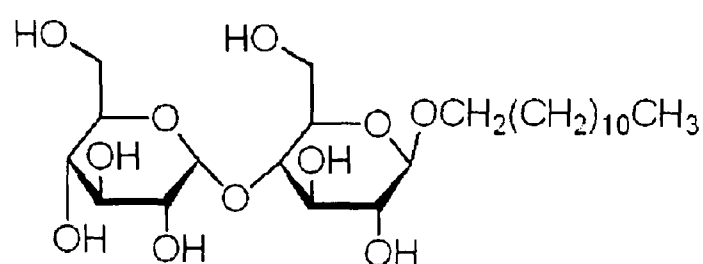
$\epsilon = 154,000 \text{ M}^{-1} \text{ cm}^{-1}$, $\lambda_{\text{max}} = 484 \text{ nm}$

Figure 5.1 Chemical structures and details of phospholipids of *POPC*, *DMPC*, *DPPC* and fluorophore of *DiOC*₁₈.

(A)

 $d_{75}\text{-DPPC}$ ($\text{C}_{40}\text{H}_5\text{NO}_8\text{PD}_{75}$, MW: 809.51)

(B)

 DDM ($\text{C}_{24}\text{H}_{46}\text{O}_{11}$,
MW: 510.62)

Sugar-based nonionic surfactant

Figure 5.2 Chemical structure and formula of (A) $d_{75}\text{-DPPC}$ and (B) DDM .

For pure *SUV* and *gD*-incorporated *SUV* experiments, a *pH* 8.0 buffer containing 10 *mM* *Tris* and 100 *mM* *NaCl* was used. For the *PLA*₂ experiment, the buffer was 10 *mM* *Tris* at *pH* 7.4 with no salt. For the indolicidin experiment, *pH* 7.12 10 *mM* phosphate buffer solution with 150 *mM* *NaCl* was prepared by dissolution of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in *UHQ DW* and titration to *pH* 7.12 by *NaOH*.

Squalane (Sigma-Aldrich, $n_D^{20} = 1.452$, 99 %) was used as an index matching fluid between the silica window ($n_D = 1.461$) and the prism in the Raman sample stage (see Figure 5.3).

5.2 Preparation of small unilamellar vesicles (*SUVs*)

The phospholipids (20 μmol) were solubilised in chloroform at room temperature. When a *DiOC*₁₈ was incorporated into the lipid bilayer for the determination of the concentration of phospholipid in *SUVs*, the phospholipids were formulated in a lipid:dye molar ratio of 200 to 1. The solvent was removed by

evaporation under a stream of dry N₂ followed by evaporation under vacuum overnight. The materials were then hydrated in Tris buffer over the gel-liquid crystalline phase transition temperature, mixed by vortexing vigorously and then sonicated with a bath sonicator (Langford Sonomatic 475H, Langford Ultrasonics, Warwickshire, UK) to yield a milky suspension of multilamellar vesicles. The multilamellar vesicles were sonicated with a 20 kHz ultrasonic horn using a 3 mm diameter titanium alloy microtip immersed to a depth of 5 cm (Model VC505, Sonics and Materials Inc., Newtown, CT). The ultrasonic intensity was 150 W·cm⁻² and was applied for fifty periods of 60 seconds each with breaks in-between so that the elevation in temperature due to heat dissipation in the sample was maintained below 10 °C. The shearing force at the interface between the vesicles and solution, from the well documented effects of cavitations and acoustic streaming (Delcampo, *et al*, 2001; Hardcastle, *et al*, 2000; Maiscuhaute, *et al*, 2001) acted to break apart the lipid producing a clear solution of SUVs along with some multilamellar vesicles (MLVs) and large unilamellar vesicles (LUVs). SUVs were purified by ultracentrifugation in a L8-55 ultracentrifuge with a 70.1 Ti rotor (Beckman Instruments, UK). Centrifugation at 30 min at 1.00×10⁵ g was used to remove titanium particles originating from the sonicator probe and 1 h at 1.66×10⁵ g for separating LUVs and MLVs. The spherical shape of the liposome was assessed qualitatively from the preservation of polarisation in scattering of vertically polarised light from a 1 mW HeNe laser. The average hydrodynamic diameter of the SUVs was evaluated to be 35 nm by photon correlation spectroscopy by Dr. J. Van Duijneveldt (University of Bristol), consistent with the formation of SUVs.

5.3 Incorporation of gramicidin into phospholipid SUVs

Phospholipids were codissolved with gD in a ratio of 25:1 in

chloroform/methanol (4:1, v/v), and the solvents were then evaporated under a stream of dry N₂ followed by evaporation under vacuum overnight. The dried *gD*/phospholipid film was redissolved in *TFE* (at 60 °C), *THF* or benzene (room temperature) for 30 mins and the solvent then evaporated under a stream of dry N₂ followed by evaporation under vacuum rotary evaporator at 18 mbar, 60 °C for 1 h. The subsequent procedures of hydration, ultrasonication, and ultracentrifugation were the same as in the preparation of pure *SUV* solutions.

5.4 UV/Vis absorption, fluorescence, and circular dichroism spectroscopy

The concentration of the phospholipids was assayed in *DiOC*₁₈-incorporated *SUVs* from the absolute absorbance at a wavelength of 490 nm by UV/Vis spectroscopy (UNICAM UV2-100). The *gD* concentration in *SUVs* was evaluated from the absorbance at $\lambda_{\text{max}} = 284 \text{ nm}$ of tryptophan (*Trp*) using the molar extinction coefficient of tryptophan $\epsilon = 5724 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The *gD* concentration was calculated as the *Trp* concentration / 3.8, because *gD* typically consists of 80 % *gA*, 5 % *gB*, and 15 % *gC*, and there are 4 *Trp* in *gA* but only 3 in *gB* and *gC*.

Fluorescence spectroscopy (CARY ECLIPSE, VARIAN) was used to check that the *gD* was present in the lipid layer and not in solution. The peak of the emission band at 332-335 nm confirmed that the *gD* in *SUV* was present in a hydrophobic environment. It corresponded with the literature (Bystrov *et al*, 1988; Urry, 1971), while in *gD* in methanol solution (more polar environment than lipid bilayer) the observed emission was at 345 nm by red-shift as explained in the Figure 6.4.

CD spectroscopy (JASCO J-720, Jasco, Inc., Easton, MD) was used to identify single-stranded channel structure and non-channel form (not single-stranded structures) in *gD*-incorporated *SUVs* according to literature assignments (Bano, *et al*, 1991, 1992; Killian, *et al*, 1988). The CD spectrum was acquired every 1 nm from $\lambda = 190 - 250 \text{ nm}$

with bandwidth of 2 *nm* at speed of 100 *nm/min* a sensitivity of 20 *mdegree*. 8 spectra were co-added to reduce the noise accumulation. The cell temperature was maintained at 20 °C by a water-jacket circulator surrounding the cell. See Chapter 6 for details of the spectral analysis.

5.5 Atomic force microscopy (AFM) (for chapter 7.1)

All images were collected with a MultiMode AFM with a Nanoscope IIIa controller (Digital Instruments Inc., Santa Barbara, CA). An E scanner (max scan size ~ 14 $\mu\text{m} \times 14 \mu\text{m} \times 4 \mu\text{m}$) and high aspect ratio triangular Si₃N₄ cantilevers (spring constant 0.12 N/m) were used to obtain images of the samples. All images were first-order flattened prior to analysis to remove sample tilt. All images were collected using the fluid tapping mode, which measures topography by intermittently contacting the surface with the tip, thereby reducing lateral forces that can damage soft samples. The AFM fluid cell and other accessories including polypropylene connectors, silicone o-rings, and silicon tubing were cleaned with 1 % (w/v) solutions of sodium dodecyl sulfate (SDS; Sigma Chemicals, St. Louis, MO) followed by final rinses with UHQ DW and drying with N₂ before use. The tip oscillation frequency was approximately 10 *kHz*, and scan rate was 1.969 *Hz*.

5.6 Cleaning and assembly procedures

To ensure a high level of cleanliness throughout all my experiments, a procedure was followed for the systematic cleaning of the fused silica window (diameter = 30 *mm*, thickness = 3 *mm*, 02 WLQ 106 (Catalog number), Melles Griot, Huntingdon, Cambridgeshire, UK) and glass prism (right angle, A = B = C = 25.4 *mm*, 01 PRS 021 (Catalog number), Melles Griot) that form the Raman sample stage (see Figure 5.3). The silica window and prism were first washed with a copious amount of UHQ DW followed by rinse of 2-5 % solution of a strong alkaline detergent, Decon 90

(Decon Laboratories Limited, East Sussex, England), in order to remove organic films bound to the solid surfaces, especially if they had been contaminated by contact with squalane of an index matching fluid to silica window. They were dried in a stream of dry N₂.

The most important surface to be clean and hydrophilic was the face of the silica window upon which the *pslb* was formed. The silica window was placed in a clean glass beaker and then made wet using “Piranha” solution (4 mL), which is a 3:1 mixture by volume of H₂O₂ (35 wt. % solution in water, Sigma-Aldrich) and conc. H₂SO₄ (Fisher Scientific, Loughborough, UK). The acid was added slowly to the hydrogen peroxide and the evolution of gas allowed to be subsided before the window was placed in the Piranha solution. The Piranha solution was then heated to 90 °C in a water bath for 45 min and allowed to cool to room temperature. The silica window was removed from the Piranha solution and rinsed with a copious amount of *UHQ DW*. The silica window was cleaned immediately before the assembly of the Raman sample stage.

Figure 5.3 presents a schematic diagram of the Raman sample stage. The window was held in within the inner circle of the *PTFE* dish with a tightly fitting O-ring and a few drops of squalane (refractive index matching fluid) was placed on the underside of the window before assembling the window and prism. The Teflon dish and optics were then inserted into brass water jacket. To prevent contamination of the silica window, the upper surface was kept wet with *UHQ DW* during the assembly process. To check for cleanliness after assembly, a Raman spectrum of the window in contact with pure water was acquired. If there were no peaks in the C-H stretching region, we measured Raman spectra of the water background with the acquisition times, excitation laser intensity, and polarisations that would subsequently be used for the lipid spectra, in order to provide good backgrounds for subtraction of the water signal from the lipid and peptide signals.

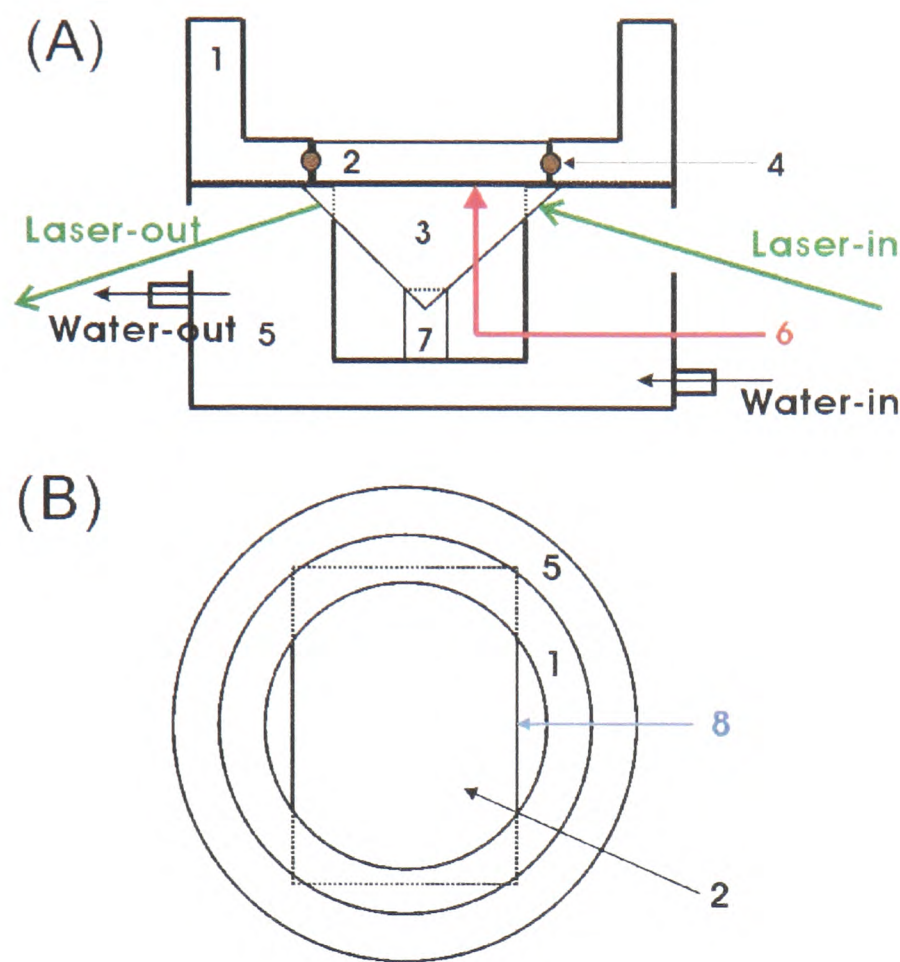


Figure 5.3 Schematic diagram of Raman sample stage of (A) side-view and (B) overview: 1. *PTFE* dish holder, 2: silica window, 3: right-angle glass prism, 4: O-ring, 5: brass water jacket, 6: squalane at interface between silica window and prism, 7: *PTFE* support of prism, 8: contact of prism to brass water jacket for heat conduction to silica window.

5.7 Formation of planar supported lipid bilayers

First the cell was stabilised at a temperature above the main phase transition temperature of phospholipid by increasing the temperature of the water circulator. Our cell required about 30 *min* of heating and an additional 30 *min* for complete thermal equilibrium. Water in the *PTFE* sample dish was removed by disposable glass pipette and the window was dried with an air blower. Then a 200 μL aliquot of the phospholipid *SUV* solution, prepared in advance, was placed on the silica window close to the centre of the objective lens which would collect Raman photons. Immediately, a fresh glass

coverslip taken from a new box was placed on top of the *SUV* droplet to generate a thin film of solution between the coverslip and the silica window. Care was taken to ensure that air bubbles were not trapped beneath the coverslip. The window was incubated for 45 *min* while maintaining the cell temperature above the temperature of the main phase transition. During the incubation period, additional buffer solution/*UHQ DW* was placed around the edge of the coverslip to suppress evaporation of water from the *SUV* dispersion and prevent the sample from drying out. After the incubation period, more drops of buffer solution/*UHQ DW* (depending on the experiment) were added to edge of the coverslip until the coverslip detached itself from the silica window. The coverslip was then removed with tweezers without it touching the silica surface. The surface was rinsed slowly and gently with buffer solution/*UHQ DW* using at least 10 times the total volume of the sample dish holder. Finally, we checked the Raman spectrum in the C-H stretching region and then compared it with reference spectra (*e.g.* of hexadecyl trimethylammonium bromide (*C₁₆TAB*) on silica, which were expected to be around half the intensity of the Raman spectrum of a phospholipid *pslb* (Greene, P. R., 2003)). If the intensity was stronger than expected, the sample was rinsed further to ensure that loosely bound vesicles were removed from the surface of the window leaving only a *pslb*.

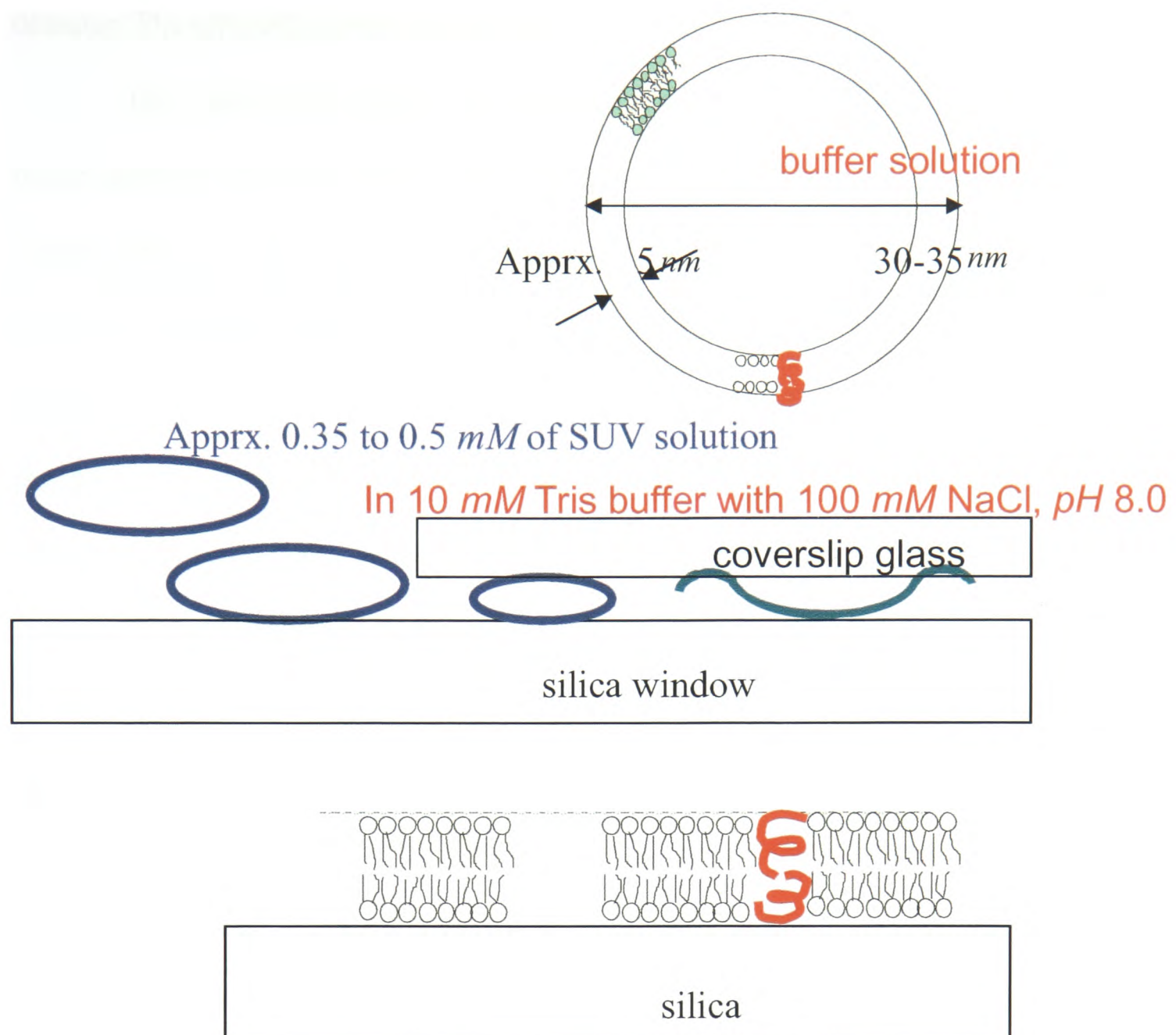


Figure 5.4 Formation of phospholipid *pslb* on a silica window by vesicle fusion.

5.8 Raman spectrometer

A commercial Raman system was used (Renishaw Ramascope System 1000, Wotton-under-Edge, U.K.) (Williams, *et al*, 1994). The scattered light was collected by a Leica DM LM microscope and directed into the Renishaw spectrometer, where it passed through holographic notch filters that remove Rayleigh scattering. A removable polarizer and a removable half-wave plate were in the path of the collected light, allowing the polarisation of Raman scattering sent to the detector to be selected. A diffraction grating was used to disperse the scattered photons onto a Peltier-cooled CCD

detector. The GRAMS software package controlled the spectrometer.

The light source used in the Raman experiments was a 2-W frequency-doubled diode-pumped Nd:YVO₄ laser, emitting light at a wavelength of 532 nm (Millennia II, Spectra-Physics). The nominal power ranged from 200 to 1800 mW in my experiments. 10 % of the laser beam was split off for use along the “internal” beam path. The “internal” beam path was guided by the laser when the spectrometer was being used as designed: the light entered the back of the spectrometer and was reflected by the holographic notch filter down the Raman microscope, where it was focused onto the sample. The remaining 90 % of the laser beam traveled along the “external” beam path, the optical path that was designed for use in *TIR*-Raman experiments, which does not involve the pump laser light passing through the internal optics of the spectrometer.

Figure 5.5 presents the Raman optical bench viewed from above. The laser beam in the “external” beam path was focused by a 100 mm plano-convex GRADIUM[®] lens (GPX-10-40, effective focal length of 40 mm) (continuous varying index lens which achieved better focusing resulting in small spot and power density) onto the water-silica interface, just above θ_c for the interface studied.

From the laser head, the light excited the laser vertically polarised, and in order to select polarisation a quarter-wave plate was placed in the beam path. The waveplate was mounted in a rotation mount in such a way that clockwise rotation of 37.5 ° based on the central position of 0 ° (anticlockwise rotation to 352 °) corresponded to *s*-polarisation, and 352 ° to *p*-polarisation (these values would change if the waveplate was remounted). The definitions of the polarisation of the excitation (*s* or *p*) and of the detected light (*x* or *y*) are shown in the Figure 5.6 (B). The vector of the electrical field in *s*-polarised incidence was parallel to the *y*-axis of the detection, while *p*-polarised excitation was parallel to the *z*-axis in the laboratory coordinates. A fixed 10× beam expander enlarged the laser beam to ~ 13 mm diameter. An adjustable 1.24× beam

expander was also placed in the beam path to allow fine adjustment of the size of the laser beam at the final focusing mirror. The mirror that directed the laser onto the sample and the focusing lens were both mounted on manual micrometer motion stages to allow changes in the incident angle as shown in the Figure 5.5 and 5.6 (A). The pump laser was directed such that it was incident at the water/silica interface just above θ_c (around 68°) ($\theta_c = 65.8^\circ$). The *TIR* spot had an ellipsoidal shape when it was optimised for Raman detection and its size along the long axis was $80\ \mu\text{m}$ and short axis $60\ \mu\text{m}$ (Figure 5.6 (C)).

The beam was incident on the prism surface at an angle of 12° to the surface normal. Off-normal incidence introduced astigmatism, *i.e.* the focal plane in the plane of incidence and perpendicular to this plane were in different places. For a 12° angle of incidence, the astigmatism was insignificant. However, if the focusing lens had a large $f/\#$, some of the light had a much higher angle of incidence and astigmatism became significant. Initially, a spot size of ca. $12\ \text{mm}$ was used at the gradium lens, giving an $f/\#$ of around 3.3. With these parameters, I had difficulty in generating a spot at the window–water interface anywhere near the diffraction-limited value. However, by reducing the $f/\#$ I was able to decrease the observed spot size even though the diffraction limited spot is larger, since astigmatism is reduced.

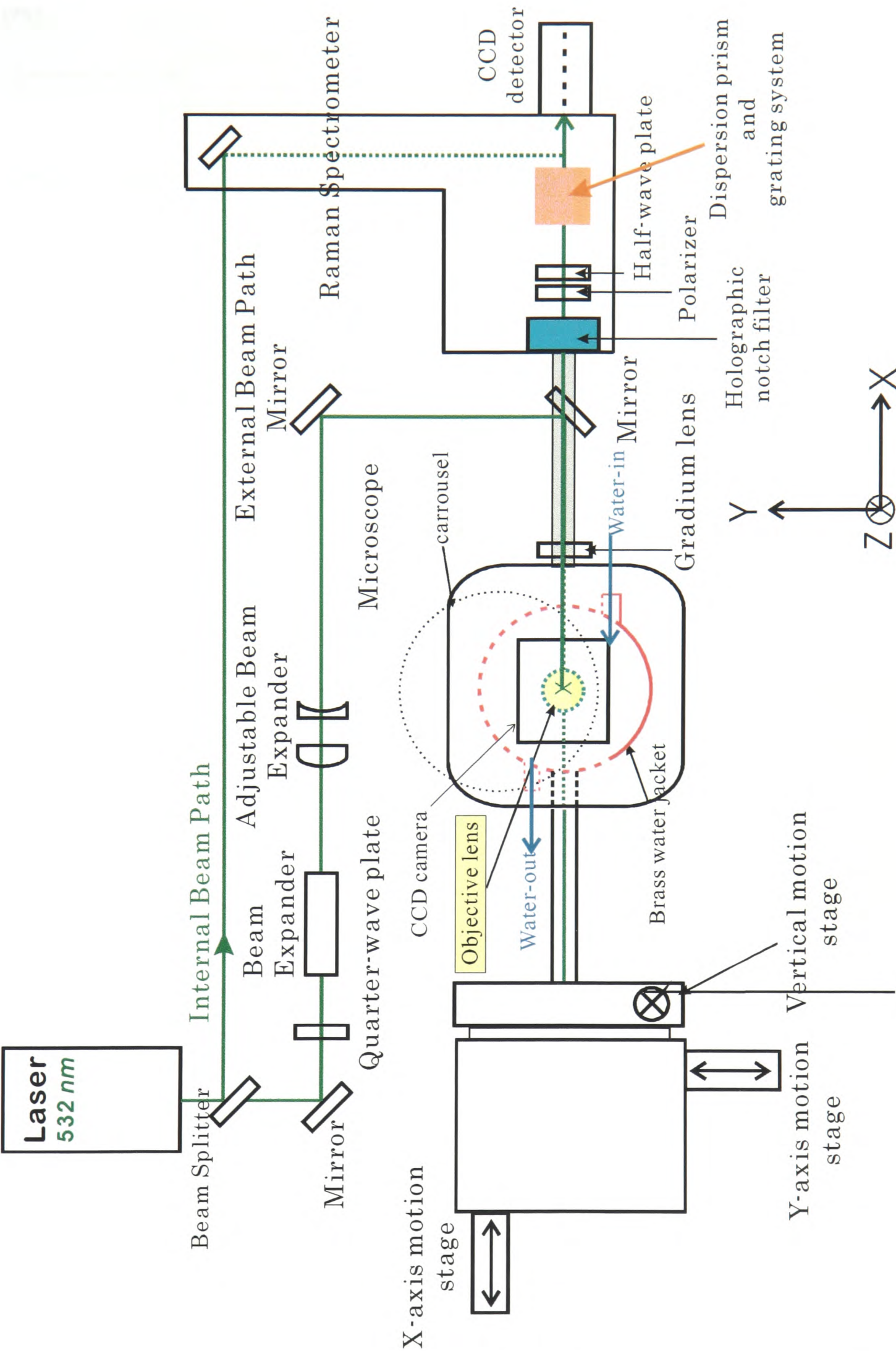


Figure 5.5 Schematic diagram of the optical path in Raman bench by overview.

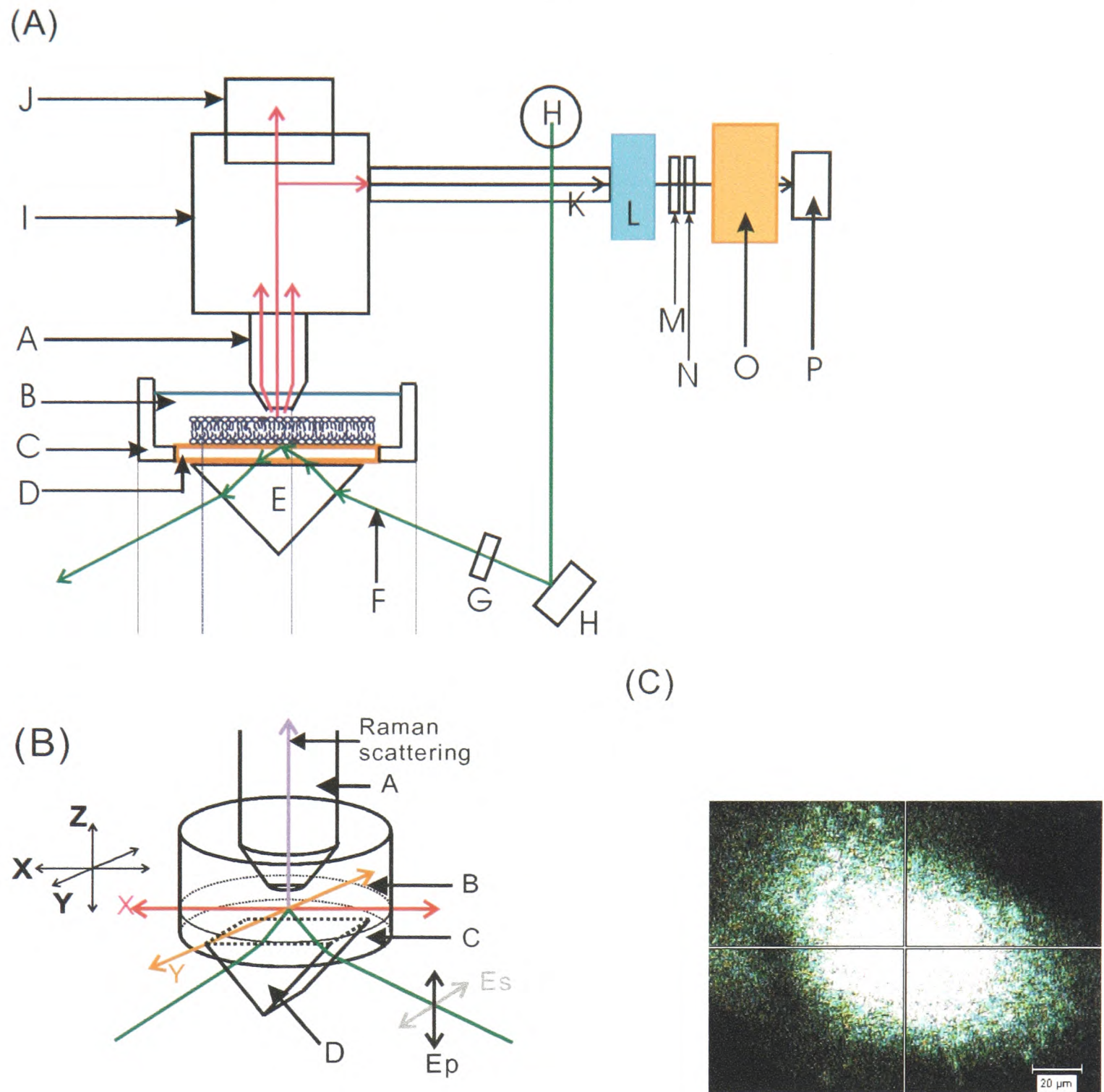


Figure 5.6 (A) Schematic diagram of Raman stage of *TIR*-Raman spectroscopy (A: objective lens; B: *UHW DW* or buffer solution; C: *PTFE* sample dish; D: fused silica window; E: boron silicate prism; F: incoming laser beam; G: GRAIUM[®] lens; H: mirror; I: microscope; J: *CCD* camera; K: optical path to monochromator and *CCD* detector); L: holographic notch filter; M: polariser; N: half-wave plate; O: dispersion prism and grating system; P: *CCD* detector. (B) Definition of laboratory coordinates and polarisation directions defined for *TIR* Raman experiments. (C) 0.2 *W* (set intensity of laser head) *s*-polarised *TIR* spot captured by *CCD* camera at water/*DPPC* bilayer/silica interface at 35 °C.

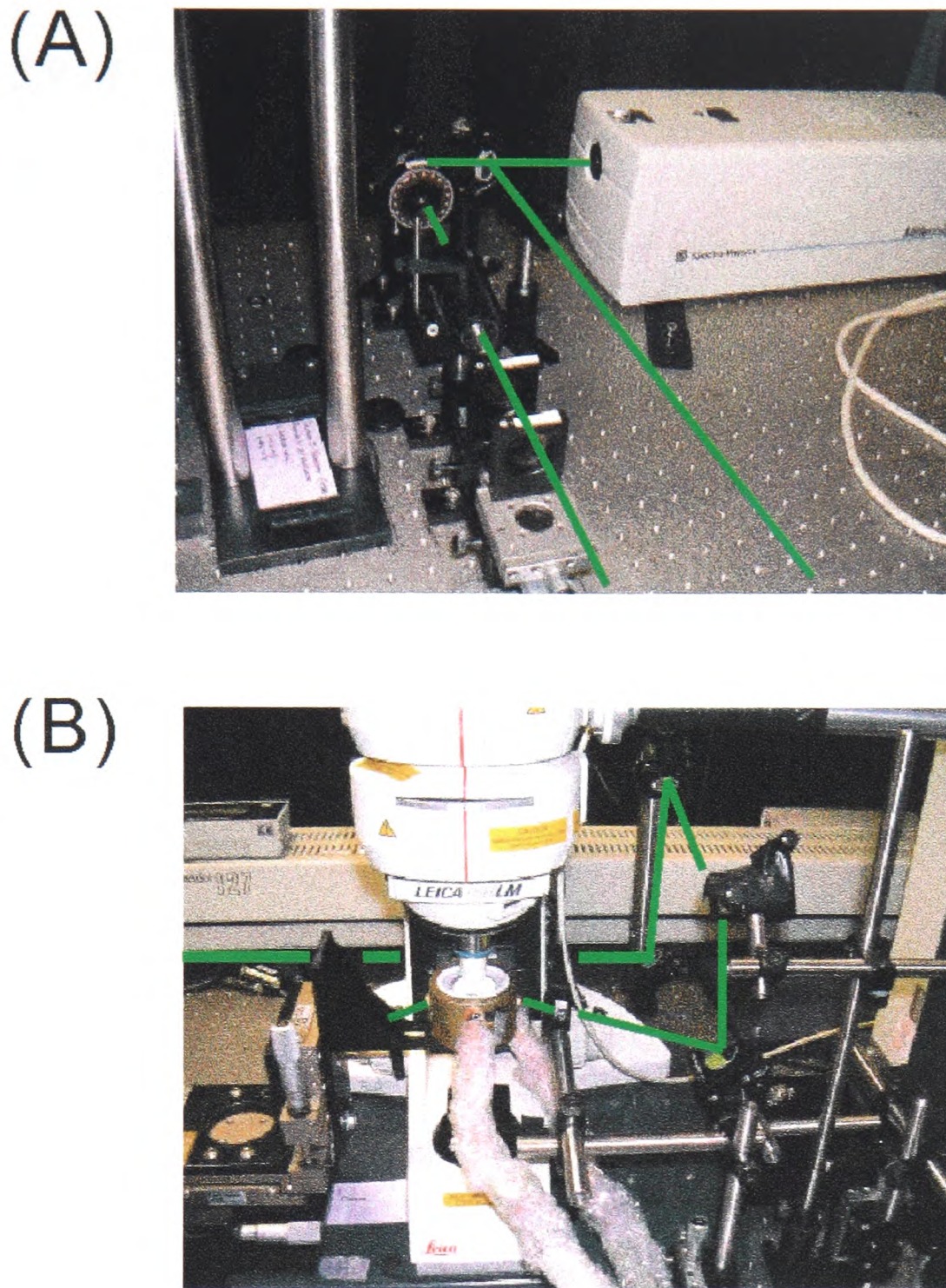


Figure 5.7 Photographs showing (A) the internal and external beam paths from laser head and (B) Raman sample stage.

5.9 Measurement of phase transitions in bulk suspensions (for chapter 8.5)

Spectra of the bulk suspension of *DMPC* vesicles were recorded in capillary tubes (1-mm diameter, ~ 2 mM lipid) in a temperature-controlled cell, using the internal beam path of the Raman spectrometer. The pump beam was focused to a 2- μm diameter spot just below the inner wall of the capillary to minimise light scattering from larger aggregates in the suspension. The temperature was measured with a thermocouple placed adjacent to the capillary tube. The nominal power was 30 mW. No polarisation selection was applied to the backscattered light.

5.10 Calibration of laser intensity

Prior to Raman measurement, the power of laser at the final prism was calibrated against the nominal power at the laser head. (Subsequently, it is the nominal power that is quoted in each experiment.) The pump laser was delivered through the external path via a beam splitter (Figure 5.5) that reduced the laser power by ca.10 % (Table 5.1). There were additional losses at each optic in the beampath so the final power was approximately 65 % of the nominal power. There was an additional 5 % loss at the entrance face of the final prism and a much smaller loss at the interface between the BK7 prism and the silica window. The reflection loss at the prism surface could be eliminated with antireflection coatings, but the spectra were not in general obtained at maximum laser power.

The Figure 5.8 plots the measured power versus the nominal power of the laser head. There is a good linear correlation up to 1.4 W nominal power.

Nominal power of laser head / W	Measured power of laser after final lens / W
0.2	0.121
0.4	0.254
0.6	0.391
0.8	0.530
1.0	0.663
1.2	0.813
1.4	0.950

Table 5.1 Calibration of the laser power for *TIR* setup.

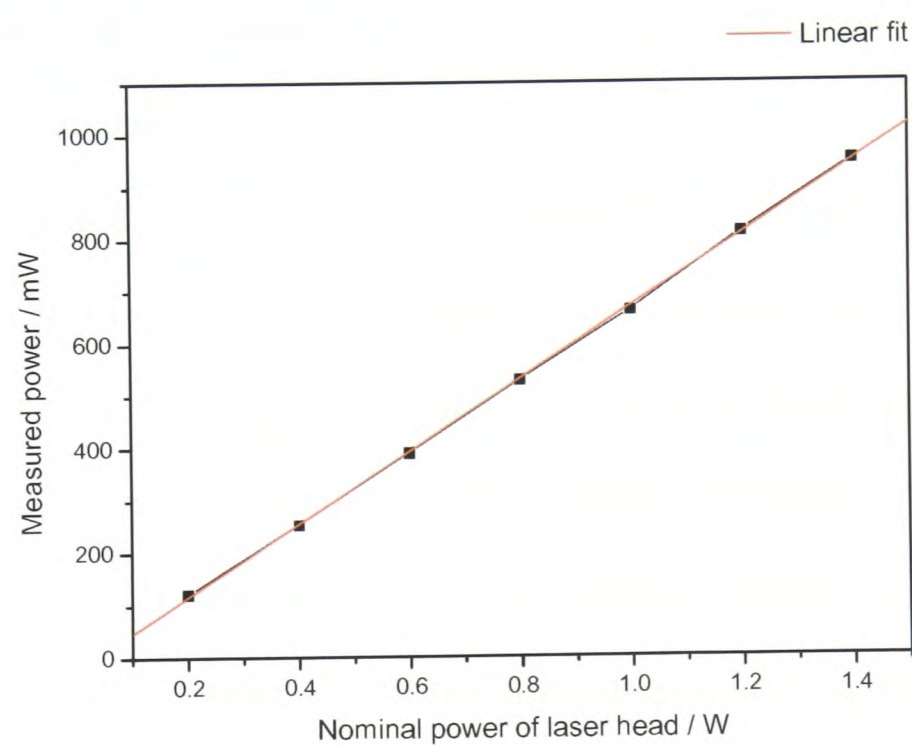


Figure 5.8 Linear calibration of the actual power of laser ahead of the prism.

5.11 Sample heating and damage (for chapter 8.1)

To test whether the pump laser caused significant heating of the lipid bilayer, we compared the spectra of *DMPC* bilayers at 0.3 *W* increments in the nominal power from 0.3 to 1.8 *W* at a temperature of 22 °C, where the Raman spectra were sensitive to small changes in temperature. The reading of the temperature while the laser shined at water-silica interface was performed by contact of a thermocouple in the vicinity of the

exposed spot on the silica window (with goggle-worn). Raman spectra were acquired for 225 s each on the same spot of the surface. Within each series, the spectra were indistinguishable to within a scale factor arising from the different power levels as explained in Figure 8.2 in chapter 8.1. We conclude that the pump laser caused no significant heating of the lipid film. To test for sample damage, consecutive Raman spectra were acquired for 30 min each on the same area of the surface. No change in the lipid spectrum was observed.

5.12 Preparation of peptide/protein solutions

Indolicidin was weighed on a 5-figure balance to nearest 0.1 mg and dissolved in pH 7.12 10 mM phosphate buffer solution with 150 mM NaCl to the desired concentration of 12 μ M.

The required amount of PLA_2 was dissolved in 10 mM Tris buffer (pH 7.4) to give a solution at 0.1 mg/mL. This solution was divided into small portions and stored in the freezer (-18 °C) for up to three months. Solutions made using lyophilised porcine pancreatic PLA_2 were made using known weights of protein. When used as a 1 M NH_4SO_4 suspension, it was diluted with the required amount of buffer. Wear gloves! PLA_2 may be lethal if it enters the blood, therefore avoid sharp objects while handling it!

5.13 Procedures for lipid-surfactant mixtures (for chapter 10 and 11)

A 1:8.6 mixture by molar ratio of DPPC to β -D-dodecyl maltoside (DDM) was prepared from 4.6 mg of the phospholipid and 27.6 mg of the surfactant (FW = 511 g mol^{-1} undeuterated), taking into account any deuteration. The mixture was dissolved in 5 mL of UHQ DW, dispersed by sonication and gently heated using warm tap water, then stirred on a magnetic hot plate at room temperature overnight (min. 6 h). This gave a clear stock solution at a concentration of 6.44 mg/mL (1:8.6, mol/mol).

This stock solution was diluted by a factor of 50 with *UHQ DW*, e.g. 100 μL + 4900 μL *UHQ DW* to obtain solution (1) with concentration of $\sim 0.1 \text{ mg/mL}$. Solution (1) was diluted by a factor of 10 with *UHQ DW*, e.g. 500 μL of solution (1) + 4500 μL *UHQ DW* to give solution (2) with concentration of $\sim 0.01 \text{ mg/mL}$. Solution (2) was diluted by a factor of 10 with *UHQ DW*, e.g. 500 μL of solution (2) + 4500 μL *UHQ* water, to give solution (3) at a concentration of $\sim 0.001 \text{ mg/mL}$. (The critical micelle concentration of *DDM* is 0.08527 mg/mL (Hines *et al*, 1998).)

To form a *pslb* of the phospholipid, solution (1) was first incubated with a clean silica substrate with occasional agitation of the solution for 60 *min*. The bulk of the solution was removed with a micropipette and was replaced with clean *UHQ DW*. The *UHQ DW* was replaced twice more (five times for *d₇₅-DPPC-h-DDM* experiment), then left for 15 *min*. *TIR*-Raman spectra were measured in a time series of each 45 *s* until the spectra did not change with time, at which point it was assumed that all the (soluble) surfactant had been removed from the lipid bilayer. In the second stage, solution (2) was incubated with the bilayer formed from solution (1) for 60 *min* with occasional stirring/agitation with a micropipette. Solution (2) was removed, replaced three times with clean *UHQ DW*, and left for 15 *min*. Again, *TIR* Raman spectra were measured in a time series until the spectra did not change. The procedure was repeated with solution (3) with 60 *mins* incubation, after which solution (3) was removed and the sample rinsed three times with *UHQ DW*.

5.14 Preparation of *d₇₅-DPPC vesicle solution*

d₇₅-DPPC (Avanti Polar Lipid, 4.0 *mg*) was dispersed in 4.0 *mL* of *UHQ DW* and bath-sonicated at 54-60 °C until the solution was homogeneous (3 times 15 *min* + extra more *min* till it looked very clear). Then the solution was diluted to a final lipid concentration 0.5 *mg/mL*. It was equilibrated at 60 °C for 10 *min* and bath-sonicated

for 15 *min* at 60 °C. We didn't do ultrasonication procedure to make *d*₇₅-*DPPC* vesicle solution because bath-sonication could make solution clear and so it was not necessary to make *SUV* solution and there was concern of possible H-D change in buffer/*UHQ DW* during ultrasonication.

For Raman experiments, the solution was loaded onto the pre-heated silica window at around 44-45 °C (the water bath circulator was set to 65 °C due to the heat loss through the hose connecting the circulator to the Raman sample stage). After incubating the vesicles with the silica window for 5 *h* at 44-45 °C, the vesicle solution was cooled down to 21 °C and then rinsed away with a copious amount of *UHQ DW*. Raman spectra were taken at intervals during the rinsing until they no longer changed with further rinsing.

Chapter 6 Reconstituted vesicles in solution

This chapter describes the characterization of the phospholipid vesicles that I used to build the supported phospholipid bilayers at the water/silica interface. The formation of the planar supported lipid bilayers was made by the direct fusion of small unilamellar vesicles (*SUVs*) from solution. This method is one of the more well-studied protocols for forming lipid bilayers mentioned in Chapter 2. Various phospholipids were considered for my Raman experiments. Biomembranes are normally in a fluid state due to the presence of unsaturated phospholipids. My early experiments used palmitoyl oleoyl phosphatidylcholine (*POPC* from egg yolk), which forms a membrane in a liquid crystalline state at room temperature. However, unsaturated phospholipids contain a strong band in the Raman spectrum from the C=C bond that obscures the Amide I vibration and some of the tryptophan ring vibrations. We therefore chose two of the most common saturated phosphatidylcholines, dimyristoyl (*DMPC*) and dipalmitoyl (*DPPC*). *DMPC* is in its liquid crystalline phase at 23.6 °C (around room temperature), while *DPPC* is in the gel phase. An unsaturated phosphatidylcholine was chosen to probe the molecular orientation of sp^2 carbon bond in the acyl chains of lipid bilayers at water/silica interface by *TIR*-Raman scattering using the polarisation technique.

As described in Chapter 5, *DMPC*, *DPPC*, and *POPC* were reconstituted as small unilamellar vesicles. The chemical and physical analysis of these vesicles is described in the following subchapters.

6.1 Phospholipid vesicles

The *SUVs* in solution were characterised in terms of the final concentrations of

the phospholipids and other constituents forming the vesicle. *UV/Vis* absorption spectroscopy was used to determine the concentration of phospholipids in a solution of *SUVs* after ultracentrifugation. Since lipids absorb only weakly in the near *UV*, a lipid soluble dye molecule, *DiOC₁₈*, was added at molar ratio of 0.5 %. Figure 6.1 shows the absorption spectrum of the *DiOC₁₈* chromophores in lipid bilayers of *DMPC*. The preparation of the *DMPC SUVs* was described in the experimental section of Chapter 5.

Assuming the formulation ratio of 200 to 1 (moles of lipid to moles of dye) is maintained in the *SUVs*, the final concentration of lipid may be calculated by application of the Beer-Lambert law:

$$A = \varepsilon \cdot c \cdot l \quad (6.1)$$

where $A = -\log_{10}(I/I_0)$ is the absorbance, I is the transmitted light intensity, I_0 is the transmitted light intensity in the absence of chromophores, ε is the molar extinction coefficient of the chromophore in $[M^{-1} \cdot cm^{-1}]$, c is concentration in $[M]$, and l is the beam pathlength of quartz cuvette in $[cm]$. *DiOC₁₈* had an absorption maximum at 491 nm, concomitant with the literature (Bhowmik, *et al*, 2001).

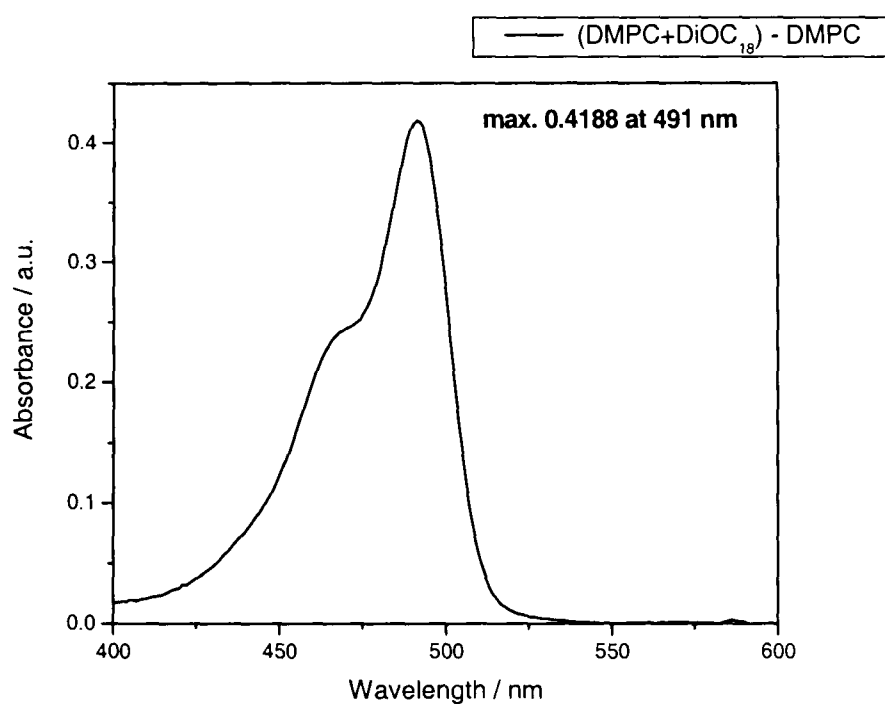


Figure 6.1 Visible absorption spectra of *DiOC₁₈* in *DMPC SUVs*. (*DMPC* of initial concentration of 1.356 g/L and 200 : 1 molar ratio of *DiOC₁₈*)

In this experiment, the final concentration of the lipids in the *SUVs* was 0.64 *mM* (~ 0.43 g/L). The yield was 32 %, and the remaining 68 % of the *DMPC* molecules were lost during the hydration by ultrasonication or the separation of the *SUVs* from multilamellar aggregates by ultracentrifugation. The statistically averaged concentration of lipid in *DMPC SUVs* was 0.55 *mM* (~ 0.37 g/L). The yield may be dependent on the condition of the tip of the ultrasonic horn, on the ultrasonic conditions in the water bath used for rehydration, on the temperature control during the hydration steps, and on the dryness after the lipid was mixed with the dye in chloroform. The average concentrations of solutions of *SUVs* of the other phospholipids were 0.35 *mM* (~ 0.27 g/L) for *POPC* and 0.50 *mM* (~ 0.37 g/L) for *DPPC*. The *POPC SUVs* were less productive than other *DMPC* and *DPPC SUVs*. One possible explanation is that the unsaturated acyl chains are vulnerable to oxidation during the harsh process of ultrasonication above the melting temperature of the lipid. With these final concentrations, one 100- μL drop contains 10 to 15 times the amount of lipid needed to cover the surface of a 30-*mm* diameter fused silica window with a lipid bilayer, based on a surface excess of $5.0 \mu\text{mol} \cdot \text{m}^{-2}$ for a *DMPC* planar supported bilayer in the liquid crystalline phase (L_α) (Lee & Bain, 2005).

The size of the *SUVs* in the solution was kindly determined for us by Dr. Jeroen van Dijnveldt at Bristol University by photon correlation spectroscopy. The vesicles of average diameter 35 *nm* were evaluated. I could not confirm the unilamellarity of the *SUVs* in solution. Although the lamellarity of *SUVs* was not confirmed by any experiment, I could predict unilamellarity considering that such a small vesicle cannot retain a multimellar nature without a large amount of bending strain.

6.2 Gramicidin D-incorporated phospholipid vesicles

Gramicidin D (*gD*) was chosen as a model channel peptide, as described in the

introduction section of Chapter 4. Here I describe both the quantification of the amount of *gD* incorporated in the *SUVs* and considered the effect of the cosolvent used for hydration on the structure and function of the peptide in the lipid bilayer. For the Raman scattering measurement of this model peptide in lipid bilayers, we want the biophysically stable conformation to be predominant. With trifluoroethanol of cosolvent, the channel form in the *SUV* could be differentiated from other nonchannel forms in *DMPC* bilayers by circular dichroism.

6.2.1 Aspects of the physical chemistry

The Figure 6.2 shows the *UV* absorption spectra of *gD* in *DMPC SUVs*. To obtain the *UV* absorption spectra of *gD* of the Figure 6.2 (B), the background from the *DMPC SUVs* alone was subtracted from spectrum of the *DMPC-gD SUVs*, shown in Figure 6.2(A).

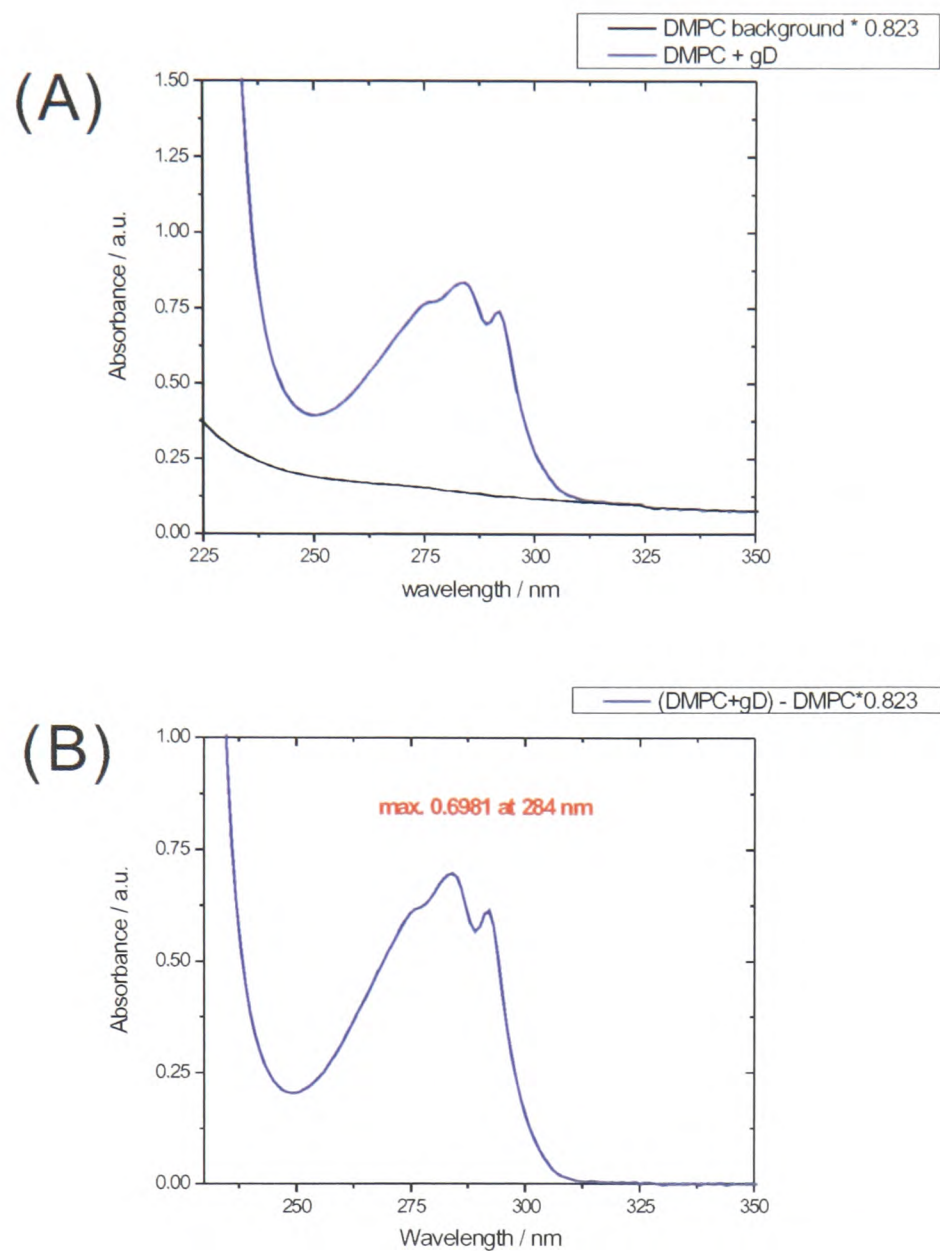


Figure 6.2 *UV* absorption spectra of (A) *DMPC SUV* and *gD*-incorporated *DMPC SUV* and (B) subtracted *gD* by *DMPC SUV*.

In the Figure 6.2(B), we see the *UV* absorption bands of tryptophan in *gD* which corresponded well to data in the literature (Yamamoto & Tanaka, 1972). It has been proposed that the *gD* absorption spectrum can be decomposed into three dominant transition moments for the electronic transitions of tryptophan: ${}^1L_a(\tilde{B} \leftarrow \tilde{X})$ at 273 nm, ${}^1L_b(\tilde{A} \leftarrow \tilde{X})$ at 285, 292 nm, and $(\tilde{C} \leftarrow \tilde{X})$ at 217 nm as shown in Figure 6.3 (Yamamoto & Tanaka, 1972).

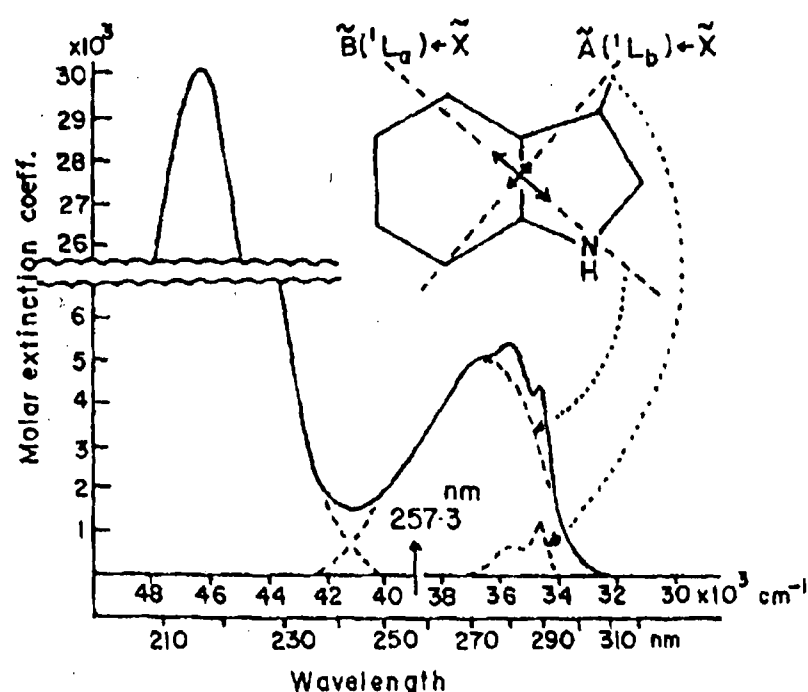


Figure 6.3 Resolution of the *UV* absorption bands in tryptophan. The figure was taken from the article by Yamamoto & Tanaka (Yamamoto & Tanaka, 1972).

We calculated the concentration of *gD* in the *DMPC SUVs* using the Beer-Lambert law in the same way as we calculated the lipid yield earlier. When trifluoroethanol was used as the cosolvent for mixing the lipid with *gD*, the concentration of *gD* in the final solution after centrifugation was typically 28–30 μM (~ 0.064 g/L). We estimate a molar ratio of 20 to 1 of *DMPC* to gramicidin in the *DMPC-gD SUV*. This value compares with a ratio of 10 to 1 in the initial formulation (initial concentration of *gD* of 0.429 g/L). This molar ratio is sufficiently high that we can confidently study *gD* in the channel form with no possibility of *gD* forming hexameric aggregates, which have been observed at lower ratios (Killian, *et al*, 1987; Spisni, *et al*, 1983).

The *UV/Vis* spectra verify the existence of *gD* in the *SUV* solution, but do not differentiate between molecules of gramicidin in an aqueous or a lipid environment. For this, we turn to fluorescence. The presence of *gD* in a lipid environment is deduced from the fluorescence spectra of *gD* in the *POPC SUV* shown in Figure 6.4 (A). The fluorescence maximum of *gD* in the *POPC SUV* is at 332–334 nm of the emission compared to 345 nm in methanol. These maxima are exactly matched with the literature

(Cox, *et al*, 1992). The emission maximum in the *gD* dissolved in methanol is red-shifted since the polar solvent stabilizes the highly dipolar excited state of the indole ring. Since the excited state is sufficiently long-lived for the Frank-Condon excited state to decay to the equilibrium excited state before fluorescence, the fluorescence spectrum is sensitive to the polarity of the environment of the indole rings.

Also Figure 6.4 (B) presents the fluorescence emission spectra of *gD* in *DMPC* *SUV* prepared using different cosolvents of benzene, tetrahydrofuran, and trifluoroethanol. The spectra are characterised by the emission band at 333 *nm* indicating that *gD* is accommodated in lipid. The effect of these various cosolvents on the solvent-dependent conformations of *gD* in lipid are considered in Chapter 6.2.2.

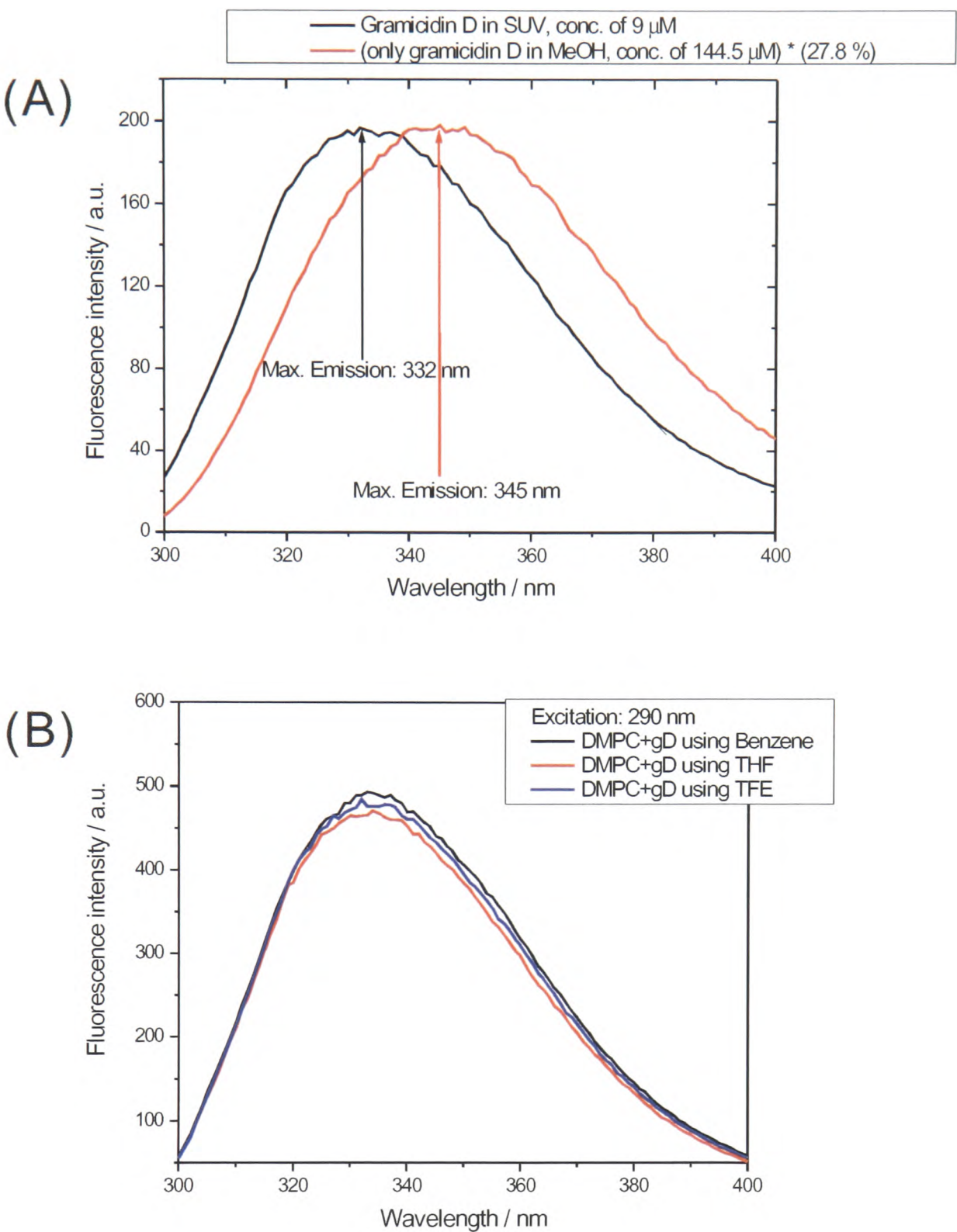


Figure 6.4 Fluorescence spectra of (A) 9- μ M gD in *POPC* SUV (in black) and in methanol (in red), (B) gD in *DMPC* SUV with different cosolvents of benzene, tetrahydrofuran, and trifluoroethanol. Excitation wavelength = 290 nm.

6.2.2 Aspects of Biophysics

The conformation adopted by gramicidin is dependent on its environment. The conformation is very different in bulk solutions of relatively hydrophobic solvents (Arseniev, *et al*, 1984; Bystrov & Arseniev, 1988; Veatch, *et al*, 1974) and in lipid bilayers (Urry, 1971; Urry, *et al*, 1971).

In organic solvents like chloroform, dioxane, ethanol, and methanol, it prefers to adopt an intertwined double-stranded β -helical form (depicted in Chapter 4). The helicity has been discovered in predominantly $\beta^{5.6}$ and $\beta^{7.2}$ forms (Arseniev, *et al*, 1984; Bystrov & Arseniev, 1988; Veatch, *et al*, 1974). However, all of the possible conformations are always coexistent in spite of any effort to isolate a certain helical form via organic solution state.

In lipid bilayers, the structural characteristics of the hydrophobic shell with an hydrophilic inner hole intrigues shaping a channel under surroundings of acyl chains. The four bulky tryptophan residues play a role of a formation of a channel (Wallace, 1991). There are also some variables precluding channel formation such as effect of the length of the acyl chain in lipid molecules (Van Echteld, *et al*, 1982). Further studies of acyl chain effect could explain the observation that unsaturated lipid in bilayer may promote channel formation (Sychev, *et al*, 1993).

Gramicidin has also solvent-dependent conformations when it is dissolved with lipid by cosolvent (Bano, *et al*, 1991, 1992; Killian, *et al*, 1988). Organic solvents frustrate discrete conformations. However, many papers report attempts to discriminate certain structures. Fortunately, gramicidin tends to make a channel form comprising head-to-head single-stranded β helices when trifluoroethanol (*TFE*) is used as a cosolvent for mixing with lipid molecules.

Another variable that can be used to make the channel form is the subsequent heating of the mixed *gD*/lipid vesicles (Bano, *et al*, 1991; Cox, *et al*, 1992; Killian, *et al*,

1988).

I chose to prepare the channel form by trifluoroethanol codissolution. For comparison I tried to make other, non-channel conformations with different solvents, but only succeeded in generating mixtures of conformations. Even with an organic cosolvent like benzene or tetrahydrofuran, it was not seemingly possible to achieve predominant intertwined double-stranded β -helices in lipid bilayers. The spectroscopic data show the structures always coexist as a mixture of non-channel forms under the influence of the likelihood of channel-forming.

Figure 6.5 shows the circular dichroism (*CD*) spectrum of *gD* incorporated in the bilayer of the *DMPC SUVs*. The circular dichroism is the difference in extinction coefficient, $\Delta\epsilon = \epsilon_L - \epsilon_R = \frac{(A_L - A_R)}{c \cdot l}$, between the left- (anticlockwise rotating) and right-handed (clockwise rotating) polarised light due to the optical activity of the peptide backbone, which tends to absorb the two plane-polarised lights differently. (Here ϵ_L , ϵ_R is the extinction coefficient by left- and right-handed polar light, A_L , A_R is the absorbance of the left- and right-handed light, c is the molar concentration of the peptide or protein, and l is the pathlength of the sample cell.) The different secondary structures of peptides or proteins have characteristic *CD* spectra, so *CD* is a useful tool for examining secondary structure qualitatively and quantitatively in some cases. The output of the *CD* instruments gives the ellipticity, $\theta = 33 (A_L - A_R)$, in degrees. *CD* spectra were plotted after converting the ellipticities of reading from the spectroscope into calculation of the mean residue molar ellipticities as shown:

$$\theta_{MRW} = \theta / (10 \times c \times n \times l) \quad (6.2)$$

θ_{MRW} is the mean residue weight molar ellipticity in the dimension of *degree* $\cdot$ $cm^2 \cdot dmol^{-1}$, c is the molar concentration of the *gD* in $mol \cdot L^{-1}$, n is the number of peptide bonds in the *gD* which is 14 peptide bonds in 15 residues in this case, and l is

the pathlength in *cm*.

With trifluoroethanol as cosolvent, *gD* in the *DMPC* membrane adopts the channel form, based on a comparison of the *CD* spectrum with the literature (Cox, *et al*, 1992; Killian, *et al*, 1988).

Figure 6.5 presents *CD* spectrum of *gD* in *DMPC* membrane when trifluoroethanol was used. Characteristic peak positions of single-stranded β -helix of channel form are observed at 229 *nm* which is indicative of the tryptophan chiroptical effects (at 228 *nm* in the literature (Sychev, *et al*, 1993)), 218 *nm* of the peptide $n \rightarrow \pi^*$ transitions (Sychev, *et al*, 1993) and $\pi \rightarrow \pi^*$ transitions. Other literature explained the positive maxima at 218 *nm* and 235 *nm* with a weak negative inflection in between (at 229 *nm*) and negative ellipticity below 208 *nm* (Killian, *et al*, 1988). In particular, the channel form has a positive ellipticity at ~ 229 *nm* (Cox, *et al*, 1992). This peak position is useful for discriminating between the channel and nonchannel form (Cox, *et al*, 1992). According to these characteristic bands in the literature, I could discriminate the channel form of *gD* in *DMPC SUV*.

The *CD* spectra with *TFE* as cosolvent show the characteristic channel form irrespective of the temperature of the solution during the measurement. According to the literature, when benzene is chosen for cosolubilisation, the non-channel forms are clearly favored in the preparation (Bano, *et al*, 1991). Although I could only obtain less than half of the concentration of *gD* in *DMPC SUVs* by cosolubilisation of benzene (Figure 6.6), it was possible to measure *CD* spectra as shown in the Figure 6.7.

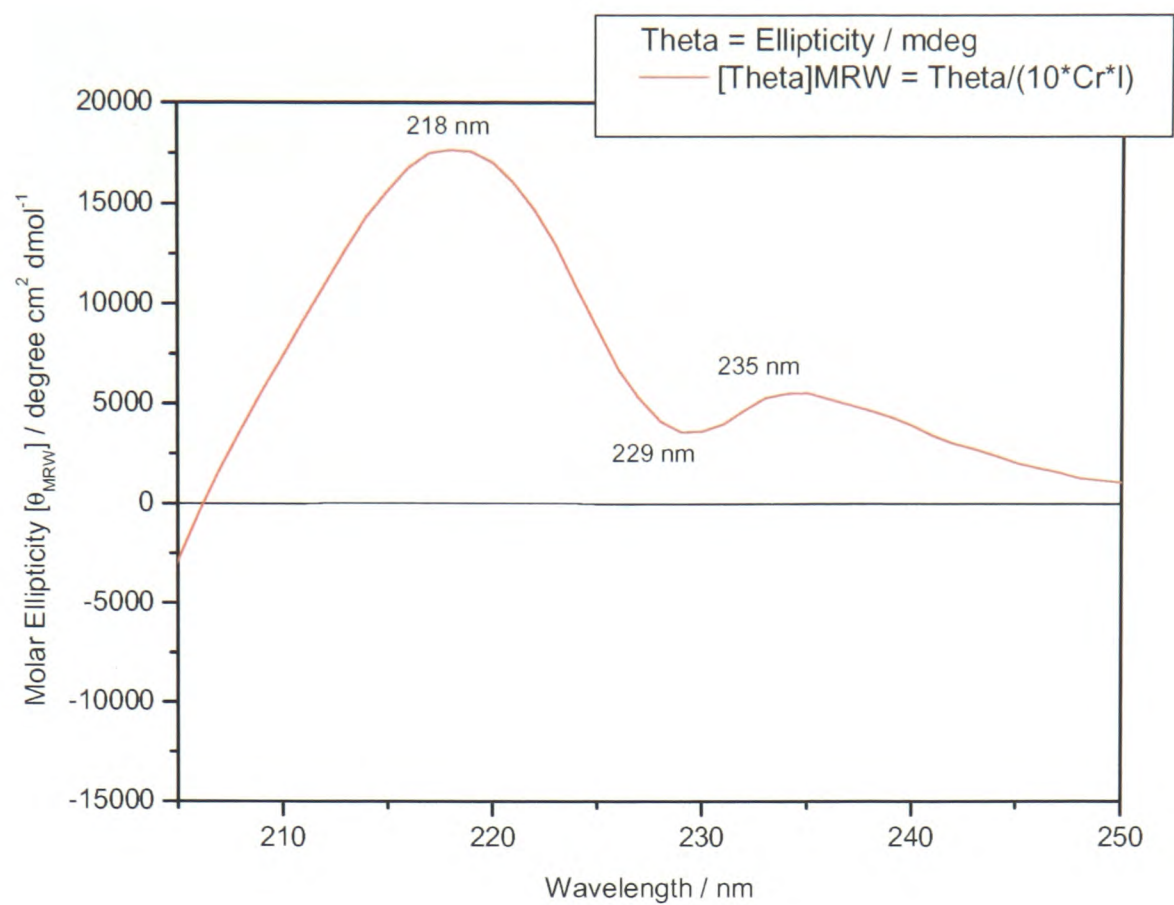


Figure 6.5 The CD spectrum of *gD* in *DMPC SUV*’s prepared with a cosolvent of trifluoroethanol. The final concentration of *gD* in *SUV* was 31.5 μ M.

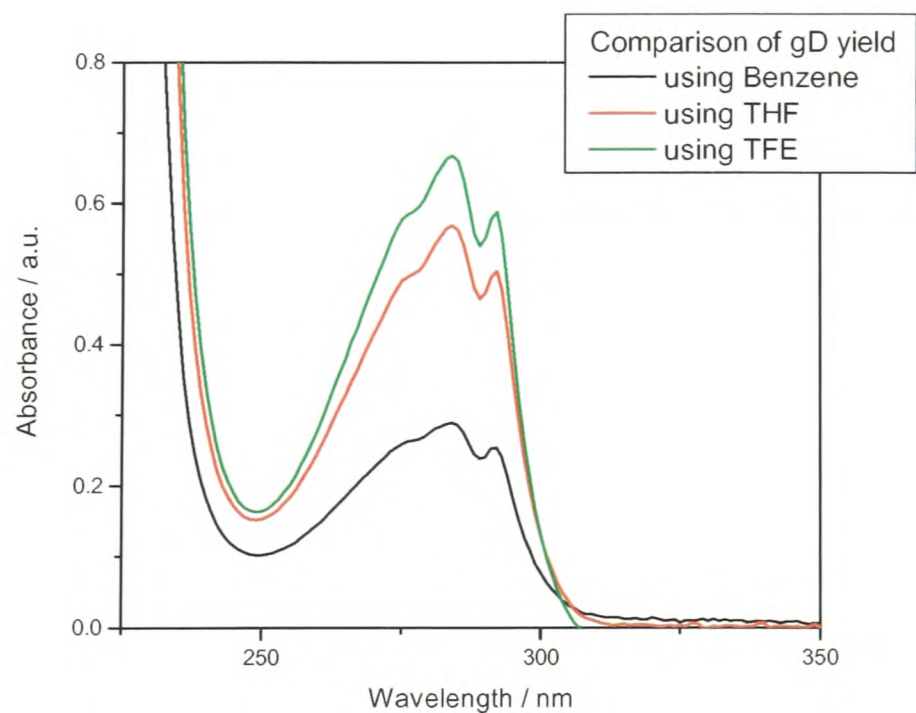


Figure 6.6 UV absorption spectra of *gD* in the *DMPC* bilayer of the *SUV*’s which were prepared using a cosolvent of benzene (in black), tetrahydrofuran (in red), and trifluoroethanol (in green). The concentration of *gD* in *DMPC SUV* co-dissolved by trifluoroethanol was 31.5 μ M calculated by Beer-Lambert law.

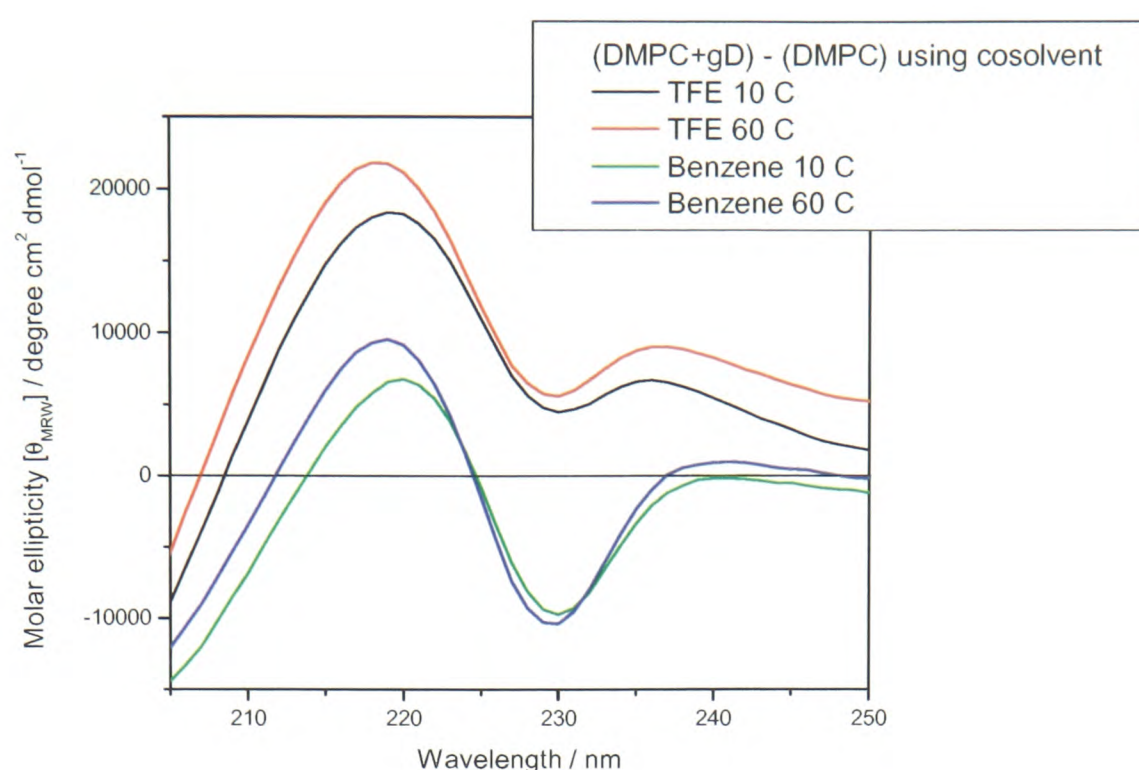


Figure 6.7 CD spectra of *gD* in the *DMPC SUV* prepared using a cosolvent of benzene (*CD* spectra were measured at the thermally equilibrium state of 60 °C indicated in red line and 10 °C indicated in black one) and *TFE* (also measured at 60 °C indicated in blue line and 10 °C indicated in green one).

In our results as shown in the Figure 6.7, the *CD* spectra of any nonchannel forms were observed as expected. When benzene was used, the spectra were characterised by a negative peak at 229 nm and a weaker positive peak at 219 nm. These spectra deviate from the typical channel form at 229 nm position. This result reveals a dynamic equilibrium between different nonchannel conformations (Killian, *et al*, 1988). In the literature, the *CD* spectra of gramicidin in the *DMPC* (1 to 10 molar ratios) have a positive curvature below 208 nm (Killian, *et al*, 1988) when ethanol is used as cosolvent. It is known that gramicidin forms the mixtures of the $\pi\pi^{5,6}$ double-stranded β helices in ethanol solution (Arseniev, *et al*, 1984), not in the phospholipid bilayer prepared by codissolution of ethanol. In the lipid bilayer the conformational transition from the $\pi\pi^{5,6}$ double-stranded β helices could be interconvertible to the right-handed

single-stranded $\pi^{6.3}$ β -helix in the *DMPC* bilayer when ethanol was used (Bouchard, *et al*, 1995). In the potentially noninterconverting mixtures used with ethanol as a cosolvent, it is possible to estimate many nonchannel conformations such as left-handed anti-parallel and parallel $\pi\pi^{5.6}$ double-stranded β -helices. In tetrahydrofuran or benzene the $\pi\pi^{5.6}$ double-stranded β -helices predominate, while still some portion of right-handed single-stranded $\pi^{6.3}$ β -helix might be possibly coexistent (Bano, *et al*, 1991; Sychev, *et al*, 1996). So generally the conformational behaviour of gramicidin in the membrane is very complex. My spectra are different from the data of ethanol-codissolution gramicidin/*DMPC* of the literature in that there was no evidence of positive curvature below 208 nm. However, in as far as a negative ellipticity at 229 nm and relatively weak positive ellipticity at 218 nm are markers of the nonchannel form, my *CD* data suggest that the non-channel form is dominant in *SUVs* formed with benzene as cosolvent, although I have not attempted to understand how much they are mixed with right-handed single-stranded $\pi^{6.3}$ β -helix of the channel form. The longer incubation (at least 12 h) by heating at 60 °C might convert the nonchannel mixtures into the stable channel (Bano, *et al*, 1991; Cox, *et al*, 1992). About the temperature effect of below or above melting point of the *DMPC* lipid molecules, I could expect more channel conformation even if the *CD* acquisition time within 10 min was done just after 10 min for thermal equilibrium at 60 °C (Bano, *et al*, 1991).

In Figure 6.8, I have more database that are well matched with the literature (Killian, *et al*, 1988), observing the *CD* spectra when using a chloroform/methanol mixture (1/1, v/v) and the change of the *CD* spectra upon heating, which is indicating that the nonchannel forms were converted into right-handed single-stranded $\pi^{6.3}$ β -helix of the channel form.

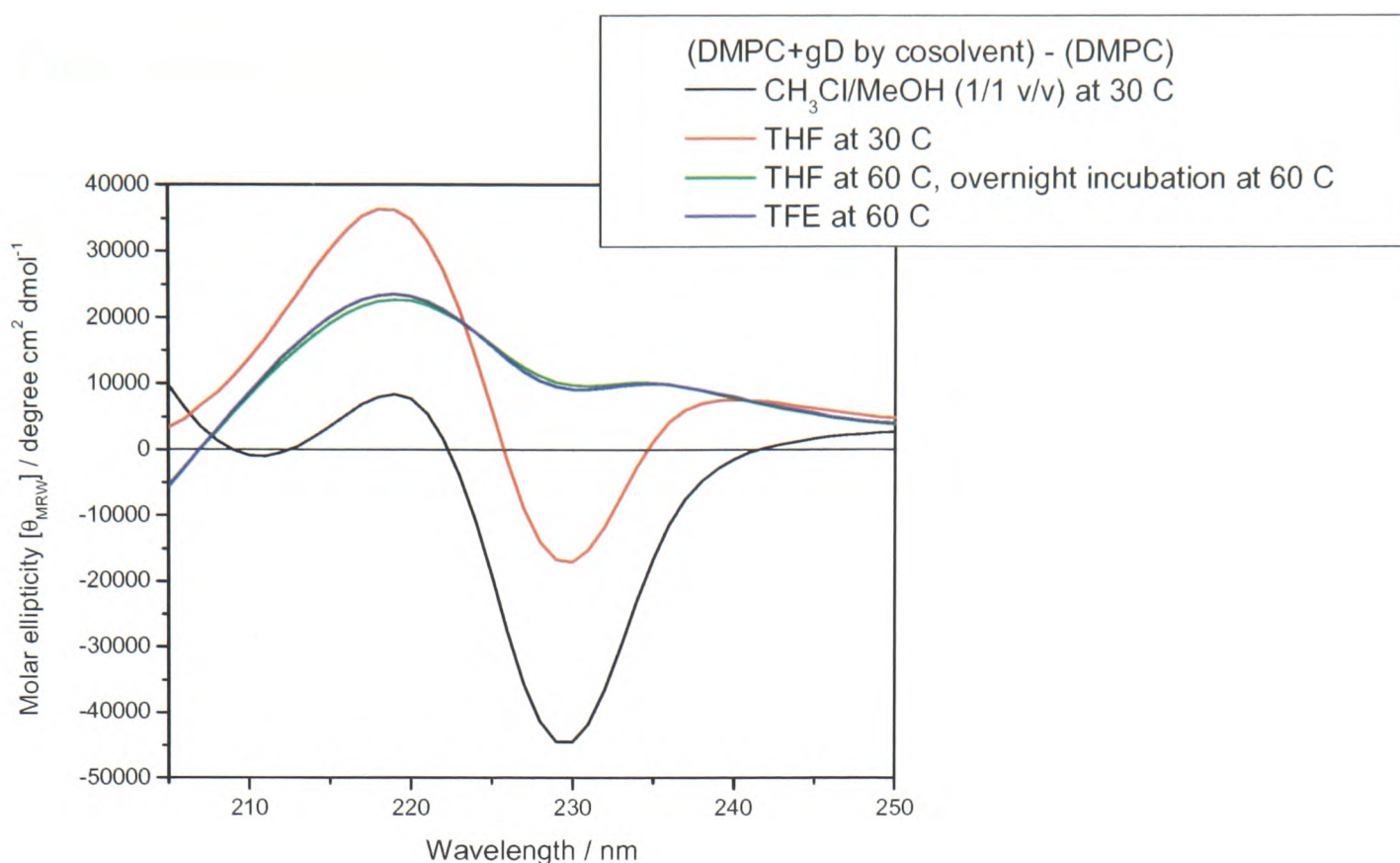


Figure 6.8 CD spectra of *gD* in *DMPC SUV* prepared using a cosolvent of chloroform/methanol (1/1, v/v) (black line), *THF* (red line), and *TFE* (blue line) The CD measurement was at 30 °C, but one sample made from *THF* was scanned at 60 °C after overnight incubation at the same temperature (green line).

The molar ratio of *gD* to *DMPC* with benzene, *THF*, and *TFE* as was calculated from the *UV* absorption spectra of tryptophan as shown in Figure 6.6. I studied whether or not the conformation of gramicidin in the lipid was dependent on the gramicidin to lipid ratio in order to take a consideration of the gramicidin-gramicidin interactions (Killian, *et al*, 1988). It is generally accepted that the higher the gramicidin to lipid ratio in the membrane, the greater are the gramicidin-gramicidin interactions and the smaller is the fraction of the *gD* in the channel form (Killian, *et al*, 1987). The calculated ratios of the Figure 6.6 data show ~ 1:50 (*gD*: *DMPC*) in case of benzene codissolution, ~ 1:25 used by *THF*, and ~ 1:20 used by *TFE*. (They all were formulated in the initial ratio of 1:10.) They are not consistent enough to directly compare with the

molar ratio effect. However these values might be useful for comparing my *CD* spectra with the reference papers (although there has been no *CD* data of benzene-dissolved gramicidin in the *DMPC* bilayer) in order to verify that my preparation is going correct. These are lower than the critical molar ratio causing the H_{II} phase formation by gramicidin-gramicidin interactions (Killian, *et al*, 1987).

6.3 Discussion

As mentioned previously, *CD* spectra well presented the single-stranded $\beta^{6.3} \pi$ -helix dimer structure of the channel form (Masotti *et al*, 1980; Urry *et al*, 1983) which differed from various conformations in organic solution, although *gD* shows polymorphism in various solvents; we used *TFE* to preferentially form helical and channel structure like a panacea. Gramidicin dissolved in organic solution expresses multiplex conformations of typically double-stranded dimer which may be parallel or antiparallel, left-handed or right-handed and has a range of residues per turn from 5.6 to 6.4 (Kovacs, *et al*, 1999; Bystrov & Arseniev, 1988). But in the heterogeneous anisotropic lipid environment, it is almost exclusively single-stranded (Kovacs, *et al*, 1999). Nevertheless it is accompanied with a fraction of other conformations depending on a cosolvent (Bano, *et al*, 1991; Killian *et al*, 1988), environment (Cox, *et al*, 1992; Salom, *et al*, 1998), and heat (Masotti, *et al*, 1980) effect in preparation. The *CD* spectra could clearly confirm the single-stranded dimeric structure only, nothing more about detailed conformation. We will investigate a secondary structure of *gD* in *DMPC* supported lipid bilayer with *TIR*-Raman data in Chapter 9.1.

6.4 Conclusion

POPC, *DMPC*, *DPPC* could be reconstituted as *SUVs* in 10 *mM* Tris at pH 8.0. The concentrations of these phospholipids were quantified by a simple Beer-Lambert law using fluorescence dye of *DiOC₁₈* with molar extinction coefficient known at maximum absorption at 491 *nm*. According to the ultrasonic and ultracentrifugation preparation (in Chapter 5.2), the final concentrations ranged from 0.27 to 0.37 *g/L*, which was sufficient to form a lipid bilayer when one 100- μ *L* drop is incubated onto the the 30-*mm* diameter silica window of under coverslip over melting temperature. They had average diameter of 35 *nm* revealed by photon correlation spectroscopy.

Also the reconstitution of *gD* into *POPC*, *DMPC* membranes in *SUVs* was successfully achieved prior to a preparation of supported lipid bilayers. A *gD* concentration was evaluated by *UV* absorption of tryptophan bands at maximum of 284 *nm*, and the *gD* in *DMPC SUV* had approximately 0.064 *g/L* and molar ratio of 20 to 1. The presence of *gD* in lipid layer was confirmed by the fluorescence emission spectra at maximum 332-334 *nm* which is well matched with the literature data.

Considering the solvent-dependent polymorphism of *gD*, I chose to predominate the channel form which has been discriminated by a trifluoroethanol dissolution, because we needed to know the secondary structure as a model membrane peptide in order to develop our *TIR*-Raman spectroscopy. The *CD* spectra validated that *gD* in *DMPC SUV* formed a channel structure, which corresponded to the literature data. Furthermore attempts to prepare *gD* in *DMPC SUVs* in nonchannel forms were made, but I could have a mixture of various of nonchannel with some portion of channel structures.

Chapter 7 Planar supported lipid bilayers at water/solid interface

This chapter addresses the characterization of supported phospholipid bilayers formed by fusion of phospholipid *SUVs* onto an oxidized silicon wafer or a fused silica prism. Atomic force microscope (*AFM*) imaging was employed to observe the process of fusion of vesicles to a flat silicon wafer (since the silica windows used in the Raman experiments are too large to be studied in our sample-scanning *AFM*). *TIR*-Raman scattering was used to follow the kinetics of adsorption onto silica windows, though without information on the spatial homogeneity of the film. Comparison of the intensity of the Raman signal from a fused vesicle layer with that from a bilayer of the cationic surfactant, *C₁₆TAB* (hexadecyltrimethylammonium bromide) was used to support the belief that the lipids form bilayers on silica. The section closes with a comparison of the Raman spectra of saturated and unsaturated planar supported lipid bilayers (*pslbs*) at the silica/water interface.

7.1 Characterisation by atomic force microscopy

The formation of *pslbs* is believed to proceed by the adsorption of intact vesicles onto the silica surface, the rupture of these vesicles to form bilayer patches and the annealing of these patches to form a continuous bilayer (Tamm & McConnell, 1985). I employed *AFM* imaging, in tapping mode, both to follow the time course of adsorption and to verify that a uniform lipid layer resulted.

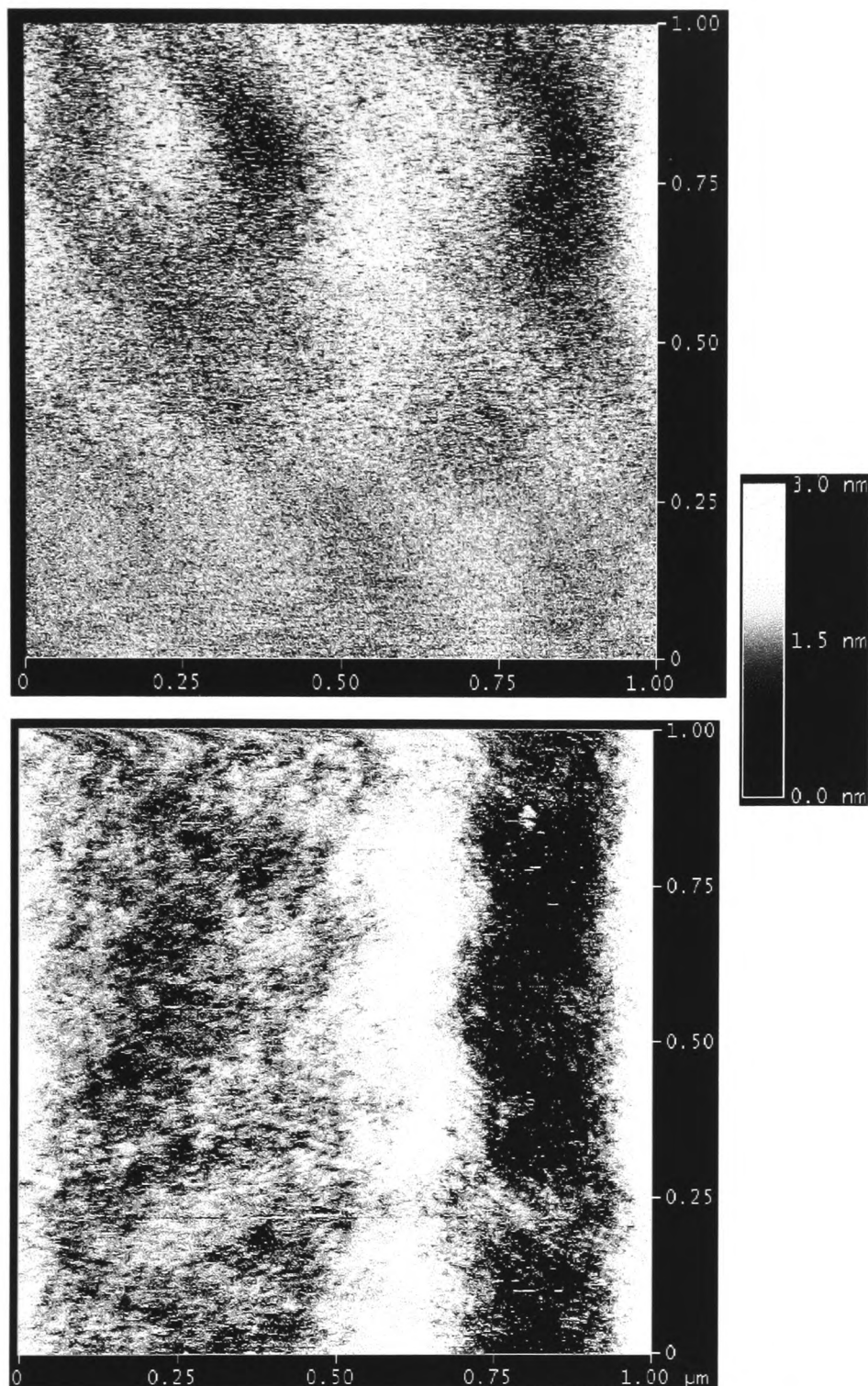


Figure 7.1 The top-view *AFM* image of *POPC* layer (upper) and *gD*-embedded *POPC* layer (lower) on a silicon wafer by fresh buffer rinsing after an incubation of 10 *min*. *AFM* measurements were done in a liquid cell by introducing the vesicle solution of $\sim 0.46 \text{ mg/mL}$ onto the silicon oxide and rinsing by the 0.1 *M* Tris buffer solution at pH 8.0 with 0.5 *M* NaCl.

According to Boxer (Salafsky, *et al*, 1996), a complete bilayer is formed on clean glass coverslip (cleaned by rinsing with distilled water and then baking at 450 °C

for 5-8 h) after ten minutes of incubation with *SUVs* under the conditions of our experiment. Figure 7.1 shows *AFM* images over a $1 \times 1 \mu\text{m}$ area for both *POPC pslbs* without (left) and with (right) incorporated gramicidin. Both images are featureless, suggesting a continuous bilayer and the absence of aggregates of gramicidin molecules. Individual gramicidin molecules in the lipid bilayer are too small to be observed at this resolution. The fused layer is dynamic in that individual lipids molecules are mobile, as many studies of fluorescence microscopy/spectroscopy have shown (Tamm & McConnell, 1985). Consequently, one would not expect to resolve individual molecules in the *AFM* images.

Figure 7.2 shows *AFM* images of the time course of the fusion of *SUVs* of *POPC* onto an oxidized silicon wafer. Tapping mode was employed, but one cannot be sure that the probe does not cause significant deformation the soft vesicles adsorbed on the surface. It was carefully controlled not to disturb the soft and dynamic interface. The *AFM* images were recorded just after buffer rinsing on different incubation time, and then they were compared. The early imaging of the 2 min-incubated *POPC pslb* featured lots of vesicles looking ruptured onto the substrate. Figure 7.3 also shows another image, together with cross-sections, taken at 2 min incubation. Doughnuts, roughly 70-75 nm in diameter, are observed, which we interpret to be adsorbed vesicles that have not yet fused to the silica surface. Their caldera shape may be an artifact arising from the lower stiffness of the central part of an adsorbed vesicle compared to the circumference.

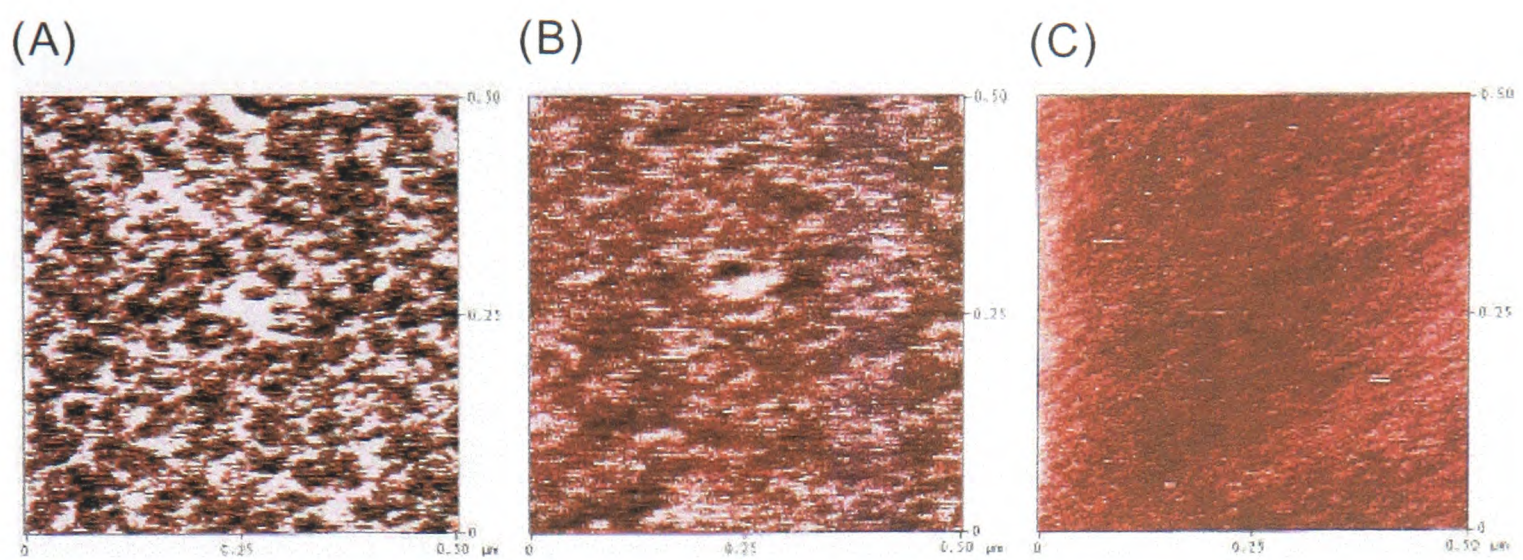


Figure 7.2 *AFM* images of time-dependent adsorption of the *POPC SUVs* (A) early stage of the fusion of 2 *min* after the vesicle solution is exposed onto the silicon, (B) 5 *min* after, (C) 30 *min* after.

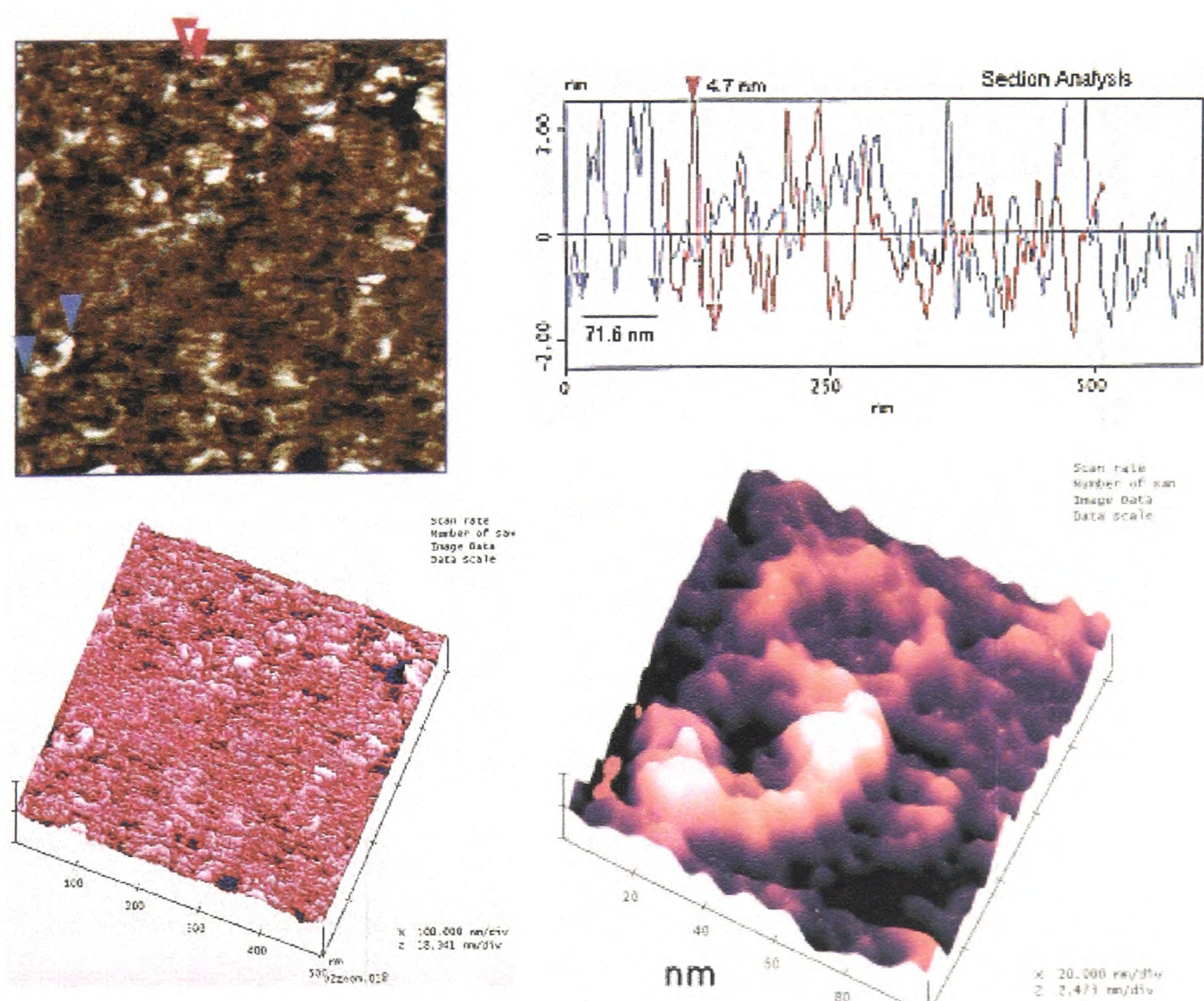


Figure 7.3 *AFM* images of very early stage of the fusion look like lots of doughnut shapes in meta-stable states.

Considering the suggested model of the fusion like vesicle adsorption ($R > R_a$)
 $\rightarrow$ fusion of surface-bound vesicle to lead to an increase of the vesicle radius to the R_r
 $(R_r > R > R_a) \rightarrow$ rupture of surface-bound vesicle ($R > R_r$) to form single-bilayer disks
as mentioned in the Ca^{2+} absent case of the literature (Reviakine & Brisson, 2000),
transient but temporarily captured image shows the time-lapse dynamic stage of the
coexistence of the bound vesicle and the bound disk (Reviakine & Brisson, 2000) while
surface-bound vesicles start to become fused. Here R is the vesicle radius, R_a is the
critical adsorption radius, and R_r is the critical rupture radius that are quoted from the
literature (Reviakine & Brisson, 2000). If the observed radius of 70-75 nm can be
estimated as similar to the final state just before rupture initiated, the equivalent radius
of a non-adsorbed vesicle can be estimated to around 35 nm, in good agreement with the
diameter measured by photon correlation spectroscopy of the *POPC SUVs*. A diameter
of 75 nm for vesicles becoming flattened is compatible with the literature (Reviakine &
Brisson, 2000). It quantifies the area compressibility modulus of the bilayer of $117 \text{ mJ} \cdot \text{m}^{-2}$
(Reviakine & Brisson, 2000). The area compressibility modulus of a bilayer has also
estimated on $\sim 100 \text{ mJ} \cdot \text{m}^{-2}$ (Seifert, 1997).

The 5 min-incubation *AFM* image showed more diffused topography due to the
surface becoming covered by the ruptured single-bilayer disks on the substrate. The 10
min-incubation *AFM* image presented a planar supported phospholipid layer at atomic
level resolution. At this stage the formation of the bilayer was not proven, since I did
not observe patches of lipid layer adjacent to patches of bare silicon, from which a
height measurement can be taken. Equally, I did not observe domains of different
height, so the number of layers is uniform over extended areas of the surface. *TIR*-
Raman spectra will be used to below to support the inference that a bilayer is formed.
The formation of the lipid layer in the *TIR*-Raman measurement was situated on a
different substrate of fused silica, compared with the silicon wafer in the *AFM* fluid cell

measurement of the *POPC* supported lipid bilayer. The stabilisation of the formation of the *DMPC* bilayer onto the fused silica window was made during the incubation under pressure of a clean glass coverslip while the water evaporation kept minimised. Incubation took nearly 45 *min* to reach the comparably relative intensity of the bilayer rather than 10 *min* onto the silicon oxide. It will be dealt with in details on the section 7.2.2.

7.2 Characterisation by *TIR Raman scattering*

7.2.1 Difference spectra

In Figure 7.4, *TIR*-Raman spectra of a supported lipid layer of *DMPC* in contact water are presented, together with the water background in the absence of lipid. They were acquired with 1.2 *W* nominal power of the laser (ca.800 *mW* prior to the prism, see Chapter 5.10) with *s*-polarised excitation and no selection of polarisation before the detector (unpolarised detection). The silica window was thermally equilibrated at 15.5 °C close to the externally focused spot. The lipid was therefore below the main phase transition temperature for bulk lipid. Experiments in the next chapter show that the laser does not significantly heat up the sample (section 8.1 of the Chapter 8). The Raman photons scattered were passed through the slit with a width of the 200 μm and imaged by the 11 pixel size onto the *CCD* of the detector. These experimental conditions for Raman acquisition will be maintained without any more mention, except the setup of the polarisation and spot temperature that were variable for each measurement. Figure 7.4 shows that the Raman spectra of the *DMPC* layer is well matched with the water background in the C-H stretching and fingerprint regions. In comparison to *ATR-FTIR*, subtraction of the water background is much more straightforward in Raman scattering. The lower background arises both from the smaller penetration depth in *TIR*-Raman and the weakness of the Raman cross-section

of water relative to that of C–H bonds. Nevertheless the penetration depth of 300 nm is still sufficient to lead to a significant peak from the O–H bending mode at 1550–1650 cm^{-1} , that can interfere with the detection of weak amide I modes from peptides incorporated into the membrane.

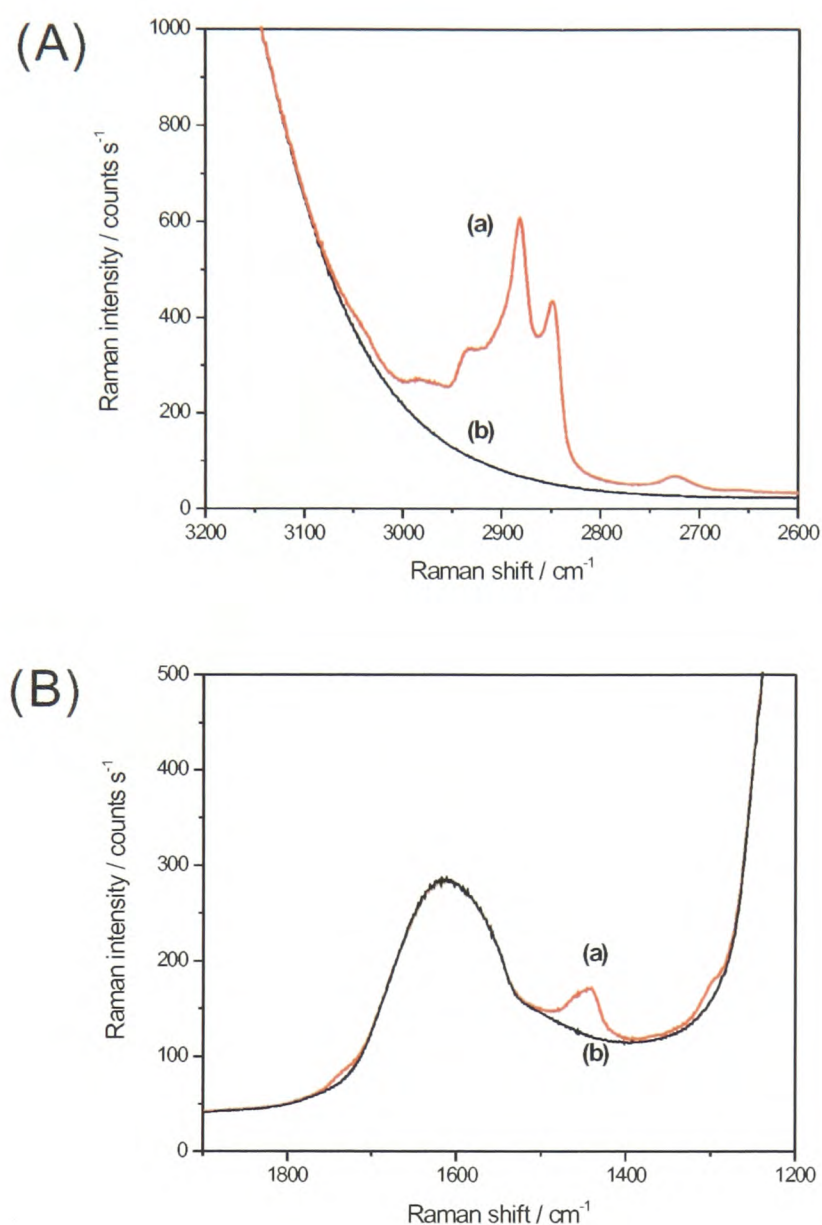


Figure 7.4 TIR Raman spectra of the supported lipid bilayers in (A) the C–H stretching and (B) the fingerprint region: (a) *DMPC pslb* in water, (b) *UHQ DW*. Temperature of the silica window = 15.5 °C

7.2.2 Time-dependent TIR-Raman spectra of fusion kinetics

The observed fusion kinetics of *POPC* vesicles onto oxidized silicon cannot necessarily be extrapolated to fused silica since the dispersion interactions between the

vesicles (*POPC* in *AFM* measurement and *DMPC* in Raman scattering) and the substrate (silicon wafer in *AFM* and fused silica in Raman measurement) are different, as is the charge of the surface. Raman scattering is sufficiently intense in the C–H stretching region to follow the kinetics of vesicle fusion by Raman scattering with 1-min acquisition times per spectrum.

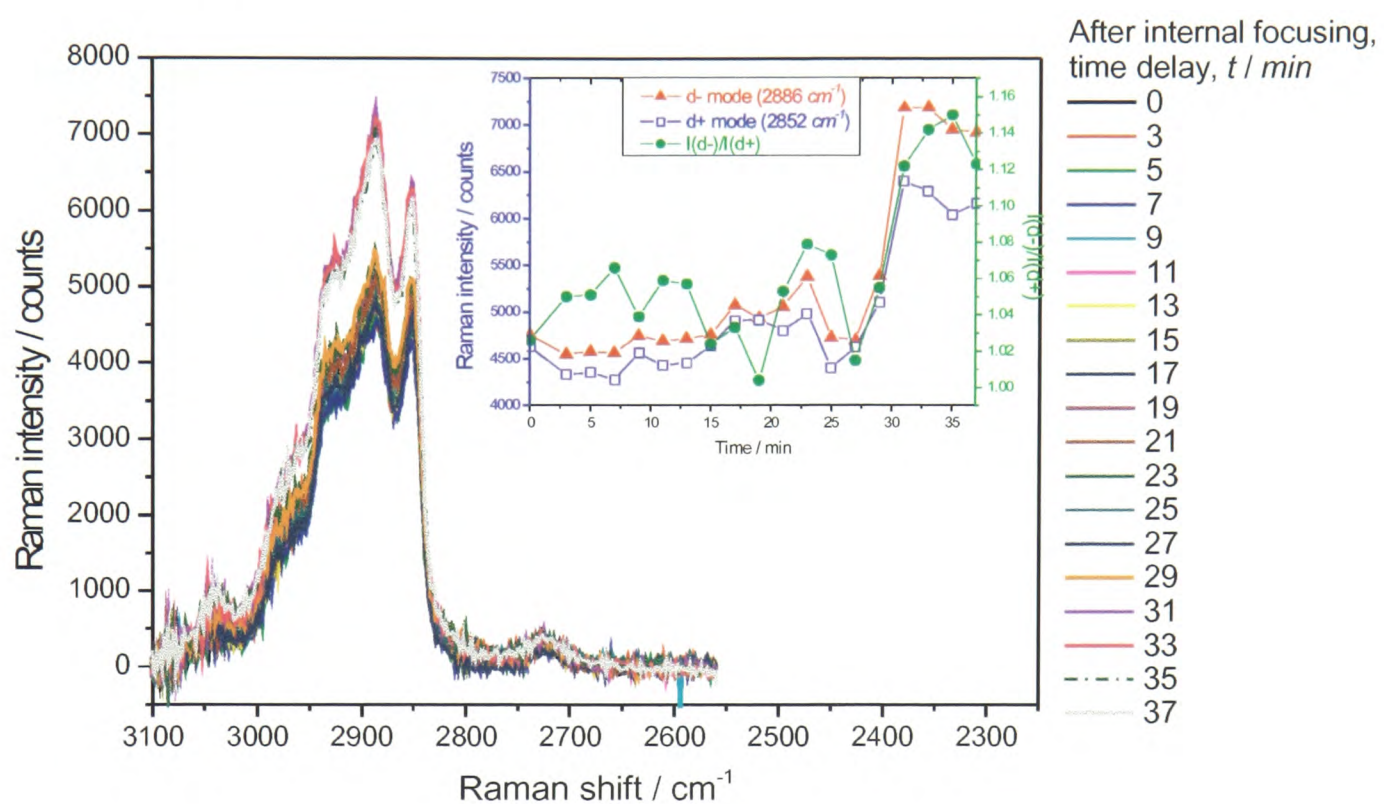


Figure 7.5 Time-lapsed *TIR*-Raman spectra of a fused silica window in contact with a *DMPC SUV* solution sandwiched under a glass coverslip during the formation of the supported lipid layer. The temperature of the silica window was 32.8 °C. $\lambda = 523$ nm, 1.2 W, 60 s acquisition, Leica $\times 100$ oil-immersion objective. Inset indicate the time-elapsd changes of d^- (CH_2 antisymmetric stretching) and d^+ (CH_2 symmetric stretching) modes and $I(d^-)/I(d^+)$ of intensity ratio of d^- to d^+ mode, which is indicative of chain ordering.

Figure 7.5 shows spectra taken at various incubation times. Due to the presence of a coverslip and oil and water films of varying thickness, the internal beam path needed to be used to focus the objective before each series of measurements. The

time delays, t , are measured from the time at which the microscope was focused, which was within around 1 *min* after the lipid solution was placed on the silica window and approximately 2 *min* after internal confocal focusing using the Leica $\times 100$ oil-immersion objective. The temperature was maintained over the main phase transition and the evaporation of water was minimised by placing drops of *UHQ DW* around the edges of the glass cover slip. The spectra in the time series fall into two distinct sets. The early spectra ($t < 30$ *min*) have the lower intensity in the C-H region than the later spectra ($t > 30$ *min*) with a lower ratio of the intensities of the antisymmetric and symmetric methylene stretches, $I(d^-)/I(d^+)$. This ratio reflects the chain ordering of the lipid chains (Larsson & Rand, 1973). The higher the ratio, the more ordered are the chains. It appears that there is a rather sudden process at which adsorbed vesicles reconstruct to form a uniform and conformationally ordered film. I will show in Chapter 8 that vesicles in the bulk give a lower value of $I(d^-)/I(d^+)$ than the *pslb* (possibly for geometrical reasons rather than chemical ones). The sudden change in the spectrum at $t = 30$ *min* may also be associated with a change from adsorbed vesicles to a planar bilayer. After $t = 30$ *min*, the spectra were very stable.

Figure 7.6, compares spectra at 7, 19 and 35 *min* with a spectrum of a fully formed bilayer in pure water after rinsing. Spectra acquired with the coverslip and oil-immersion objective cannot be compared quantitatively with those acquired the water immersion objective, so the first three spectra have been multiplied by a scale factor to overlay the 35-*min* spectrum with the spectrum of the *pslb* in *UHQ DW*. The near-coincidence of the 35-*min* spectrum and the spectrum after rinsing suggests that no further changes occur upon rinsing of the adsorbed lipid with *UHQ DW*. With this result, I used an incubation time of 45 *mins* in subsequent experiments. The reproducibility of the spectra obtained with this procedure provided good evidence that the fusion process had proceeded to completion within this time.

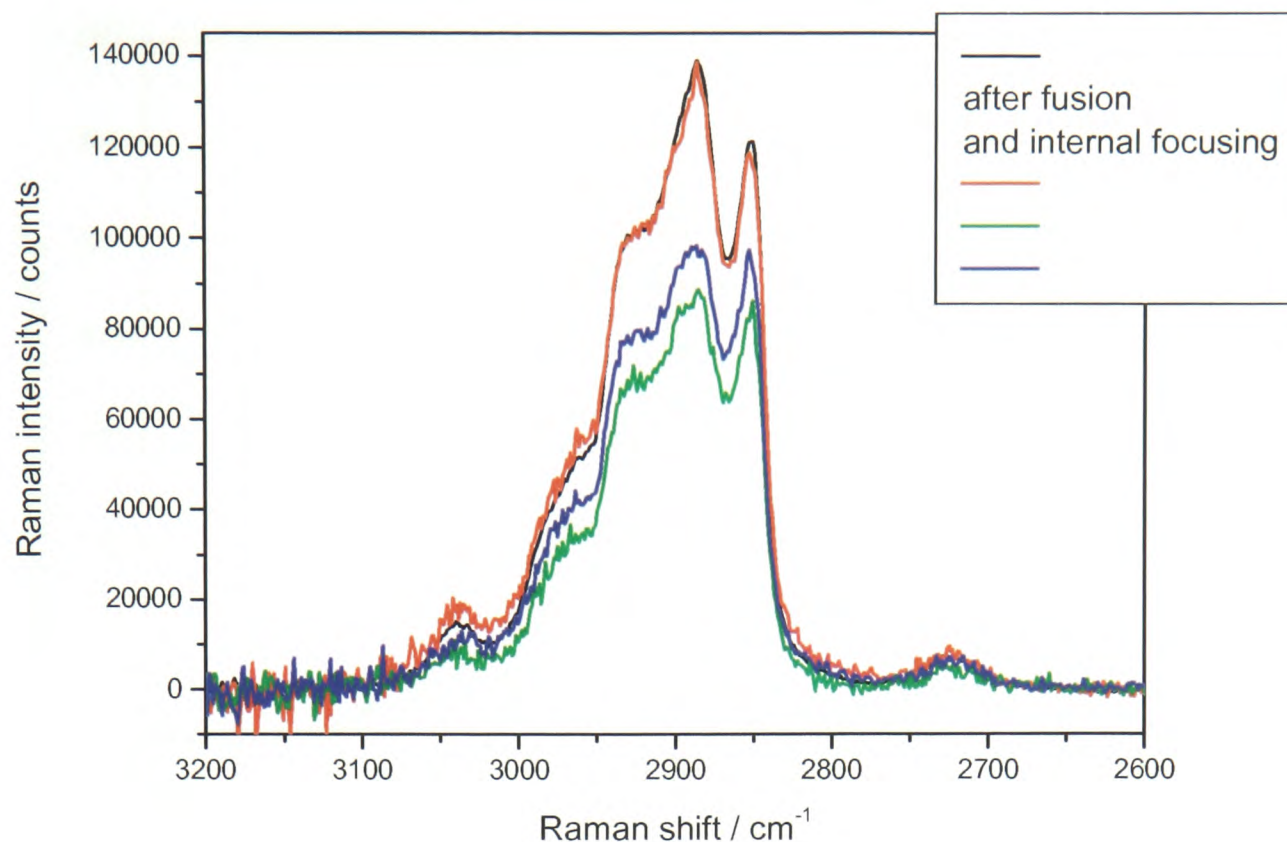


Figure 7.6 Comparison of the time-dependent Raman spectra during the incubation under the coverslip glass, with the supported lipid bilayer after rinsing with *UHQ DW*. The 7- min (green), 19-min (blue), and 35-min (in red) spectra are the same as in Figure 7.5. They were scaled in a factor of 19.5 for comparison with the supported lipid bilayer (black) in water. For the latter spectrum, the temperature of the silica window was 32.0 °C, nominal power = 1.2 W, acquisition time = 450 s, Zeiss 40× water-immersion objective, *s*-polarised excitation, unpolarised detection.

7.2.3 Lamellarity of the supported phospholipid layers

To establish that the lipid layers fused onto the silica window are indeed bilayers, I compared the Raman spectra of the *pslbs* with those of a cationic surfactant.

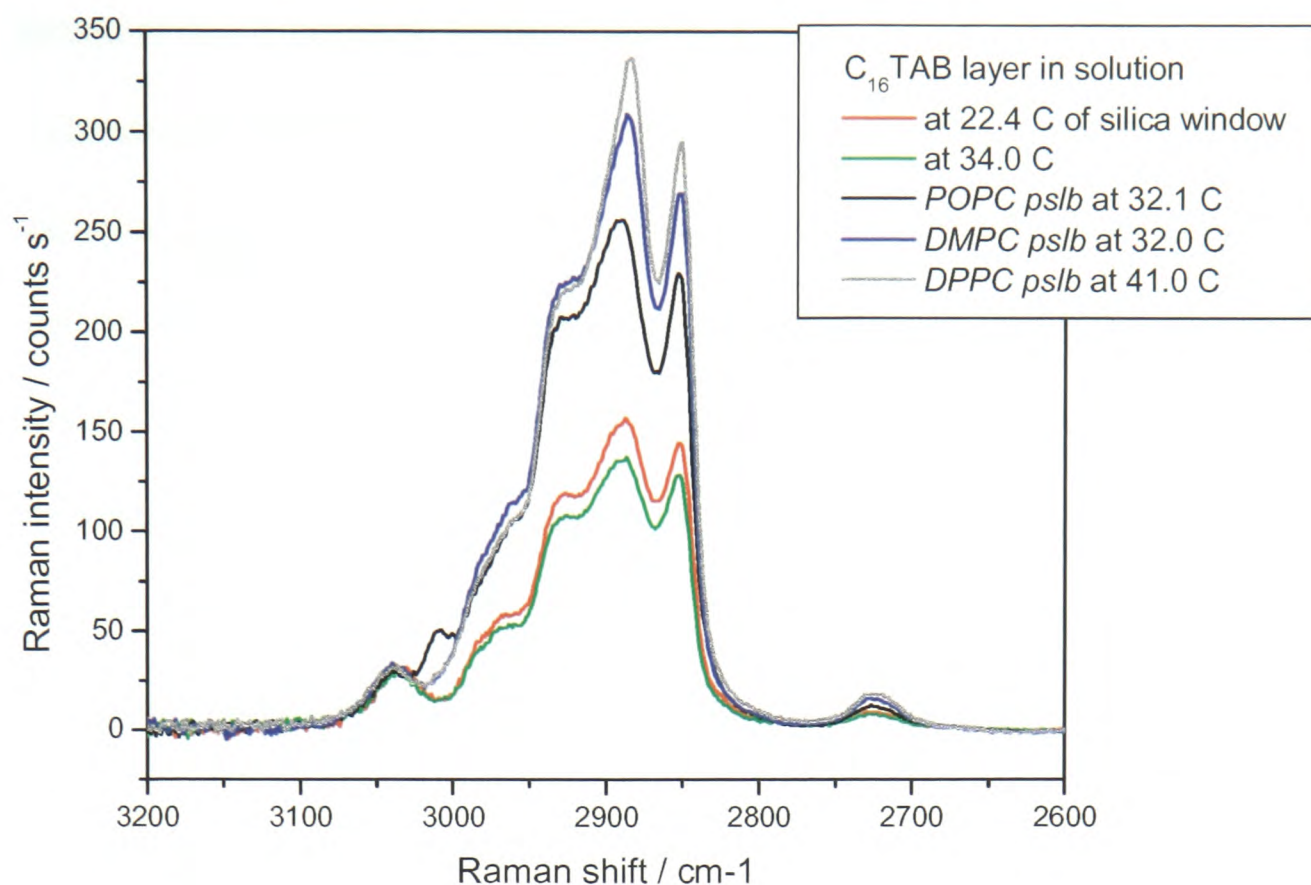


Figure 7.7 Comparison of *TIR*-Raman spectra of supported phospholipids layers of *DMPC* (blue), *DPPC* (grey) and *POPC* (black) with the adsorbed layer of *C₁₆TAB* (in red and green). All measured at 523 nm, 1.2 W, 450 s acquisition, by Zeiss 40× water-immersion objective, *s*-polarised excitation, unpolarised detection.

In Figure 7.7, Raman spectra of fused lipid films of *POPC*, *DMPC*, and *DPPC* are compared with layers adsorbed from a solution of the surfactant hexadecyltrimethylammonium bromide (*C₁₆TAB*) at water/silica interface just above the critical micelle concentration of 0.92 mM. Atkin *et al.* (Atkin, *et al.*, 2003) have reported that *C₁₆TAB* adsorbs onto silica with a surface excess of $4.4 \mu\text{mol}\cdot\text{m}^{-2}$ at 24.5 °C. If we make the crude assumption that the integrated area is proportional to the number of C-H bonds in the molecule times the surface excess, we estimate that the coverage of the lipids is $5.0 \mu\text{mol}\cdot\text{m}^{-2}$ in case of the *DMPC* supported lipid bilayer in the liquid crystalline phase (L_α) and $5.25 \mu\text{mol}\cdot\text{m}^{-2}$ in the *DPPC* supported lipid bilayer in L_α .

These results of the surface excess are in good agreement with the ellipsometric measurement on a lecithin bilayer adsorbed from mixed micelles of lecithin with dodecylmaltoside (Tiberg, *et al*, 2000), and with measurements of areas per molecule in bulk lamellar phases (Lis, *et al*, 1982). The bilayers were deposited in the L_α phase, and the surface coverage remained constant thereafter since the lipids are insoluble and the supernatant is pure water. These calculations are not exact, since the intensity of the Raman signal depends on the orientation of the molecules and the substituent groups as well as the coverage, but are sufficiently accurate to confirm that the average thickness approximates to a bilayer rather than a multilamellar film.

7.2.4 Polarised TIR-Raman spectra of DMPC and POPC *pslb's*

Figure 7.8 shows the polarised TIR-Raman spectra of the planar supported lipid bilayer of *DMPC* measured at 27.0 °C. The assignment of the peaks is well-established. In the C-H stretching region (Figure 7.8(A)), the symmetric (d^+) and antisymmetric (d^-) methylene stretching vibrations appear at 2851 and 2882 cm^{-1} , respectively (Aslanian, *et al*, 1986; Lhert, *et al*, 2002). The shoulder at 2928 cm^{-1} is assigned to a Fermi resonance of the symmetric methyl stretch (r^+_{FR}) and the peak around 2972 cm^{-1} to the antisymmetric methyl stretch (r^-) of the fatty acid chains (Aslanian, *et al*, 1986). The band at 3042 cm^{-1} is assigned to the antisymmetric stretching mode of the choline methyl of the polar headgroup (Lhert, *et al*, 2002).

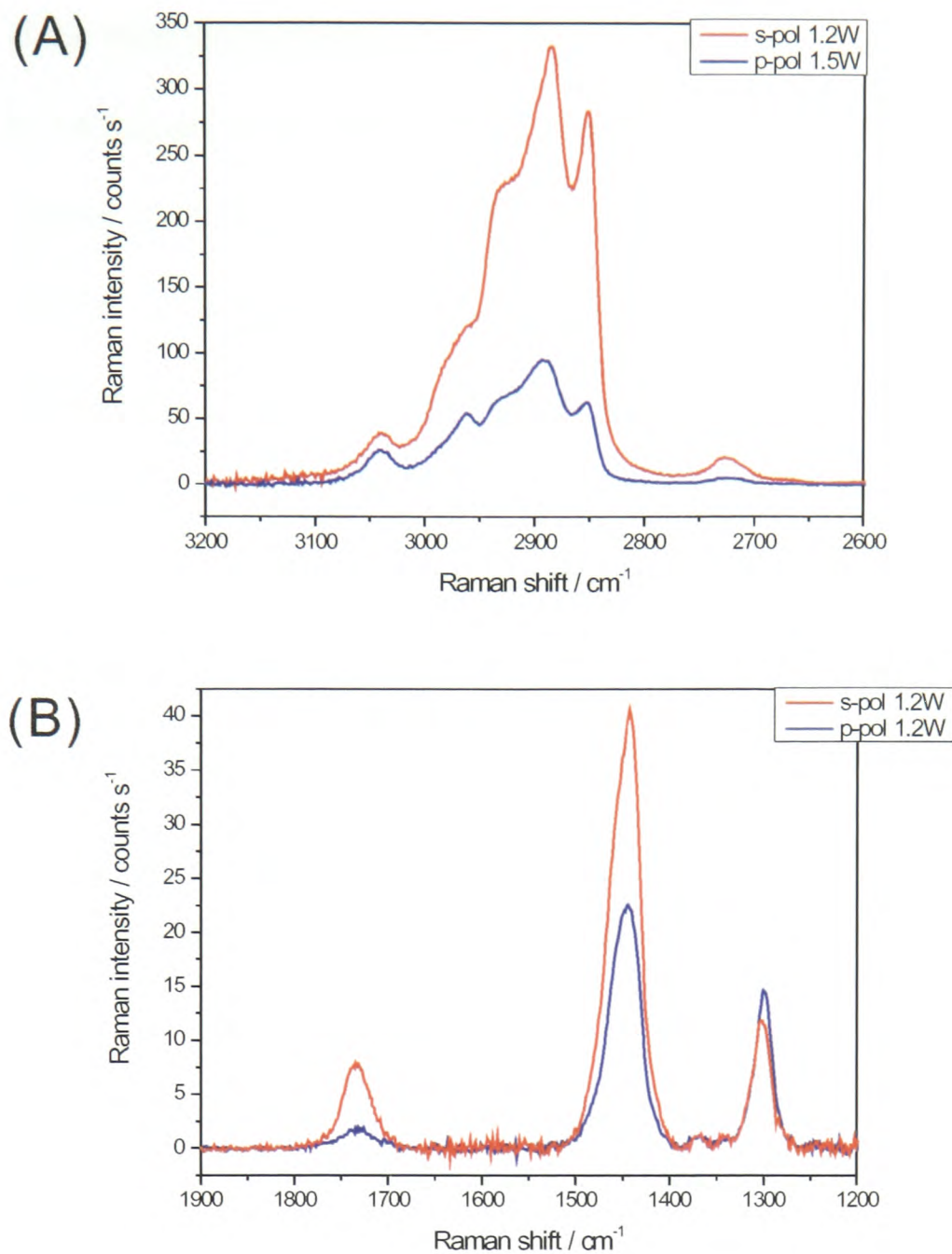


Figure 7.8 Polarised *TIR*-Raman spectra of a supported lipid bilayer of *DMPC* in (A) the C-H stretching and (B) the fingerprint region; *s*-polarised pump (red), *p*-polarised pump (blue); unpolarised detection

In the fingerprint region of Figure 7.8(B), the characteristic lipid bands are also observed: the Fermi doublet of the methylene scissoring mode at 1441 and 1453 cm^{-1} , the in-plane methylene twisting mode at 1298 cm^{-1} (Aslanian, *et al*, 1986; Gaber & Peticolas, 1977), and the ester carbonyl stretch at 1734 cm^{-1} . The phosphate bands below 1200 cm^{-1} are obscured by the strong Si-O bands from the silica substrate.

Figure 7.9 compares the polarised *TIR*-Raman spectra of the planar supported

lipid bilayers of *DMPC* and *POPC*. In the bilayer of *POPC*, the lipid chains are more fluid and the methylene modes are consequently shifted to higher frequencies: 2855 and 2893 cm^{-1} (Aslanian, *et al*, 1986; Gaber & Peticolas, 1977; Snyder, *et al*, 1982). *POPC* shows vinylic C-H vibrations at 3010 cm^{-1} from the oleoyl chain. In the fingerprint region, *POPC* is characterised with a strong band at 1658 cm^{-1} arising from the *cis* carbon double bond in the oleoyl chain as well. In the C-H stretching region, both *s*-polarised spectra of *DMPC* and *POPC* were measured above the main phase transition temperature at 35.0 °C and 20 °C, respectively. The value of the ratio $I(d-)/I(d+)$ of the *DMPC* in Figure 7.9(A) is lower than Figure 7.8(A): the temperature variation will be addressed in detail in the Chapter 8. The overall similarity in signal strengths, particularly in groups that are not so sensitive to acyl chain conformation, such as the ester carbonyl and the choline, suggests that the coverage of the two lipids are similar.

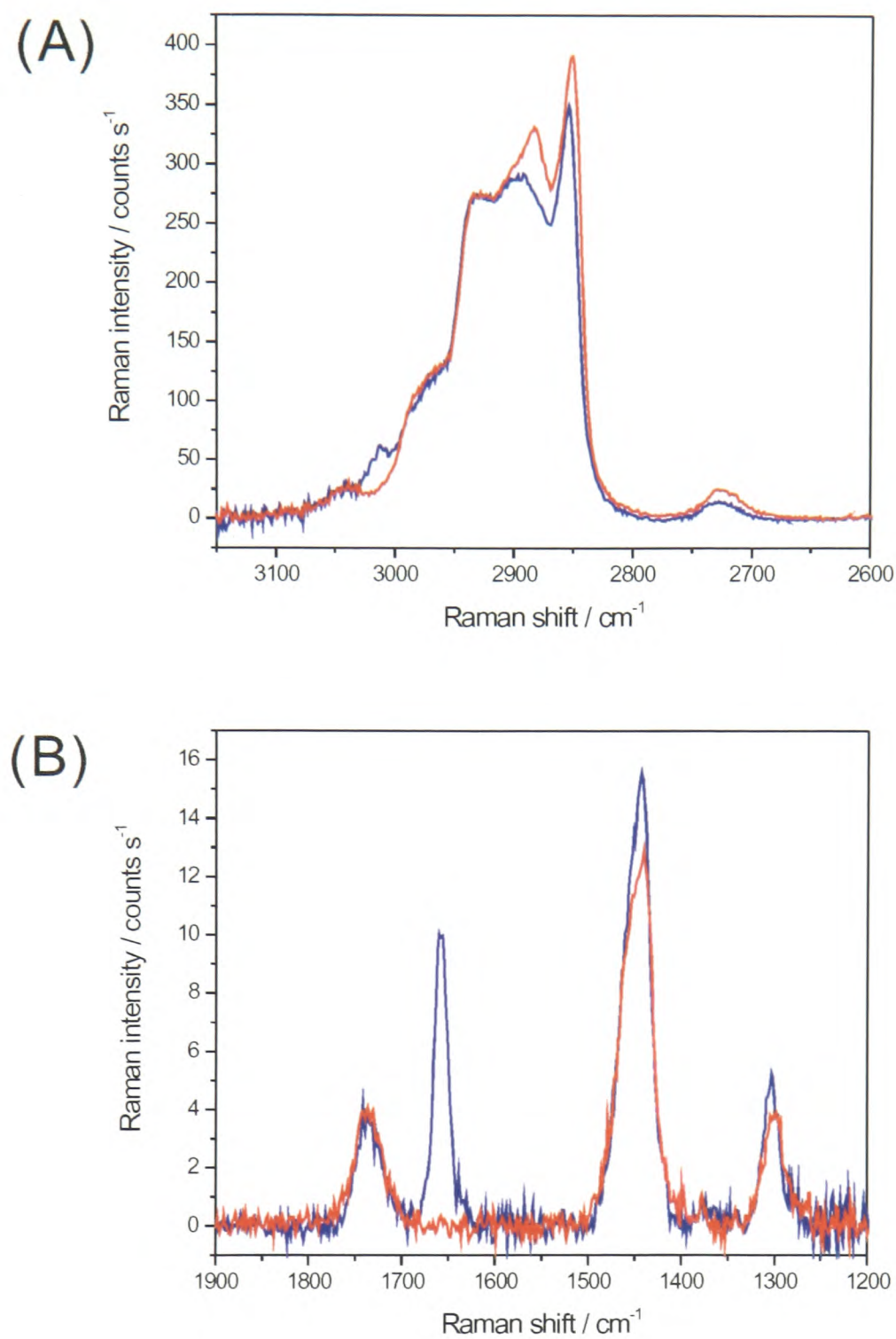


Figure 7.9 Polarised *TIR*-Raman spectra of supported lipid bilayers of *DMPC* (red) and *POPC* (blue) at the silica/water interface: (A) *sy* spectra of *DMPC* and *POPC* in the C-H stretching region; (B) *sy* spectra of *DMPC* and *POPC* in the fingerprint region. Acquisition times were 225 *s* for the C-H stretching and 600 *s* for the fingerprint region.

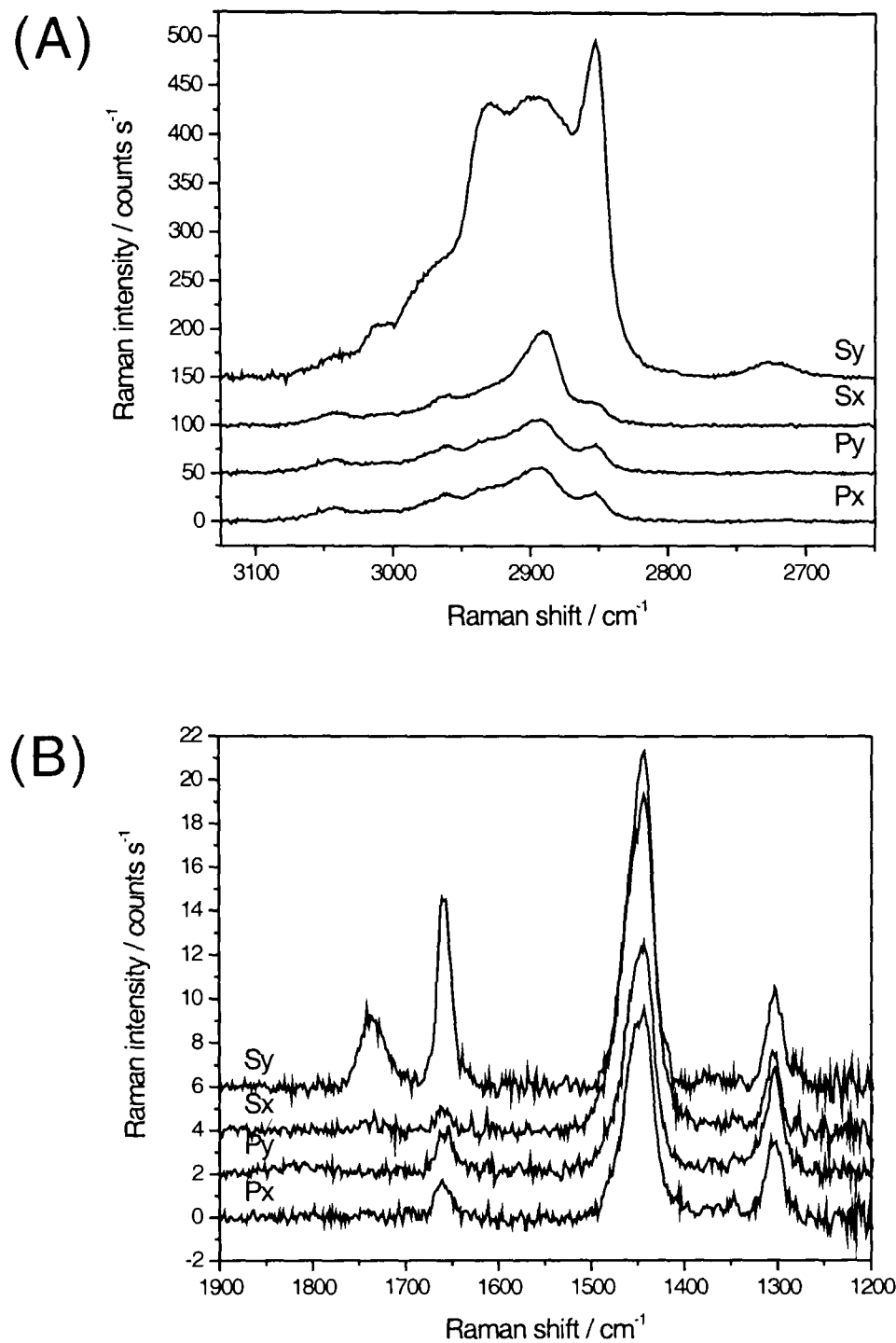


Figure 7.10 (A) *sy*, *sx*, *py*, *px* spectra of *POPC* in the C-H stretching region; (B) *sy*, *sx*, *py*, *px* spectra of *POPC* in the fingerprint region. Acquisition times were 225 s for *sy*, 600 s for *sx*, *py*, *px* spectra in the C-H stretching region, and 600 s for *sy*, 1200 s for *sx*, *py*, *px* spectra in the C-H fingerprint region.

Figure 7.10 shows the four orthogonal polarization combinations for *POPC* bilayers: *sx*, *sy*, *px*, *py*, where *s* and *p* label the polarisation of the pump laser and *x* and *y* the polarisation of the collected Raman signal. For a uniaxial monolayer and a pump laser incident at the critical angle, θ_c , the *px* and *py* spectra should be the same (to

within a scale factor due to the different transmission efficiencies of the polarisation optics). Experimentally, the incident angle is about 3° above θ_c , in order to reduce the penetration depth of the evanescence wave (Greene & Bain, 2004), so these two spectra are not quite identical. For isotropic layers, the *sx* and *py* spectra would be identical at all angles of incidence. While some differences are observed in the *py* spectrum – the symmetric methylene band is stronger and antisymmetric methylene band weaker – these results may be contrasted with the case of a crystalline layer (such as a Langmuir-Blodgett monolayer of the fatty acid, zinc arachidate) in which the CH_2 modes disappear entirely from the *p*-polarised spectra at the critical angle (Haydock, 2002). I want to deal with some comparisons of the polarised spectra related with the chain orientation of phospholipid in *pslb* in discussion of the Chapter 7.3.

We can be more interested the polarised spectra presented in the Figure 7.8 and 7.10 as long as the orientation of the phospholipid in lipid bilayer is probed by a combination of polarisation in incidence and detection axes.

It could be generally accepted that the plane where the C-H bonds exist is more parallel to the electrical field of the *s*-polarisation incident to water/silica interface. It is not surprising that the antisymmetric methyl stretch ($\bar{\nu}$) in the choline head group induced relatively similar intensity of the *s*-polarised spectra to the *p*-polarised due to the randomly oriented in the 3-*D* space with no preference of particular direction parallel to the plane of incidence. The intensities of the ester carbonyl stretching mode and the methylene scissoring mode are bigger in the *s*-polarised spectra than the *p*-polarised. It means that the averaged vibrations of these bonds are more in-plane of the surface, contrast to the case of the methylene twisting mode with their orientation more erected to the surface.

In the Figure 7.10, we can observe the variation of the polarised Raman intensity of C=C mode as a function of different polarisation of incidence and detection.

7.3 Conclusion

Planar supported lipid bilayers (*pslbs*) were formed by *POPC*, *DMPC*, and *DPPC* by the incubation under glass coverslip method (which was introduced in Chapter 5.7). *AFM* measurements presented *in situ* images from the vesicle rupture of the early stage to the formation of the lipid layer which became flattened at a molecular level after 30 *min*. The Raman measurement also revealed that the intensity of the CH_2 stretching mode became saturated after 30 *min* under the coverslip over the temperature of the main phase transition of lipid and the intensity was exactly matched with the fully formed lipid bilayer. To confirm bilayer formation, we compared the Raman intensity of the *pslbs* with the cationic surfactant *C₁₆TAB* and the intensity roughly doubled when compared to *C₁₆TAB*, which indicated the formation of a lipid bilayer.

Subtraction of the water background is much more straightforward in Raman scattering and it provided a perfect method for removal of water signals to obtain the Raman spectra of the *pslb* in the C-H stretching and fingerprint region, although the penetration depth of 300 *nm* is still sufficient to lead to a significant peak from the O-H bending mode at 1550-1650 cm^{-1} .

Raman spectra of *POPC*, *DMPC*, *DPPC* *pslbs* could be qualitatively identified according to the literature, and furthermore the polarised spectra were presented to give information of the orientation in a specific molecular vibration such as vinylic stretching in *POPC* *pslb*.

Chapter 8 Phase transitions in planar supported lipid bilayers by polarised *TIR*-Raman microspectroscopy

The phase transitions in single-component phospholipid bilayers at the water/solid interface are of interest to membrane biophysicists due to their extensive application in biological research in nano- and microtechnology. The major challenge in investigating structural changes in single planar supported lipid bilayers (*pslbs*) is one of sensitivity. Early studies by *L. K. Tamm et al* (Tamm & McConnell, 1985) employed fluorescence recovery after pattern photobleaching (*FRAP*) to measure lateral diffusion coefficients in bilayers deposited by the Langmuir-Blodgett (*LB*) technique onto the glass, quartz, and silicon. Conventionally differential scanning calorimetry (*DSC*) has been involved in the measurement (Naumann, *et al*, 1992; Shaw, *et al*, 2003; Yang & Appleyard, 2000). More understandings of the conformational changes in acyl chains of 2-*D* leaflets during the phase transition have been made by nuclear magnetic resonance (*NMR*) (Gaede, *et al*, 2004; Naumann, *et al*, 1992), and sum-frequency vibrational spectroscopy (Liu & Conboy, 2004). (The work by Naumann *et al* is not applied to the planar supported lipid bilayer but the spherical supported vesicles.) Macroscopic visualisation of the phase transitions of *pslbs* has been also recently achieved by atomic force microscopy (*AFM*) (Charrier & Thibaudau, 2005; Feng, *et al*, 2005; Kaasgaard, *et al*, 2003; McClain & Breen, 2001; Seantier, *et al*, 2004; Xie, *et al*, 2002). Their attempts revealed that the transition ranged much broader by ~ 8 °C than in unilamellar vesicle and the main melting point was upshifted to ~ 28 °C (Charrier & Thibaudau, 2005; Tokumasu, *et al*, 2003; Xie, *et al*, 2002). However, the explanation of the broader width and the temperature shift has been still controversial (Charrier & Thibaudau, 2005; Xie, *et al*, 2002).

This chapter describes the application of *TIR*-Raman scattering to the study of the phase transitions in *POPC*, *DMPC*, and *DPPC* *pslbs* at the water/silica interface. There is no precedent for the use of Raman scattering to study *pslbs*, though Raman has been used to study liposomes. In early Raman works by *H. Van Dael et al* (Van Dael, *et al*, 1982), *SUVs* of *DMPC* were studied. Their Raman measurements on *SUVs* (below 100 nm of diameter) of *DMPC* revealed a broad transition (from 10 to 27 °C) in contrast to a narrow width in the case of *LUV* (above 100 nm of diameter) and *MLV*. The broadening of the transition in the *SUV* was validated by thermal analysis although the width of the transition was not exactly in accord in the different techniques. In early *DSC* work by *P. W. M. Van Dijck et al* (Van Dijck, *et al*, 1977), the main phase transition of liquid crystalline phase (L_α) to gel phase (L_β) in the *SUVs* was found to be broader than in an *LUV* or *MLV*. (These phases are schematically described in Figure 2.2.) Koynova and Caffrey reported, by *DSC*, that the chain melting transition of *SUVs* with diameters below 30-50 nm was considerably broader than that for *MLV* (cf. $\Delta T_{1/2} = 3.5$ °C for *SUVs* vs. ~ 0.1 °C for *MLVs*) (Koynova & Caffrey, 1998). Further, the enthalpy change of the transition in *SUVs* was smaller than that in *MLVs*. The difference was attributed to a sensitivity of the enthalpy level of the gel phase lipid to vesicle size (Koynova & Caffrey, 1998). However, the phase transition still appears broader by Raman spectroscopy than by *DSC* (Koynova & Caffrey, 1998; Van Dael, *et al*, 1982; Van Dijck, *et al*, 1977). Our Raman spectra of *DMPC SUVs* with a diameter of 35 nm, as shown in this chapter, were consistent with early data by *H. Van Dael et al* (Van Dael, *et al*, 1982); to the best of my knowledge, this is the only publications reporting Raman spectra of supported phospholipids bilayer. (There are other recent publications of phospholipids bilayers in vesicle solution using Raman tweezers (Sanderson & Ward, 2005) and sum-frequency vibrational spectroscopy (Liu & Conboy, 2004).) It could consolidate to apply for our Raman setup with *TIR* geometry and polarisation to

measure the phase transition of *pslbs* of *DMPC*, *DPPC*, and *POPC*. As expected, the results of *DMPC pslb* had broader transition and higher ($I(d^-)/I(d^+)$) indicative of chain ordering (Lhert, *et al*, 2002) than *SUV* in solution. The analysis of the peak shift characteristic of the phase transition presented monotonic changes in *DMPC* but two regions of different peak shift in *DPPC* that might indicate ripple phase ($P_{\beta'}$) of an intermediate as a pre-transition state. ($P_{\beta'}$ is depicted in Figure 2.2 of Chapter 2.) It can be issued that $P_{\beta'}$ phase in *DPPC pslb* at water-solid interface might exist as discussed in the literature (Tamm & McConnell, 1985).

8.1 Local heating by laser

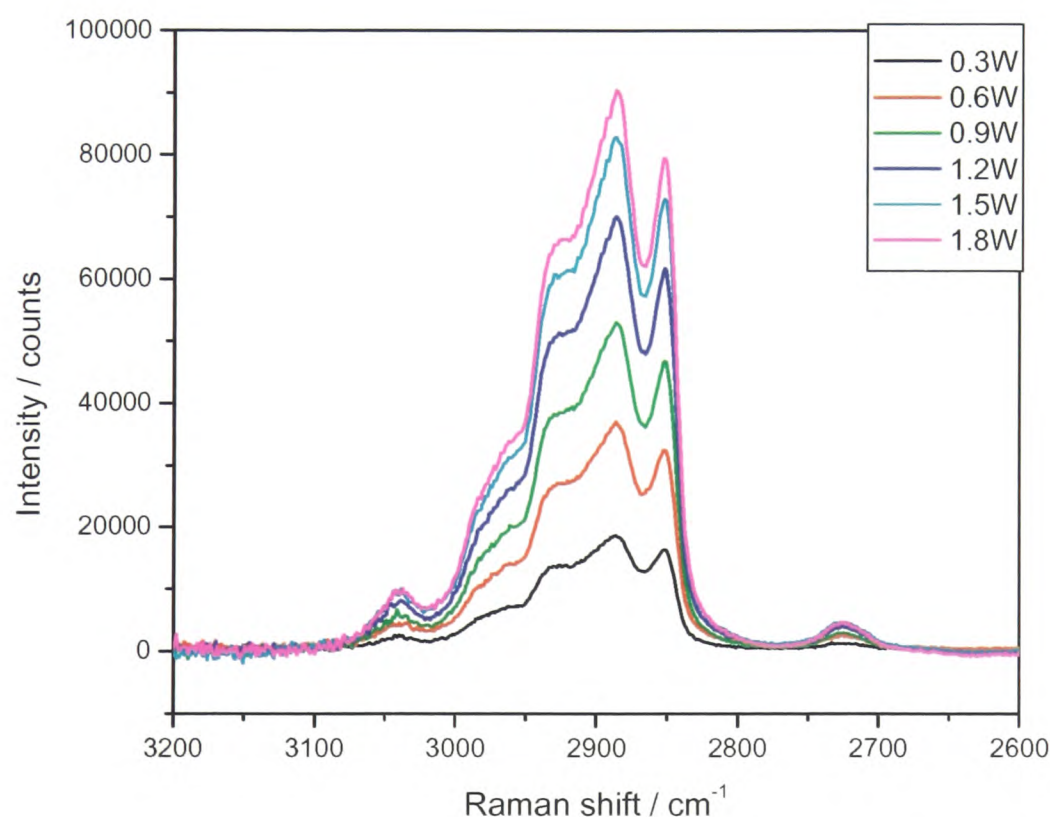


Figure 8.1 Power-dependent *TIR*-Raman spectra of *DMPC* measured at 20.5 °C in the silica window for 225 seconds in *s*-polarised excitation of 532 nm and unpolarised detection with the 200 μm slit width and 11 pixel size of the *CCD* detector: 0.3 W (in black), 0.6 W (in red), 0.9 W (in green), 1.2 W (in blue), 1.5 W (in light blue), and 1.8 W (in pink).

Before I started measuring the phase transition behaviour of *DMPC*, *DPPC*, and *POPC* *pslbs*, it was necessary to check whether the focused laser spot caused any local heating of the sample. A *DMPC* *pslb* was prepared on fused silica. *TIR*-Raman spectra were acquired for different (nominal) intensities of laser from 0.3 W to 1.8 W, as shown in Figure 8.1. Spectra were acquired at 20.5 °C, which is below the expected main phase transition temperature (~ 28 °C) of supported lipid bilayer which has been most recently reported by temperature-controlled *AFM* in the literature (Charrier & Thibaudau, 2005). Figure 8.2 shows that the 0.3 W spectrum can be exactly overlapped with the 1.2 W and 1.8 W spectra if it is multiplied by a factor of 3.76 or 4.86, respectively. The data presented later show that the Raman spectrum is very sensitive to temperature around 20 °C, so any heating of the sample would be immediately evident in a comparison of spectra taken with different laser powers. The Raman spectra did not change with time of exposure to the laser beam either, confirming the absence of photodamage. (The phospholipid bilayer is robust in respect of photodamage compared with marker bands such as amide I and tryptophan ring modes in peptides/proteins.)

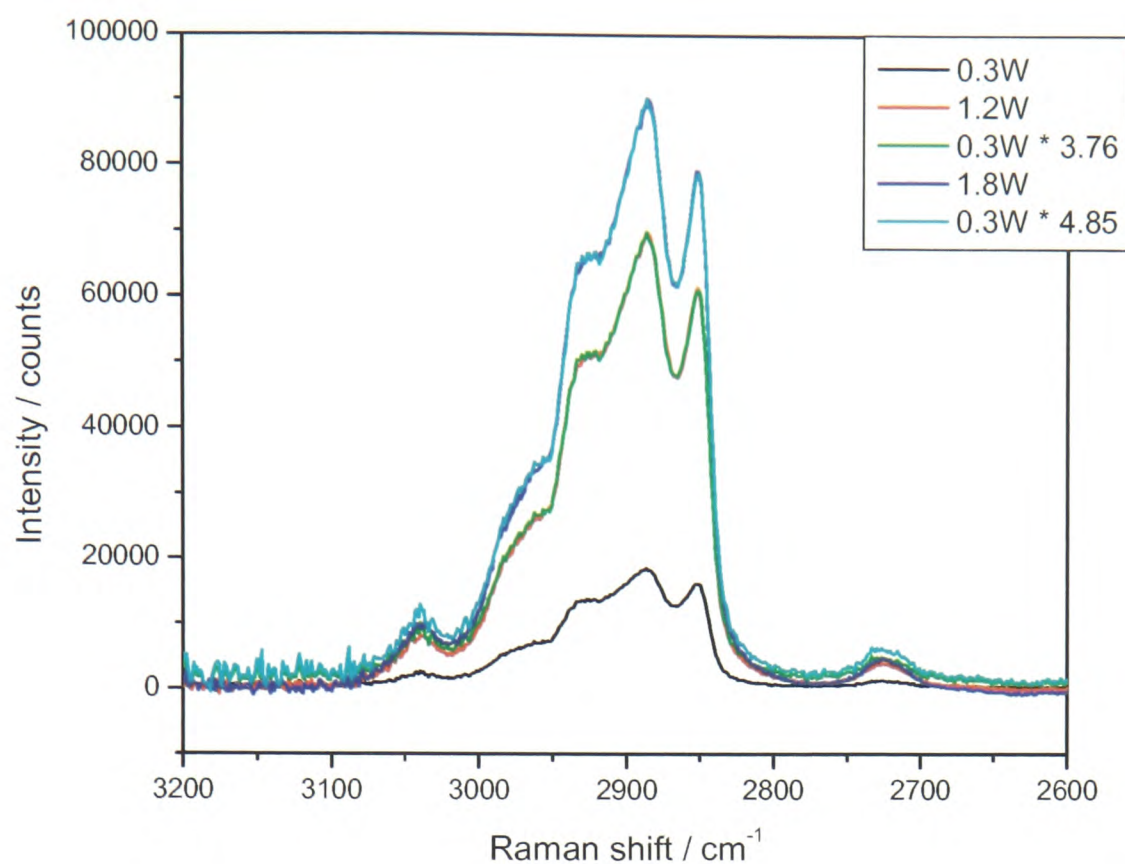


Figure 8.2 Comparison of 0.3 *W* TIR-Raman spectra of *DMPC* with 1.2 *W* and 1.8 *W* measured at 33.5 °C in the silica window in *s*-polarised excitation of 532 *nm* and unpolarised detection with the 200 μm slit width and 11 pixel size of the *CCD* detector: 0.3 *W* (in black), 1.2 *W* (in red), and 1.8 *W* (in blue).

8.2 Temperature-dependent polarised TIR-Raman spectra of DMPC *pslb*

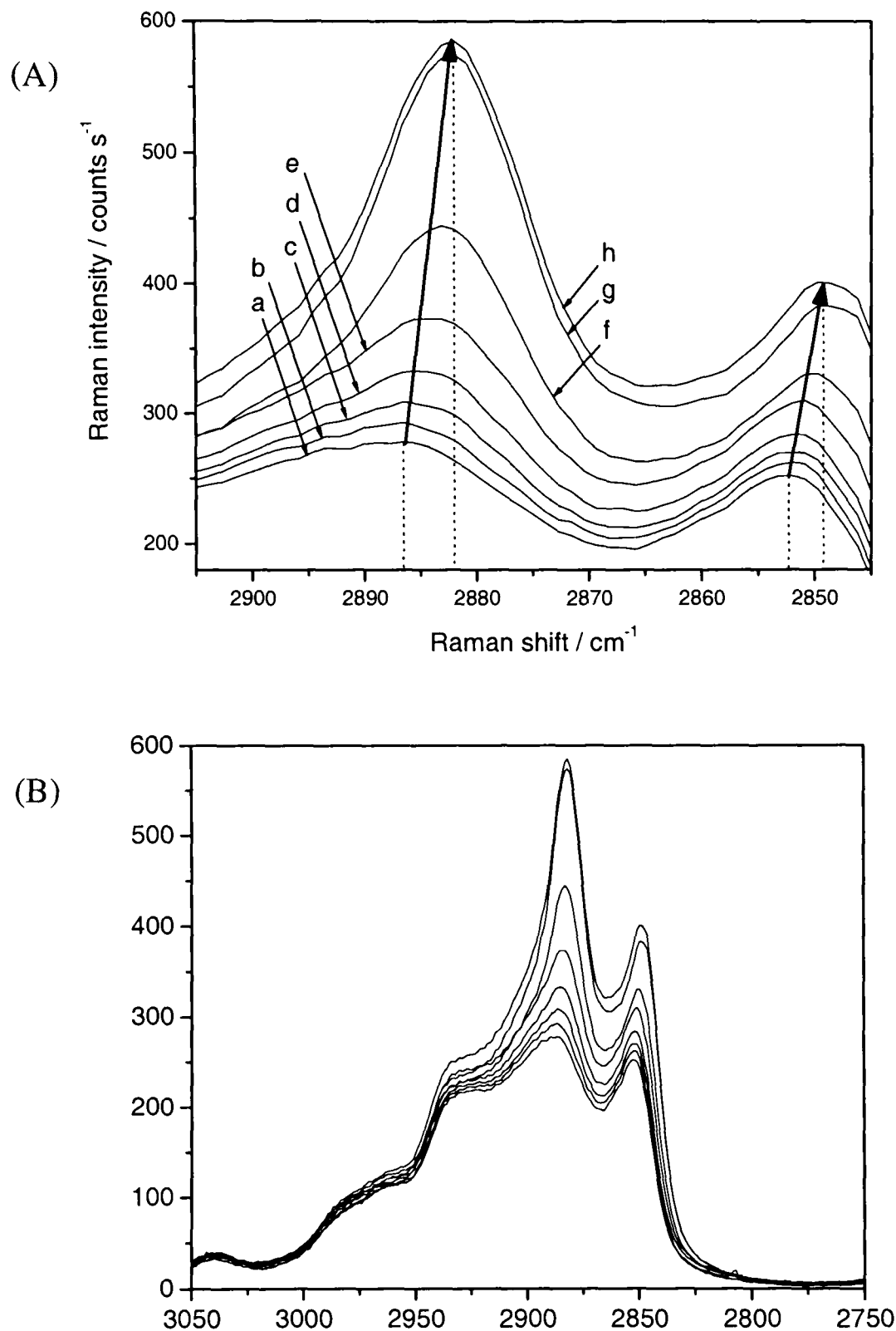


Figure 8.3 Temperature-dependence of the *s*-polarised Raman spectra of DMPC *pslb* (A) in the methylene vibration modes of C-H stretching region and (B) in the full range of C-H stretching region, 1.2 W, *s*-polarised excitation, unpolarised detection: (a) 42.0 °C, (b) 38.1 °C, (c) 32.0 °C, (d) 26.6 °C, (e) 23.3 °C, (f) 20.8 °C, (g) 16.8 °C, and (h) 13.2 °C. The arrows in (A) direct a decrease of temperature.

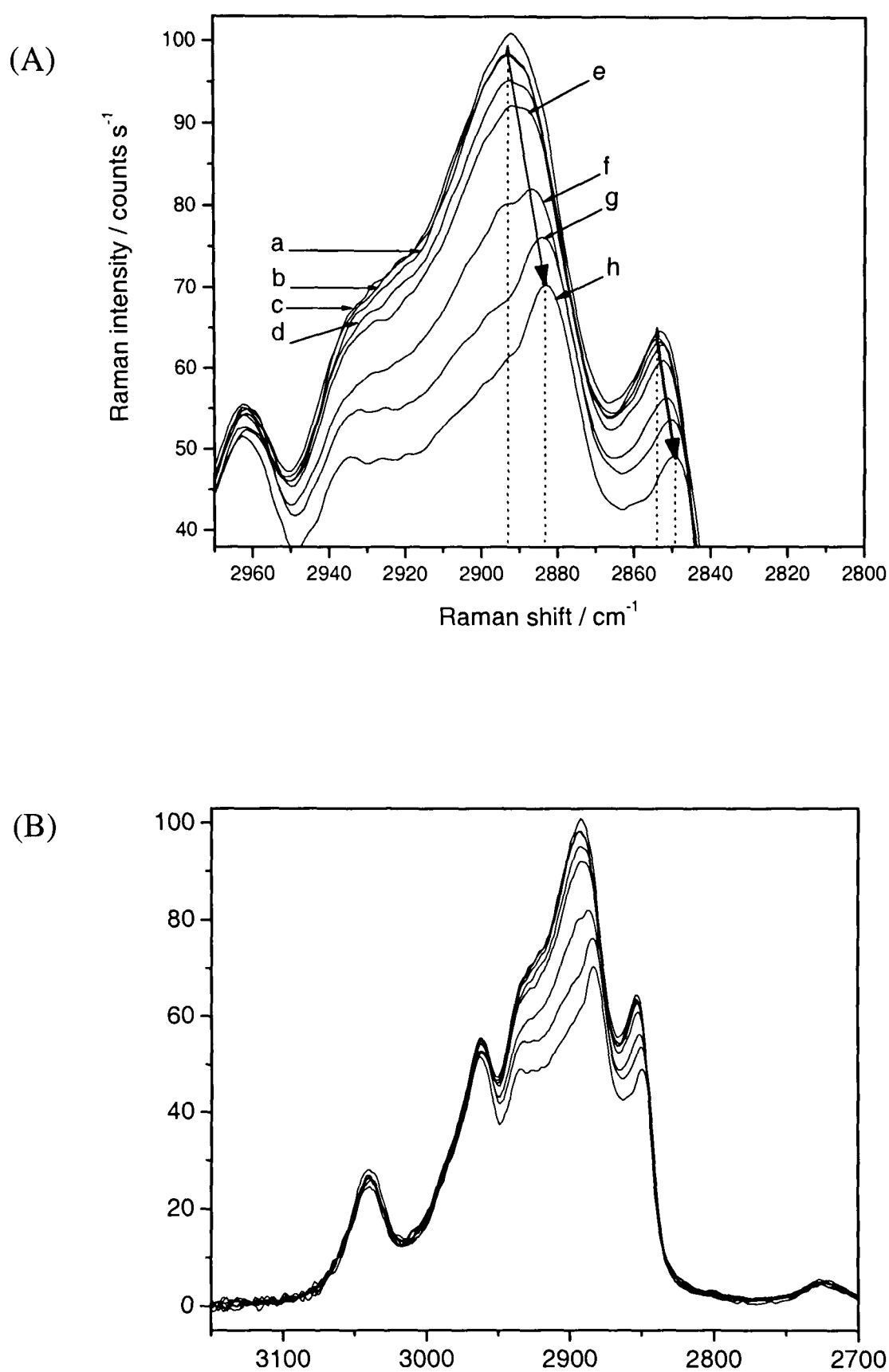


Figure 8.4 Temperature-dependence of the *p*-polarised Raman spectra of *DMPC pslb* (A) in the methylene vibration modes of C-H stretching region and (B) in the full range of C-H stretching region, 1.5 W, *p*-polarised excitation, unpolarised detection: (a) 42.0 °C, (b) 38.1 °C, (c) 32.0 °C, (d) 26.6 °C, (e) 23.3 °C, (f) 20.8 °C, (g) 16.8 °C, and (h) 13.2 °C. The arrows in (A) direct a decrease of temperature.

Figure 8.3 and 8.4 show the *s* and *p*-polarised spectra of *DMPC pslb* as a function of temperature. Both spectra have temperature-dependence in a broad range of temperatures (13.2 to 42 °C) below and above the main melting point. (Recall that a value of 22.2 ± 2.0 °C was found for the main phase transition in *SUV* by *DSC* (Koynova & Caffrey, 1998) and a value of 28 ± 4.0 °C in supported lipid bilayer by *AFM* (Charrier & Thibaudau, 2005)). In the *s*-polarised spectra of Figure 8.3, the intensities of both the d^- antisymmetric and d^+ symmetric methylene vibrations increase as the temperature of the *pslb* is decreased. The elevation of scattering intensities implies that the axes of the acyl chains becomes more upright (assuming that the number of lipid molecules within the focal spot remains constant as the temperature is changed (Charrier & Thibaudau, 2005)). The Raman intensity is maximised when the plane of methylene vibration is parallel to the *E*-field of the *s*-polarised excitation in the evanescence wave generated by *TIR*, in other words, the out-of-plane of methylene vibration which is parallel to the axis of the hydrocarbon is perpendicular to this *E*-field. (An *s*-polarised light probes Raman components solely in the plane of the surface, while a *p*-polarised light predominantly samples tensor components containing the *z*-direction (normal to the surface plane)). It means that lowering the temperature makes the acyl chain more perpendicular to the surface and more ordered. Charrier (Charrier & Thibaudau, 2005) noted that the transition in a supported single bilayer of *DMPC* on mica occurs at variable surface tension and nearly constant surface density (to within ~2 %), in contrast to a free-standing bilayer of a giant vesicle in solution for which the transitions occur at constant tension and variable surface density (~ 12 % difference (Needham & Evans, 1988)). The temperature-dependent changes are observed not only in the intensities of both d^- and d^+ mode but also in the intensity ratios of d^- to d^+ modes, $I(d^-)/I(d^+)$, and the peak shift. As the temperature decreased from (a) to (h), $I(d^-)/I(d^+)$ increased and the frequencies of both d^- and d^+ modes shifted downward. These trends

are characteristic of an increase in chain ordering at lower temperatures. I comment on fact that chain ordering is accompanied by a reduction in the volume per chain. If the area per molecule remained constant on the surface, one would expect the chains to tilt more. In fact they tilt less. One way of resolving this paradox might be if some part forms islands of the more densely packed phase.

In Figure 8.4, the intensities of the *p*-polarised spectra show exactly the opposite tendency to the *s*-polarised spectra. They decreased as the temperature of the bilayer was decreased. This result is consistent with a decreasing chain tilt with decreasing temperature.

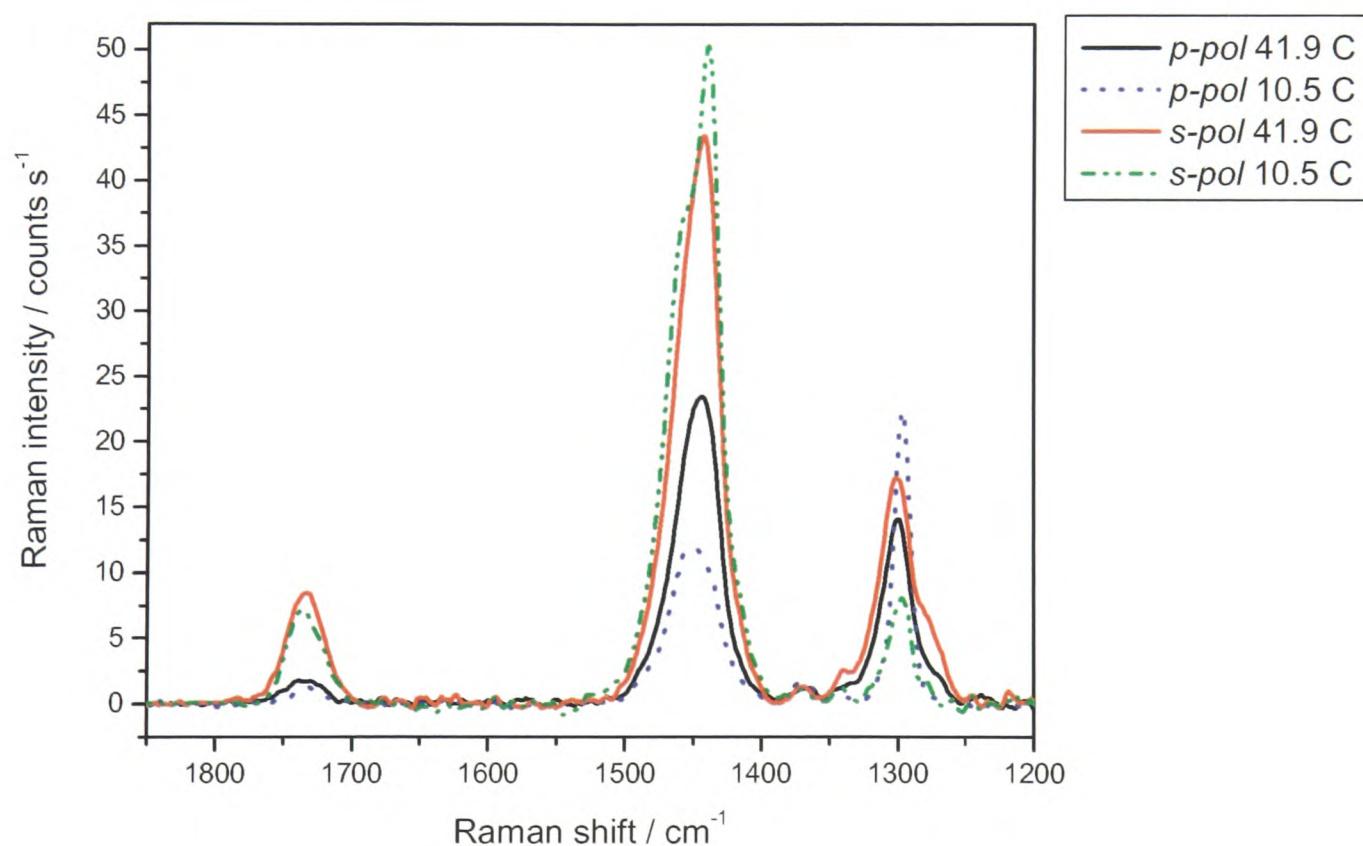


Figure 8.5 Temperature-dependence of the Raman spectra of *DMPC pslb* in the fingerprint region, 1.2 *W* unpolarised detection: *p*-polarised excitation, 41.9 °C (solid line in black); *p*-polarised excitation, 10.5 °C (dotted line in blue), (c) *s*-polarised excitation, 41.9 °C (solid line in red), (d) *s*-polarised excitation at 10.5 °C (dash-dot line in light green)

We have also studied the fingerprint region as a function of temperature. Figure 8.5 shows *s* and *p*-polarised spectra at 41.9 °C and 10.5 °C. The carbonyl band at 1734 cm^{-1} is little changed by temperature. In the *s*-polarised spectra, the methylene scissoring mode around 1450 cm^{-1} is slightly stronger at 10.5 °C (dash-dot line) than at 41.9 °C (bold solid line), while in the *p*-polarised spectra it is much weaker at 10.5 °C (dotted line) than 41.9 °C (thin solid line). The scissoring mode involves motions of C-H bonds perpendicular to the plane of the hydrocarbon backbone. The polarisability change associated with this motion is greatest in the plane of the C-H bonds and least along the chain axis. The observed intensity variations therefore indicate that the chains become more upright at lower temperatures. The inference is confirmed by the temperature-dependence of the methylene twisting modes around 1300 cm^{-1} . This mode is polarised predominantly along the chain axis (it corresponds to a mode assigned to B_{3g} symmetry in crystalline polyethylene) and therefore exhibits the opposite temperature dependence to the scissoring mode. It is stronger at low temperature in the *p*-polarised spectrum and weaker in the *s*-polarised spectrum.

Figure 8.6 shows the twisting mode of *DMPC pslb* in more detail. In addition to the variation in intensity noted above, the linewidth decreases markedly with a decrease in temperature and the frequency shifts to lower wavenumbers. The decrease in linewidth is indicative of a smaller range of conformations and/or reduced mobility in the *pslb*. By analogy with the C-H stretching modes, I suggest that the red-shift in the twisting mode is also a marker of increased chain ordering. Another interesting feature is the disappearance of the shoulder at 1270 cm^{-1} at lower temperatures in both polarisations. This particular peak has not been assigned with confidence, but it is plausible that it is associated with gauche defects, since it disappears simultaneously in both polarisations and concurrently with chain ordering as indicated by other conformational markers.

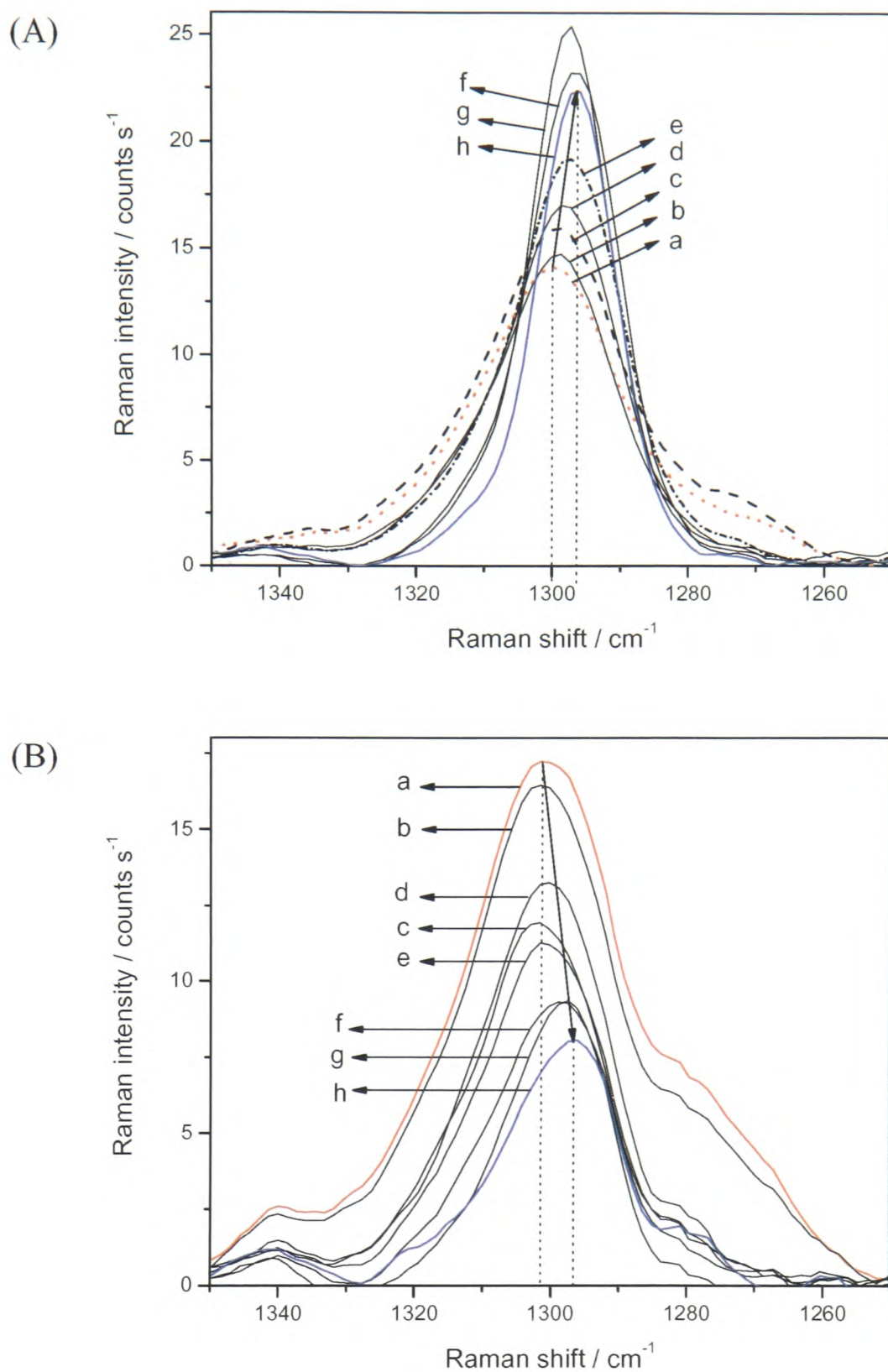


Figure 8.6 Temperature-dependence of the Raman spectra of *DMPC pslb* in the twisting mode around 1300 cm^{-1} of the fingerprint region, 1.2 *W*, unpolarised detection (A) *p*-polarised excitation, (B) *s*-polarised excitation, (a) 41.9 °C, (b) 36.6 °C, (c) 27.5 °C, (d) 24.3 °C, (e) 22.6 °C, (f) 20.2 °C, (g) 18.1 °C, and (h) 10.5 °C. The arrows indicate decreasing of temperature.

8.3 Temperature-dependent polarised TIR-Raman spectra of *DPPC pslb*

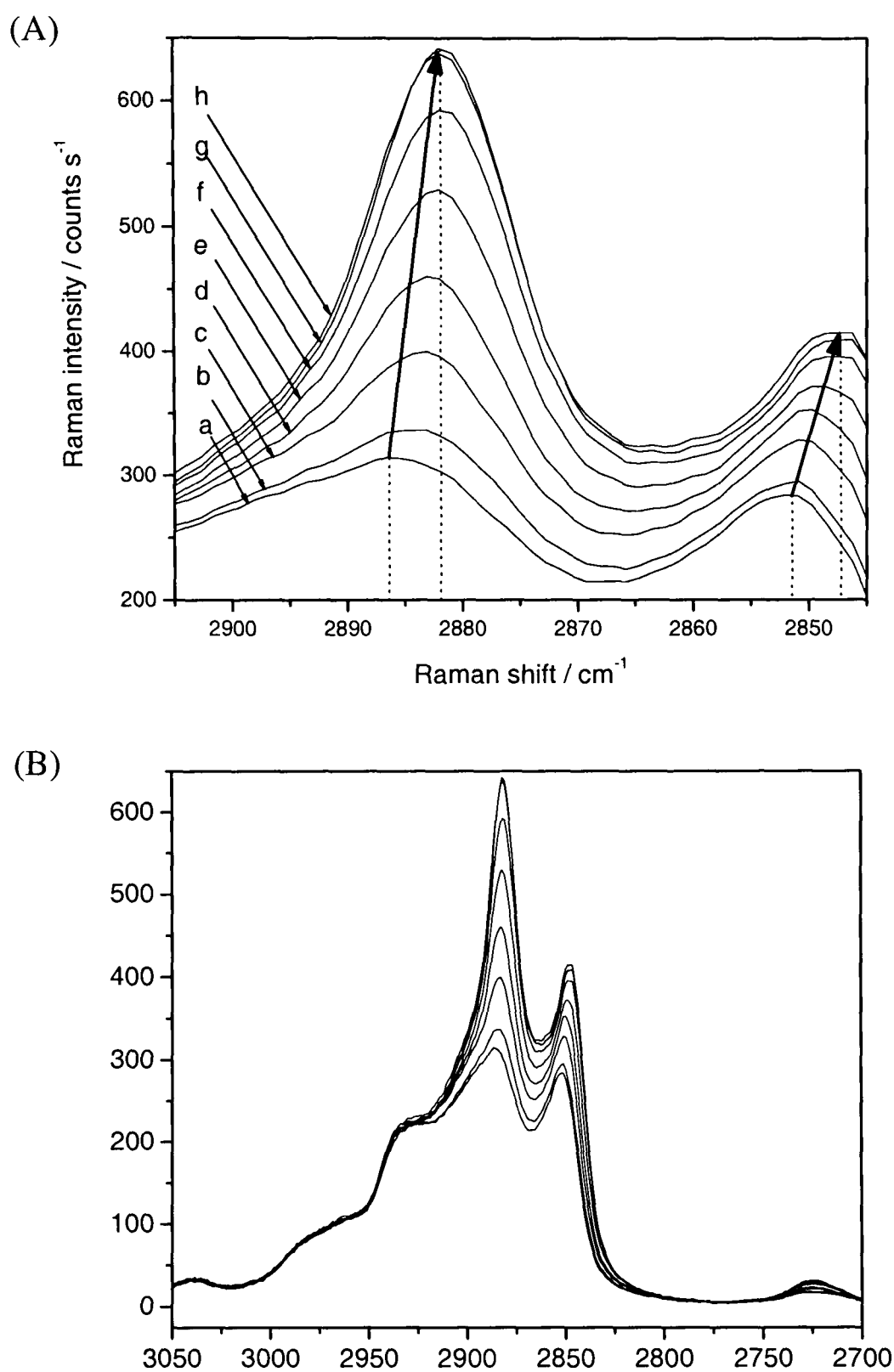


Figure 8.7 Temperature-dependence of the Raman spectra of *DPPC pslb* (A) in the methylene vibration modes of C-H stretching region and (B) in the full range of C-H stretching region, 1.2 W, *s*-polarisation of excitation, unpolarisation of detection: (a) 44.5 °C, (b) 41.0 °C, (c) 39.0 °C, (d) 37.3 °C, (e) 32.8 °C, (f) 26.8 °C, (g) 22.6 °C, and (h) 19.3 °C. The arrows direct decreasing of temperature.

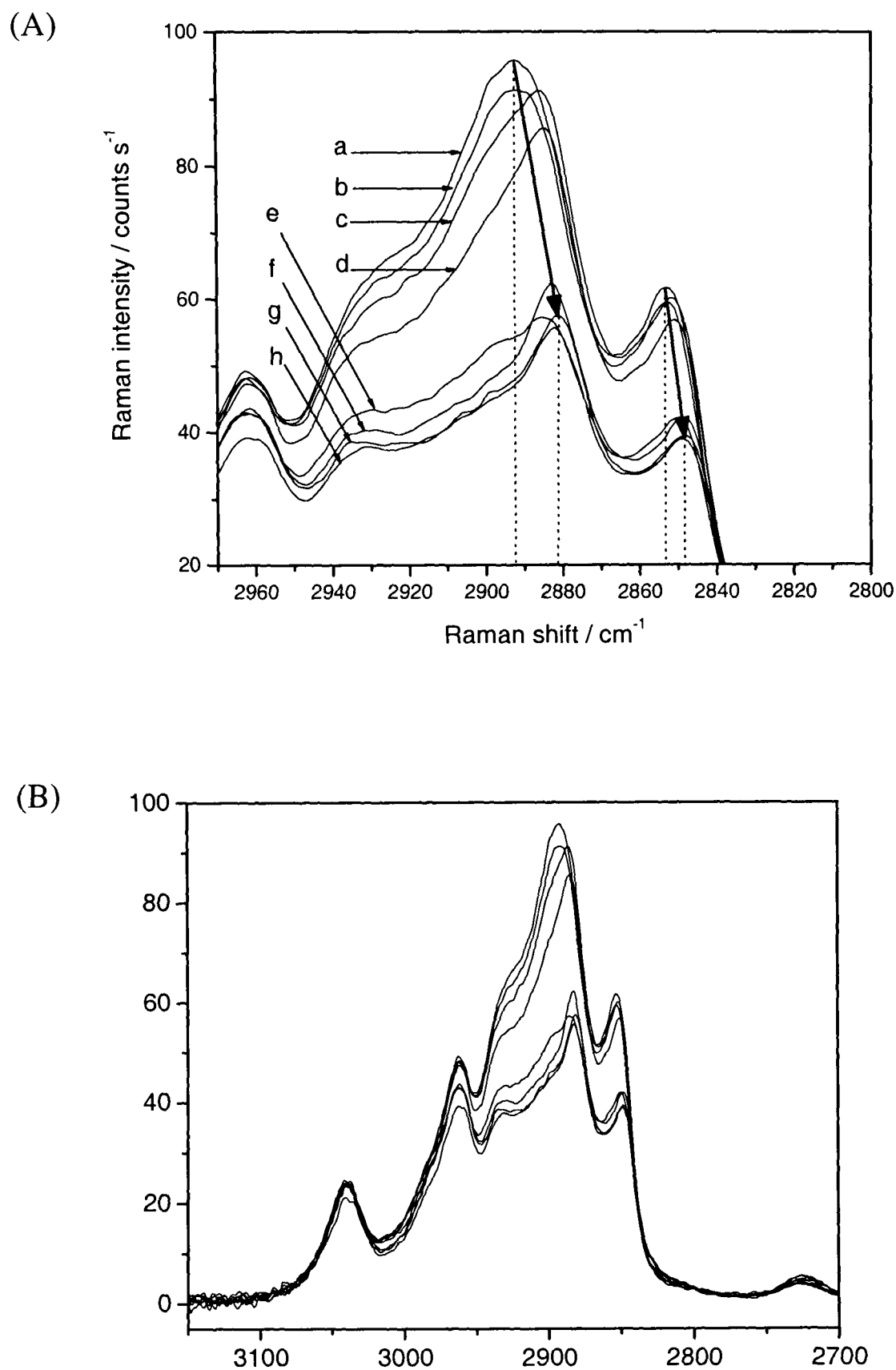


Figure 8.8 Temperature-dependence of the Raman spectra of *DPPC pslb* (A) in the methylene vibration modes of C-H stretching region and (B) in the full range of C-H stretching region, 1.5 W, *p*-polarisation of excitation, unpolarisation of detection: (a) 44.5 °C, (b) 41.0 °C, (c) 39.0 °C, (d) 37.3 °C, (e) 32.8 °C, (f) 26.8 °C, (g) 22.6 °C, and (h) 19.3 °C. The arrows direct decreasing of temperature.

Figures 8.7 and 8.8 present *s* and *p*-polarised *TIR*-Raman spectra of *DPPC pslb* in the temperature range from 19.3 °C to 44.5 °C. The reference temperature of the main phase transition is 41.0 °C in *SUV* (Marsh, 1990). For the supported lipid bilayer on mica a value of 40.4 °C was determined by *DSC* with two higher transitions below 45 °C (Yang & Appleyard, 2000) or 45.5 °C by magnetic acoustic code *AFM* (Feng, *et al*, 2005). The existence of pre-transition ($P_{\beta'}$) in supported lipid bilayer of *DPPC* is unresolved: it will be briefly discussed in relation to our Raman spectra in the section of 8.6. I tried to scan as broad a range of temperatures as possible to include these reference temperatures, but the maximum temperature achievable is limited by heat losses from the sample cell and the tubing connecting the cell to the circulator. Additionally, we were concerned about using the microscope objectives above their operating temperature, under the limitation of heating on the water-silica interface. The intensities of the *s*-polarised spectra increased while the intensities of the *p*-polarised spectra decreased as the temperature decreased. In addition, the methylene peaks shifted to lower frequencies and the ratio $I(d^-)/I(d^+)$ increased at lower temperatures. These are the same trends as the *s*-polarised spectra of *DMPC pslb* has as they were explained. The axes of the hydrocarbon chains of the acyl groups of the lipid molecules become more perpendicular to the plane of surface and the lipid molecules become more ordered while the temperature passes through the melting point. The *p*-polarised spectra have the exactly contrary results to *s*-polarised spectra in their temperature-dependencies. It is the same tendency as the *p*-polarised spectra of *DMPC pslb*. Taken into account that the $I(d^-)/I(d^+)$ is evaluated in 1.54 below the main transition and therefore it is high enough to be in liquid crystalline phase ($I(d^-)/I(d^+)$ is equal to 0.7 when the chains are completely fluid (liquid state), and equal to 2.2 in the case of a perfect crystal state (Gaber & Peticolas, 1977; Lhert, *et al*, 2002), it is ascertained that the lipid molecules experience L_{α} to $L_{\beta'}$ phase transition. The L_{α} to $P_{\beta'}$ phase transition

will be considered in 8.5 & 8.6.

8.4 Temperature-dependent polarised TIR-Raman spectra of *POPC pslb*

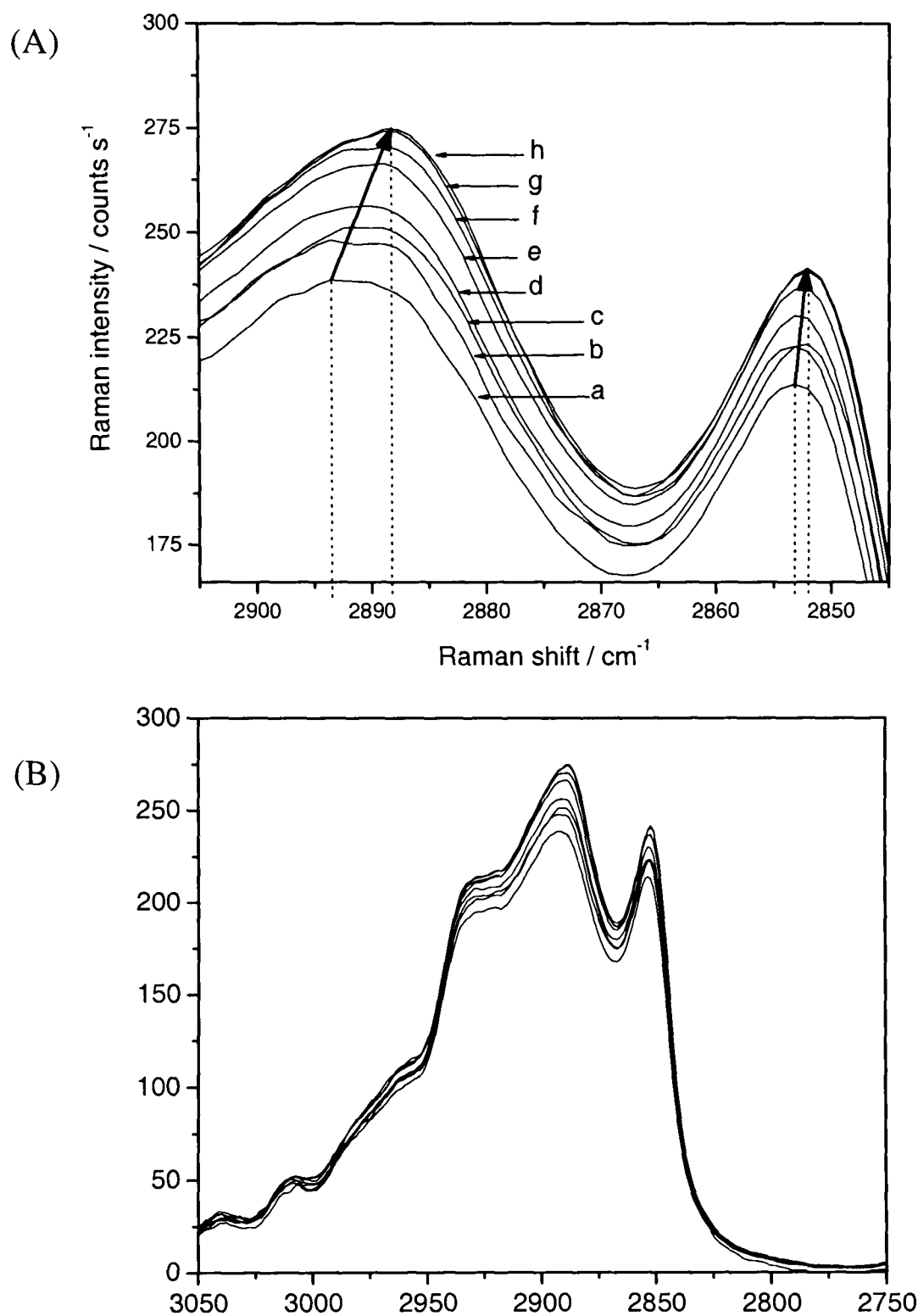


Figure 8.9 Temperature-dependence of the Raman spectra of *POPC pslb* (A) in the methylene vibration modes of C-H stretching region and (B) in the full range of C-H stretching region, 1.2 W, *s*-polarisation of excitation, unpolarisation of detection: (a) 41.0 °C, (b) 37.5 °C, (c) 32.1 °C, (d) 26.2 °C, (e) 22.9 °C, (f) 20.0 °C, (g) 17.2 °C, and (h) 13.9 °C. The direction of the arrows goes from higher to lower temperature.

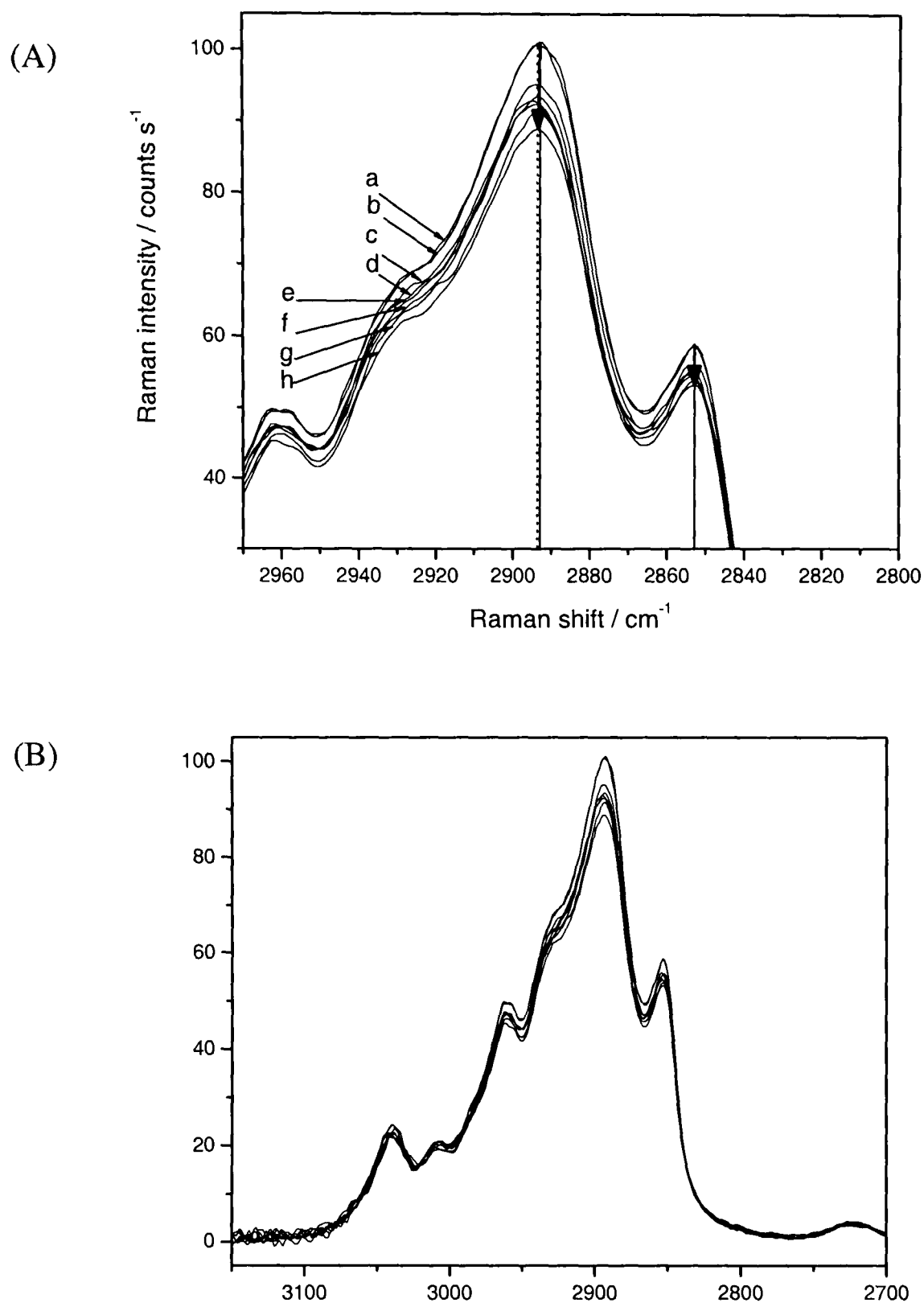


Figure 8.10 Temperature-dependence of the Raman spectra of *POPC pslb* (A) in the methylene vibration modes of C-H stretching region and (B) in the full range of C-H stretching region, 1.5 W, *p*-polarisation of excitation, unpolarisation of detection: (a) 41.0 °C, (b) 37.5 °C, (c) 32.1 °C, (d) 26.2 °C, (e) 22.9 °C, (f) 20.0 °C, (g) 17.2 °C, and (h) 13.9 °C. The direction of arrows goes to lower temperature.

In Figures 8.9 and 8.10, the *s* and *p*-polarised spectra of *POPC pslb* are presented over the temperature range 14–41 °C. In the *s*-polarised spectra, the intensity increased by only about 15 % as the temperature was lowered, compared to a factor of two changes in *DPPC*. The *p*-polarised spectra showed a corresponding decrease, indicative a small untilting of the chains. The ratio $I(d^-)/I(d^+)$ is almost independent of temperature: the chains do not become more ordered at lower temperatures because *POPC* remains in its fluid L_α phase.

8.5 Phase transition behaviour of *DMPC*, *DPPC*, and *POPC pslb*'s

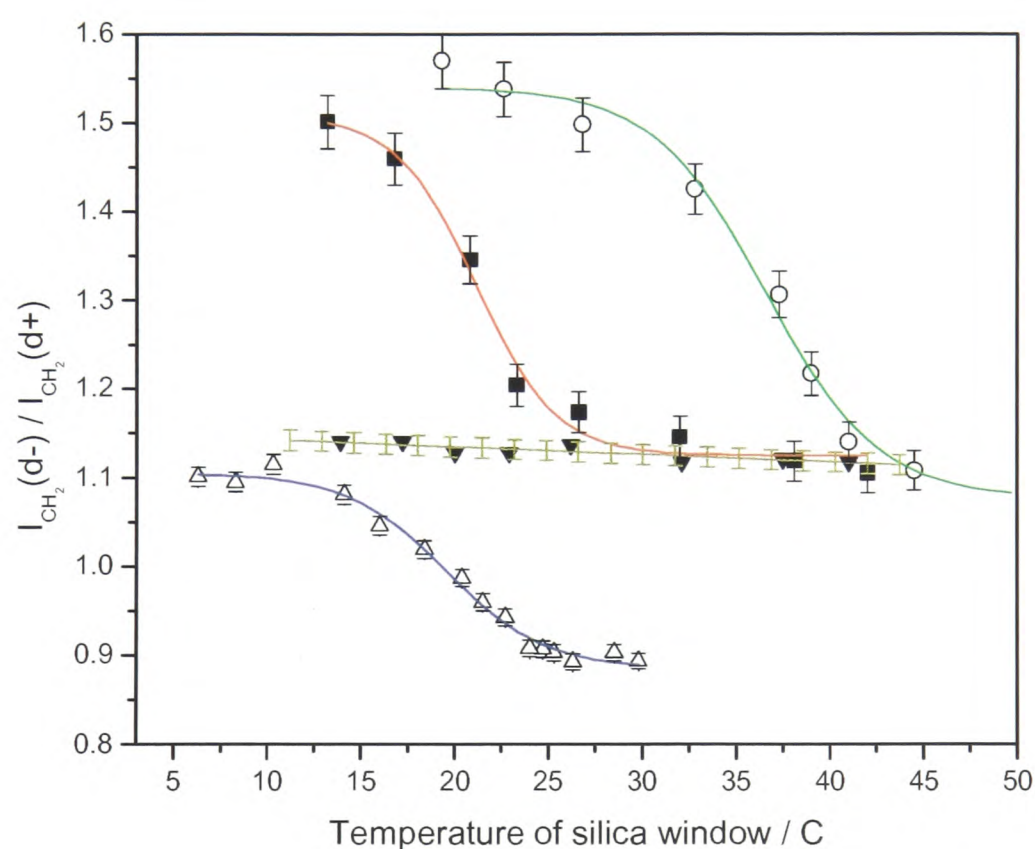


Figure 8.11 Variation in $I(d^-)/I(d^+)$ with the temperature for *pslb*'s of (■) *DMPC*, (○) *DPPC*, and (▲) *POPC* and for (△) *DMPC* vesicle in a bulk suspension. Laser power was 1.2W, *s*-polarised excitation, unpolarised detection.

For a quantitative description of the thermotropic behaviour of the *pslbs* of *DMPC*, *DPPC*, and *POPC pslbs*, the ratio $I(d^-)/I(d^+)$ and the peak frequencies of the d^-

and d^+ modes were compared. These empirical markers are characteristic of the chain ordering in the Raman spectra of lipid chains. A higher intensity ratio and lower vibrational frequency are indicative of increasing chain ordering. Figure 8.11 presents the plot of the $I(d^-)/I(d^+)$ of the *pslbs* as a function of temperature. The spectra were obtained with *s*-polarised excitation and unpolarised detection for the *pslbs*. In *DMPC*, the ratio $I(d^-)/I(d^+)$ of *pslb* is compared with *SUV*. Even though the polarisations of the excitation and scattering fields are the same in both experiments, the absolute value of $I(d^-)/I(d^+)$ in the bulk suspension of *DMPC SUV* cannot be compared directly with *pslb*, because the normal to the bilayer is randomly oriented in the suspension while it is perpendicular to the excitation field in the *pslbs*. Nevertheless, changes in $I(d^-)/I(d^+)$ with temperature can be correlated. Ideally, one would deconvolute the spectra in Figure 8.3-4, 8.7-10 to obtain the linewidths and peak frequencies of the individual peaks. However, the d^- mode overlies a complex band comprising Fermi resonances of the d^+ mode with overtones of bending modes (Gaber & Peticolas, 1977; Snyder, *et al*, 1978), as well as the r^+ mode of the methyl group (and its Fermi resonances), all of which may also change with temperature. Consequently, spectral decomposition cannot be undertaken with any great degree of certainty (Gaber & Peticolas, 1977). As a result we have adopted the simpler approach of fitting the regions around 2850 and 2880 cm^{-1} with a Savitzky-Golay procedure and extracting the peak intensity and peak position from these local fits. For *POPC* bilayers, $I(d^-)/I(d^+)$ is almost constant at a value of 1.15, decreasing only very slowly with increasing temperatures. Both *DMPC* and *DPPC* show the same ratio $I(d^-)/I(d^+)$ as *POPC* at the highest temperatures measured. As the temperature was lowered, however, $I(d^-)/I(d^+)$ increased in an approximately sigmoidal fashion, leveling out at a value of 1.5-1.6 at low temperatures. The vesicular suspension of *DMPC* showed very similar thermal behavior to the *pslb*, but the absolute values of $I(d^-)/I(d^+)$ were much lower, ranging from 0.9 at high temperature to 1.1 at low

temperature. The range of slow transition is well in accordance with the literature data from 10 to 27 °C (Van Dael, *et al*, 1982). The onset of the steep part of the curve in the *pslbs* was around 41 °C for *DPPC* and 26 °C for *DMPC*. For more details of these transient regions, we plotted the frequencies of d^- and d^+ modes of *DMPC*, *POPC*, and *DPPC* as shown in Figure 8.12-14, respectively. For *DPPC* and *DMPC*, the peak frequencies show a sigmoidal increase with temperature, the change being more pronounced in the d^- than the d^+ mode. For *POPC pslbs*, the peak frequencies in the *p*-polarised spectra are temperature-independent, while the *s*-polarised spectra show a smaller decrease with decreasing temperature than do *DMPC* and *DPPC*. These observations are consistent with *POPC* remaining in a fluid phase with only small changes in structure with temperature. However, the *DMPC* and *DPPC pslbs* have temperature dependence of both d^- and d^+ modes and the higher peak shift in d^- mode is observed than d^+ mode in both lipids. They experience variation of temperature during the phase transition. It implies that higher difference of the peak shift in d^- mode than d^+ mode is a characteristic evidence showing the main phase transition in the Raman spectra.

For the d^- mode of *DMPC pslb* in Figure 8.12, the sigmoidal behaviour of the peak shift by the *p*-polarised excitation is different from the *s*-polarised, considering broader transition (around 10 °C in the *p*-polarised, but 15 °C in the *s*-polarised) and higher transition temperature in the *s*-polarised curves. This was expected to be maintained in the temperature dependence of the peak shift of *DPPC pslb*. Actually our observation of temperature is technically limited within 50 °C due to problematic insulation causing heat loss through the connection with the temperature-controlled water-bath circulator. So the maximum observed temperature is around 45 °C at the laser spot on the water-silica interface. It would be interesting to measure higher temperature over 45 °C, because there is still on-going slow increase above a range

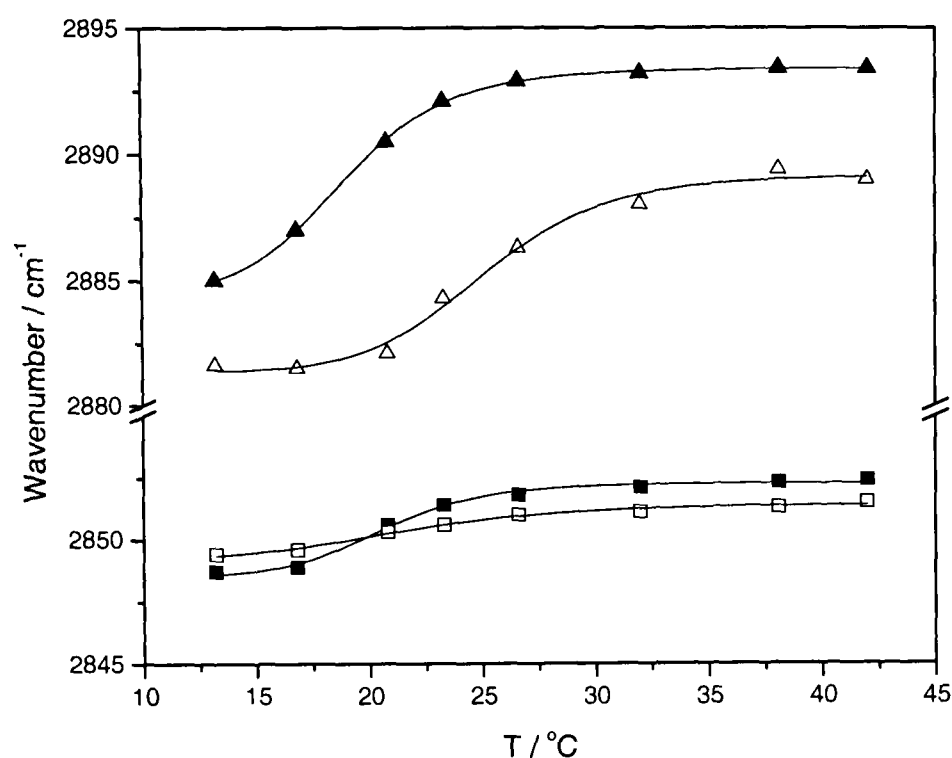


Figure 8.12 The temperature dependence of the vibrational frequencies of the d^- and d^+ modes of *DMPC pslb*. (▲) d^- , p -polarised excitation; (△) d^- , s -polarised excitation; (■) d^+ , p -polarised excitation; (□) d^+ , s -polarised excitation, with unpolarised detection.

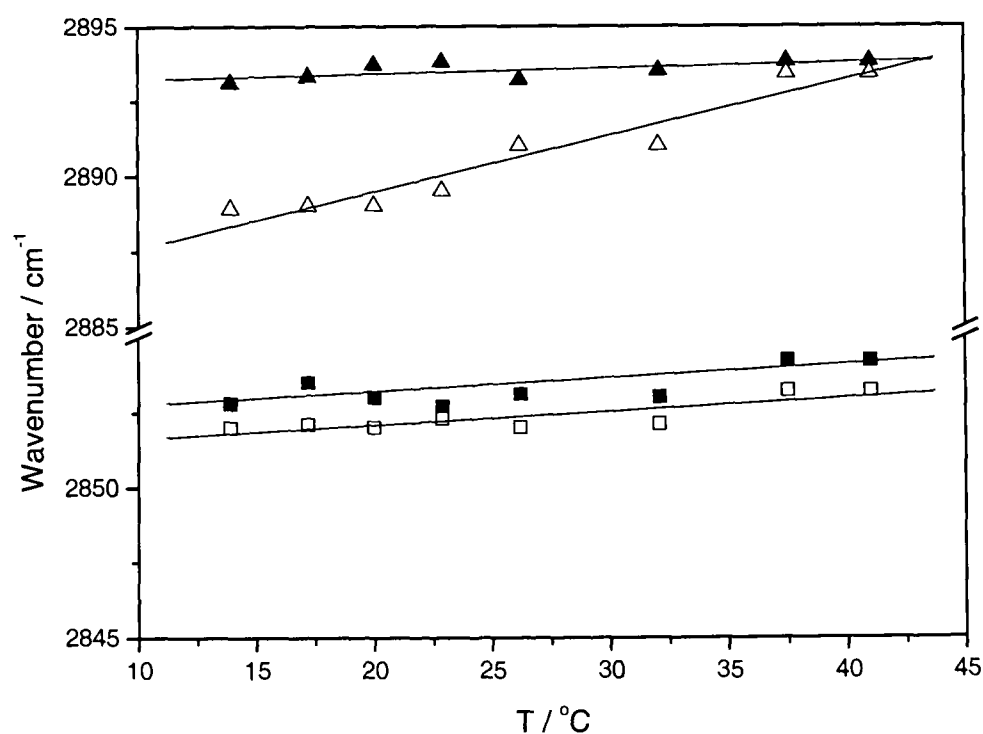


Figure 8.13 The temperature dependence of the vibrational frequencies of the d^- and d^+ modes of *POPC pslb*. (▲) d^- , p -polarised excitation; (△) d^- , s -polarised excitation; (■) d^+ , p -polarised excitation; (□) d^+ , s -polarised excitation, with unpolarised detection.

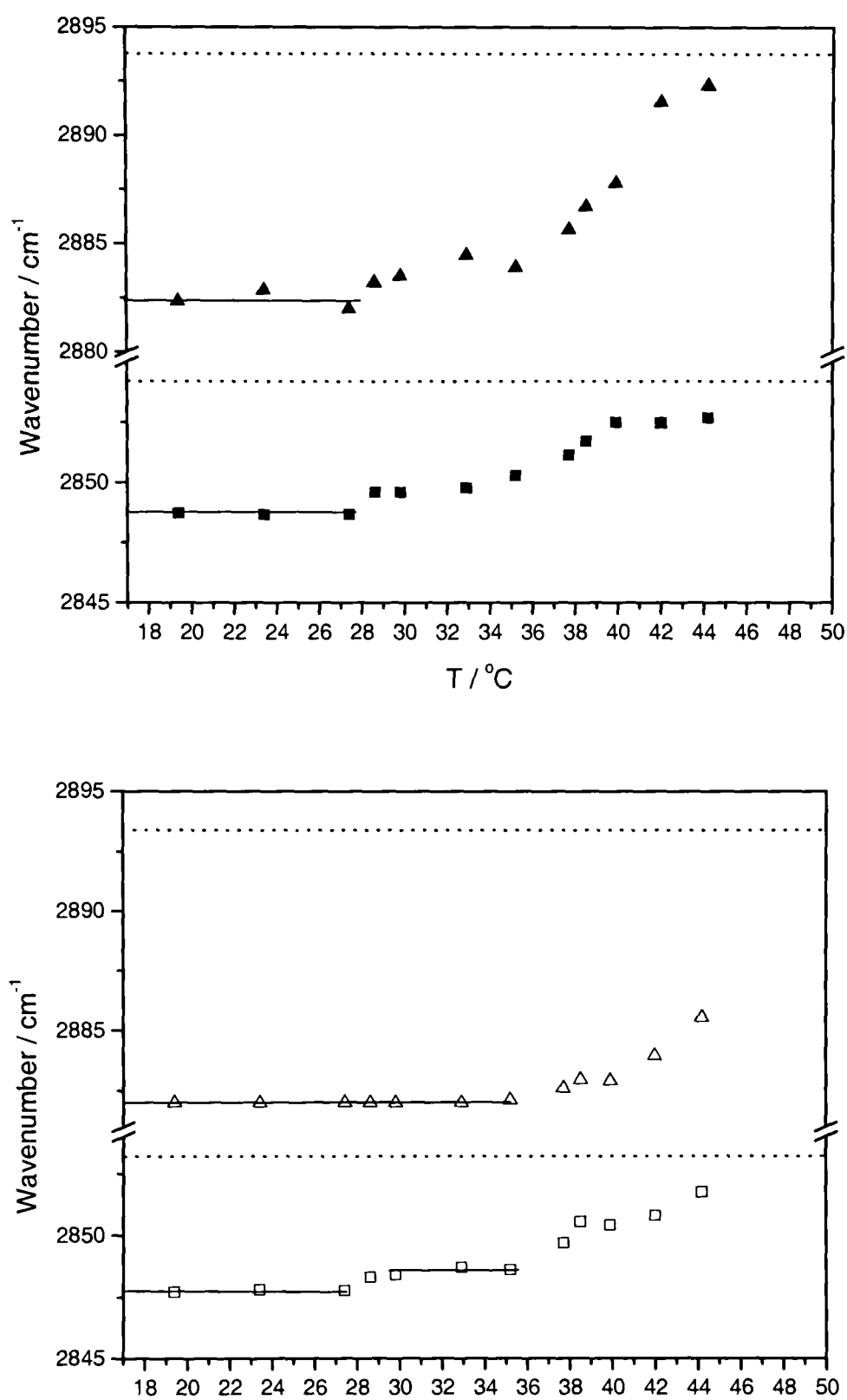


Figure 8.14 The temperature dependence of the vibrational frequencies of the d^- and d^+ modes of *DPPC pslb*. (▲) d^- , p -polarised excitation; (△) d^- , s -polarised excitation; (■) d^+ , p -polarised excitation; (□) d^+ , s -polarised excitation, with unpolarised detection. The dashed lines indicate the wavenumbers of the *POPC* vibrations measured in 41.0 °C.

including the expected temperature of phase transition, (in case of *DMPC pslb* in d^- mode, the s -polarised spectra have the half way of the transition of 26 °C with

broadness of around 20 °C higher than the *p*-polarised spectra have of 19 °C); the decelerating increase of sigmoidal curve may be extended over the temperature of our observation. In the peak shift of the *p*-polarised spectra, the frequencies level out above 25 °C, with an onset of increasing curvature around 15 °C, but in the *s*-polarised spectra, the frequencies are increased from around 20 °C to 35 °C. It is hard to speculate the main phase transition temperature in temperature-dependent peak shift of two different polarised spectra, because it is abstract what is more discernibly related to the temperature dependence by the different polarised spectra in either d^- or d^+ mode.

For the *DPPC pslb* (Fig. 8.14) the peak frequencies showed a more complex temperature variation. Below 28 ° they were independent of temperature. From 28 °C to 36 °C, the frequencies were constant at a slightly higher value. Above 36 °C, the peak frequencies increased rapidly, especially in the *p*-polarised spectra, until they approached the values observed in *POPC* (except for the d^- mode in *s*-polarised spectra). The intermediate region showing the plateau will be considered in the discussion of this chapter.

For the *POPC pslb*, (Fig. 8.13) the peak frequency was almost independent of temperature, except for the d^- mode in *s*-polarised spectra which showed a gradual increase with temperature. The peak frequencies in *POPC* are close to the values reached in the high-temperature limit of the *s* or *p*-polarised spectra of *DMPC* and *DPPC*.

8.6 Discussion

The existence of $P_{\beta'}$ in *pslb* on mica has been strongly speculated by *FRAP* (Tamm & McConnell, 1985) and *AFM* experiments (Charrier & Thibaudau, 2005). But it has also been a controversial issue in *DSC* (in planar supported lipid bilayers) (Yang & Appleyard, 2000) and *NMR* studies (in spherical supported bilayer) (Naumann, *et al*, 1992). This intermediate phase may be detectable by Raman scattering in multilamellar dispersions (Gaber & Peticolas, 1977), but has never been observed in attempt by the Raman spectra of *pslb* at a water/silica interface. The Raman data in this chapter can be regarded as supporting evidence for the existence of $P_{\beta'}$ in *pslb*. As far as it can be discerned, the prolonged phase in the temperature range between 28 °C and 36 °C as shown in Figure 8.14. It is not possible to confirm this pre-transition phase in *pslb* by our Raman data but more extensive measurement would need to be taken to consolidate this possibility.

The broadness of the phase transition of *pslbs* is also worthy of more consideration based on our Raman data. As noted in early Raman works by *H. Van Dael et. al.* (Van Dael, *et al*, 1982), the phase transition in Raman spectra was broader in *SUV* than in *LUV* and *MLV*. For *LUV* and *MLV*, Raman data were consistent with *DSC* data, showing a narrow transition. However, *SUVs* even by *DSC* had a broader transition (Van Dijck, *et al*, 1977). The *pslb* at the water/silica interface in our Raman measurements also presents a broad phase transition as observed in *SUV*. Our results are well corresponded with the temperature dependence of the CH_3 intensity by sum-frequency vibration generation (Liu & Conboy, 2004) leading to speculation that the heterogeneity of the response to the heating between two leaflets of the bilayers might delay the reaction of sharp phase transition in supported lipid bilayers on mica. Recently *AFM* measurements (Charrier & Thibaudau, 2005; Feng, *et al*, 2005) suggested that the van't

Hoff formalism at a constant surface tension transition of both leaflets that has been applied to free standing bilayers in vesicles might not be suitable to explain the large transition temperature widths observed for supported lipid bilayers on mica because the interaction of the inner leaflet lipids with the mica substrate was strong enough to modify the density in the inner leaflet by the gain of energy due to the adsorption of lipids on the substrate; the lipids in the lower leaflet at higher density are more resistant to the lipid expansion than upper leaflet during the transition. So as depicted in the literature (Feng, *et al*, 2005), a model was proposed to describe the asymmetrical melting to decouple the phase transition for the two leaflets in Figure 8.15. This model may be considered as a good framework to substantiate the broad phase transition of *pslbs* on silica that was also represented by our Raman data.

The shoulder within the twisting mode disappeared between between 36.6 °C and 27.5 °C in Figure 8.6 (B) showing the temperature-dependent Raman spectra of *DMPC pslb*. We have considered this to be due to gauche defects. In other words, the gauche defect that normally exists above the melting temperature is extinguished when the temperature lowered from 36.6 °C to 27.5 °C. Therefore considering the validity of this result, the most probable temperature of the main transition in *DMPC pslb* is rather 28 °C (which has been estimated by comparable *AFM* work recently) than 24 °C (Charrier & Thibaudau, 2005; Tokumasu, *et al*, 2003; Xie, *et al*, 2002).

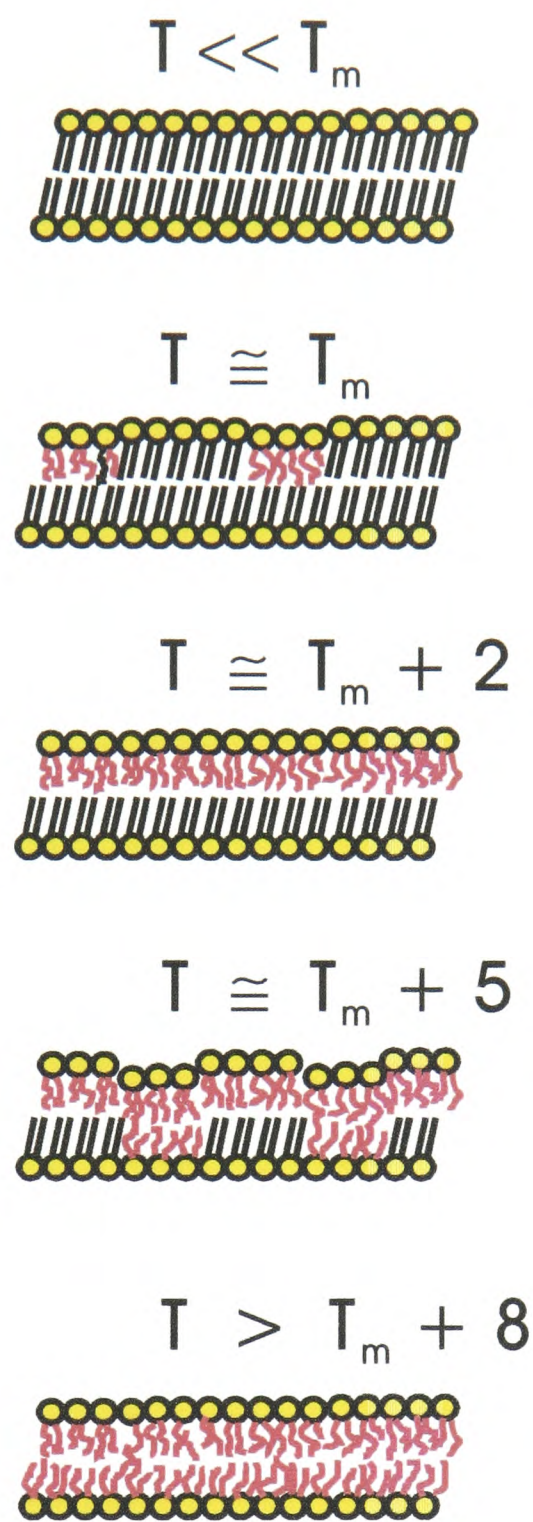


Figure 8.15 Schematics of supported lipid bilayers during phase transition. This model was proposed by Feng, *et al* (Feng, *et al*, 2005).

8.7 Conclusion

In this chapter (with Chapter 7), we can examine thermal behaviours of *DMPC*, *DPPC*, *POPC* *pslbs* using their high quality polarised *TIR*-Raman spectra, which could be acquired at water/silica interface. The local orientation and chain packing of the lipid chains does not change sharply at the temperature of the main lipid phase transition ($L_\alpha \rightarrow L_\beta$), as reported the lamellar vesicles according to an extensive number of publications (see the review article of phase transitions of phosphatidylcholines in vesicle solution (Koynova & Caffrey, 1998)). Instead, Raman spectra point to a gradual structural change over a 10 °C range below the main calorimetric phase transition. This continuous behaviour has implications for experiments carried out in the gel phase of *pslbs*, since the local structure and dynamics may depend on temperature even several degrees below the calorimetric phase transition.

The markers of chain ordering in the Raman spectra of lipid chains, $I(d^-)/I(d^+)$, present that a supported lipid bilayer in the fluidic state (L_α) has a degree of chain ordering similar to the bulk lamellar bilayer in a gel state (L_β). The temperature-dependent *s* and *p*-polarised Raman spectra in CH_2 stretching and bending modes exhibit trends consistent with each other in terms that chains experience going upright as temperature decreases.

Also, the blue shifts of the peak frequencies of the d^+ and d^- modes of *DMPC*, *DPPC*, and *POPC* have been observed. For *POPC*, the frequencies increase slowly and linearly with the temperature. For *DMPC*, the frequencies show a sigmoidal increase with temperature, the change being more pronounced in the d^- and the d^+ modes. The frequencies level out above 25 °C. For *DPPC*, below 28 °C, the frequencies are independent of temperature. Above 28 °C, the peak frequencies rise until at the highest temperatures measured they are within 2-3 °C of the values observed in *POPC* (except

for the d^+ mode in *s*-polarised spectra). The increase in frequency is not completely smooth, however, but is concentrated in the range 28-30 °C and 35-44 °C, with an indication of a plateau-in-between in *DPPC pslb*. Our Raman results indicate a broad main phase transition generally corresponding to the observations by other techniques such as *NMR* (Naumann, *et al*, 1992), *AFM* (Charrier & Thibaudau, 2005), and sum-frequency vibration generation (Liu & Conboy, 2004).

Chapter 9 *TIR*-Raman spectroscopy of peptides interacting with planar supported lipid bilayers

In Chapter 7 and 8 I showed that *TIR*-Raman spectroscopy can be used to study structural changes in planar-supported lipid bilayers at the silica/water interface. In this chapter, I show that *TIR*-Raman scattering also has the sensitivity to detect peptides either incorporated in or interacting with a *pslb*. The focus of the chapter is on the model channel-forming peptide, gramicidin D (*gD*), which was incorporated into phospholipid vesicles before fusion of the vesicles to the silica surface. I also show that the reversible binding of a soluble antimicrobial peptide, indolicidin, to a *pslb* can be observed by *TIR*-Raman spectroscopy. Inferences on the structure of the peptide have been drawn for both systems.

While *ATR-FTIR* has been used extensively to study peptides and proteins adsorbed at the solid/water interface (Tamm, 2002; Vigano, *et al*, 2001), I am aware of no previous reports of Raman spectra of peptides or proteins in *pslbs*. Raman research has centered mainly on peptides/proteins in solution in the absence of lipid due to limitations of sensitivity. Raman spectroscopy has a number of advantages over *ATR-IR*, not least the much weaker water background from the O-H bend in the region of the structure-sensitive Amide I bands of peptides.

S. Asher (Beeler, *et al*, 2004; Pimenov, *et al*, 2005) and *P. Carey* (Carey, 1999) have been at the forefront of developments in *UV* resonance Raman spectroscopy of soluble proteins. *Thomas'* works have special requirement for a well-oriented and ordered (crystal-like) solid structure in which the molecular coordinates are well-specified relative to the laboratory frame of reference, like oriented fibres of a

filamentous bacteriophage (Tsuboi, *et al*, 2005). *P. Carey* has primarily investigated amino acids with extended π -electron systems because these are highly polarisable and give rise to a strong Raman scattering, while saturated systems such as carbohydrates are less amenable for Raman analysis (Carey, 1999). Neither *Thomas* nor *Carey* applied resonance Raman scattering to peptides in lipid bilayers.

We were interested in studying membrane peptides in an environment as close as possible to the environment in nature. We also used a *pslb* on a solid substrate rather than vesicles in solution in order to specify the orientation information of the peptide with respect to the laboratory axes. Vesicles in solution are isotropic, which reduces the amount of information that can be obtained from polarized Raman scattering. In a *pslb*, the polarisation both the incident excitation and scattered radiation are well-defined in the reference coordinates of the lipid monolayer on the solid substrate. Polarised spectra are therefore sensitive to the orientation of a particular vibrational mode with respect to the interface.

9.1 Gramicidin D

The choice of *gD* as a model peptide has been justified in Chapter 4, where I also provide background information on the structure of this unusual peptide. The experimental techniques for preparing and characterizing vesicles containing *gD* and their fusion to the silica surface were described in Chapter 5 and 6. This chapter describes the acquisition and analysis of *TIR*-Raman spectra of *gD* in the bulk phase, in multilamellar vesicles and in a *pslb*.

9.1.1 Raman scattering of *gD* in a cast film

Prior to studying *gD* in a *pslb*, we required our own reference data of *gD* both on its own and in a phospholipid environment. Raman spectra of both *gD* and lipid/*gD* dispersions have been reported in early publications (Aslanian, *et al*, 1986; Iqbal & Widekamm, 1980; Naik & Krimm, 1984; Rothschild & Stanley, 1974; Short, *et al*, 1987), though the papers lack explanations of the peaks in the spectra. While the spectra in a *pslb* may differ from those in a cast film of *gD* or a *POPC/gD* dispersions, due to the sensitivity of the structure of *gD* to the preparation conditions, the bulk spectra were useful in the assignment of the spectra from the *pslbs*.

Figure 9.1 presents Raman spectra in the C–H stretching region (2800–3100 cm^{-1}) of *gD* alone in a film cast onto a silica window from a solution in 1:4 (v/v) *MeOH:CHCl₃*. Five 100- μL drops of the solution were added sequentially to the window, allowing the solvent to evaporate between each addition. The resulting film on the surface had a radius of 1.5 *cm*. The Raman spectra were acquired in *TIR* geometry with an Olympus 50 $\times$ objective in air (not aqueous solution, in denatured state).

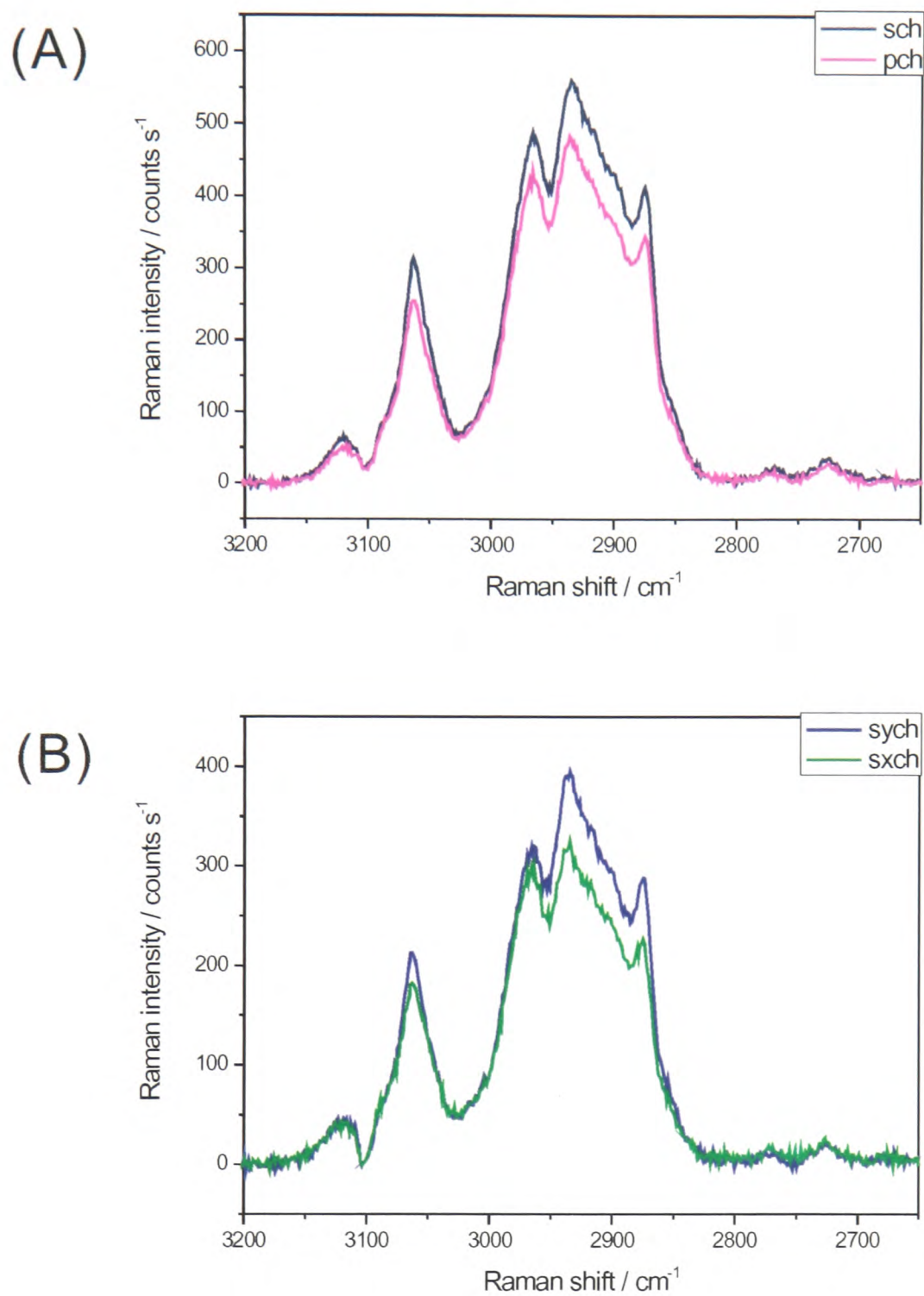


Figure 9.1 Raman spectra of *gD* of cast film in C-H stretching region, *TIR* geometry, 532 *nm*, 0.3 *W*, 1×20 *secs*, 100 μm slit width, 5 pixels, room temperature, Olympus 50×: (A) *s-pol* (in dark blue) and *p-pol* (in pink), un-*pol* detecton; (B) *s-pol* excitation, *y-pol* detection (in blue), *s-pol* excitation, *x-pol* detection (in green).

In the C–H stretching region (2800–3100 cm^{-1}), the 2935 cm^{-1} and 2967 cm^{-1} are attributed to the CH_2 and CH_3 antisymmetric stretching, respectively, and the 2875 cm^{-1} band to CH_2 symmetric stretching vibrations occurring in the 2 *Alas*, 4 *Leus*, and 4 *Vals*. (Howell, *et al*, 1999) (recall that *gD* is a very hydrophobic peptide). The 3063 cm^{-1} is assigned to aryl C–H stretching of the aromatic amino acids like *Trp* (Aslanian *et al*, 1986; Chen, *et al*, 1973). The assignment of the peak at 3120 cm^{-1} is unclear. Figure 9.2 (A) shows the spectra by *s* and *p*-polarised incidence both with unpolarised detection. Figure 9.1 (B) presents the spectra by *s*-polarised incidence with x and y-axis detection. The spectra have the same features in the region irrespective of the polarisation. The similarity of the *s* and *p*-polarised spectra for an isotropic film is unsurprising since the angle of incidence is well-above the critical angle for total internal reflection in air, so both spectra contain an admixture of on and off-axis components of the Raman tensor. The similarity of the *sx* and *sy*-spectra is more surprising, since one would expect these to be different even for an isotropic film.

. Figure 9.2 shows the fingerprint region. The strong and broad peak centered at 1670 cm^{-1} is assigned to the amide I which is similar to the Raman spectra of model polypeptides with antiparallel β -hydrogen bonding. (Krimm & Abe, 1972; Spiro & Gaber, 1977). (Parallel β -sheet is referred at 1660 cm^{-1} in the literature.)

The amide III band, which arises predominantly from N–H in-plane bending, is sensitive to hydrogen bonding and is an important indicator of secondary structure. Generally, strong hydrogen-bonding raises the amide III frequencies (Chen & Lord, 1974). The main amide III band at 1234 cm^{-1} confirms the existence of an antiparallel β -pleated sheet conformation (Spiro & Gaber, 1977). The two weaker at 1286 cm^{-1} and 1307 cm^{-1} suggest that a significant fraction of α -helical conformations (Chen & Lord, 1974; Spiro & Gaber, 1977).

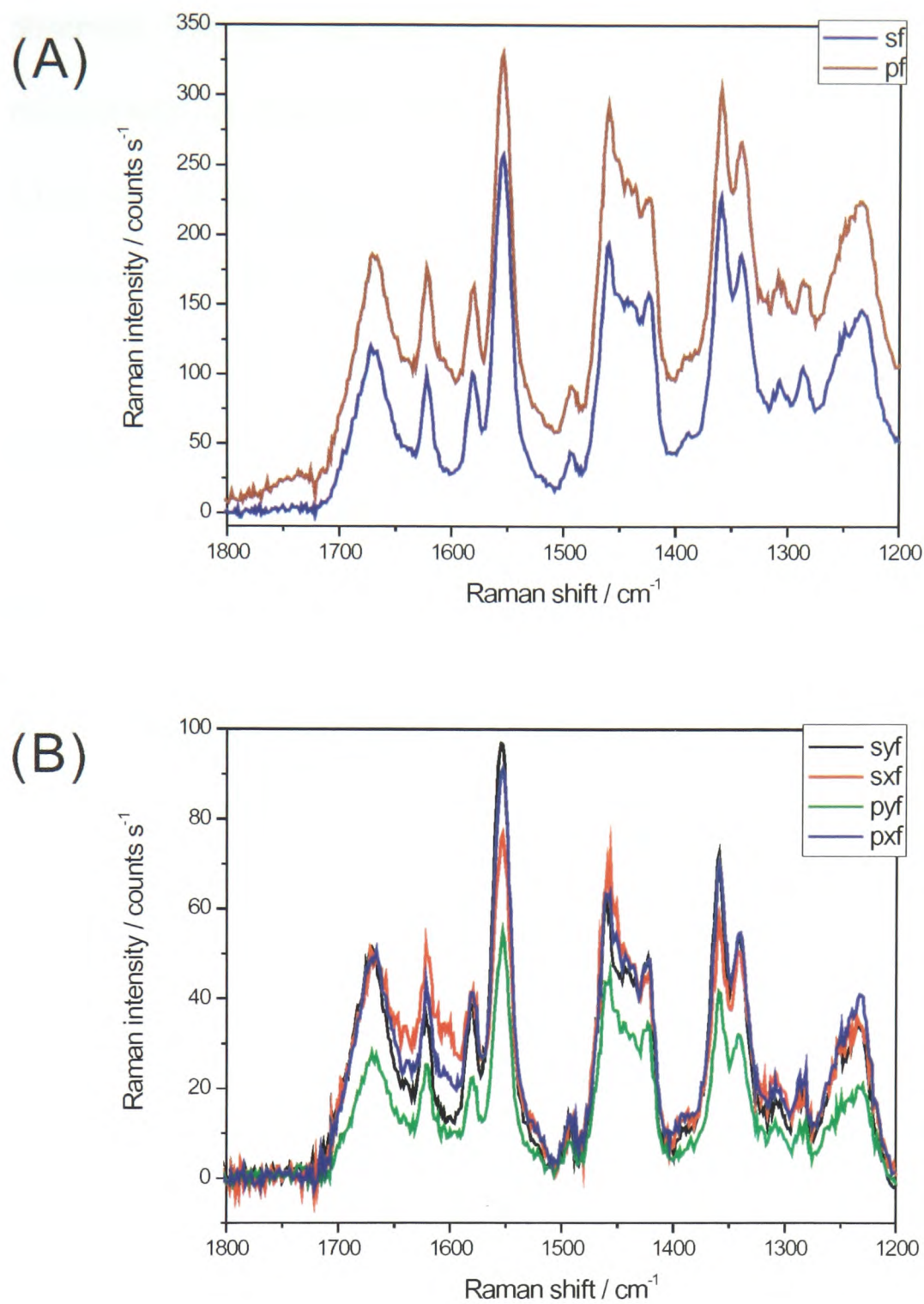


Figure 9.2 Raman spectra of *gD* of cast film in fingerprint region, 532 nm, 0.6 W by *s-pol* and *sy-pol* (0.9 W by *p-pol*, *sx-pol*, *py-pol*, *px-pol*), 1×20 s in *s-pol* (1×40 s in *p-pol*, 2×40 s in *sy-pol* and *sx-pol*, 1×60 s in *py-pol*, *px-pol*), 100 μm slit width, 5 pixels, room temperature, Olympus 50×: (A) *s-pol* (in blue) and *p-pol* spectra (in brown); (B) *sy-pol* (in black), *sx-pol* (in red), *py-pol* (in green), and *px-pol* (in blue). Axis of detection was un-pol in *s-pol* and *p-pol*.

A number of peaks assignable to various modes of the *Trp* ring vibration are observed. The ω_1 , ω_2 , and ω_3 modes are centered at 1622, 1580, and 1553 cm^{-1} , respectively, as reported in the literature (Miura, *et al*, 1989). Two peaks at 1460 and 1424 cm^{-1} , superimposed on a broad band from methylene scissoring vibrations, are assigned as ω_5 and ω_6 of *Trp* indole ring vibrations (Maryuama, 1995).

The 1360 cm^{-1} and a deep minimum between the 1342 and 1360 cm^{-1} is strongly believed to the *Trps* in the polypeptide are “buried” in hydrophobic regions (Aslanian, *et al*, 1986; Yu, 1974). The small peak at 1390 cm^{-1} has also been assigned to a buried *Trp*. (Iconomidou, *et al*, 2000).

9.1.2 Gramicidin D with POPC in a cast multistack film

To generate our own reference Raman spectra of *gD* in a lipid environment, spectra were acquired in a *TIR* geometry from a thick film of *gD* and *POPC* cast onto a silica window. The solvent was *MeOH:CHCl₃* = 1:4 (v/v) with initial concentration ratios of *gD:POPC* = 1:10, 1:25, and 1:50 (mol/mol). Figure 9.3 (A) shows *s* and *p*-polarised spectra with unpolarised detection in the C-H stretching region. We do not expect to have a well-ordered structure in this cast film, but there may be some preferential alignment of multistacks of lipid parallel to the silica surface. The assignments of *gD* in the cast film with *POPC* will assist in the analysis of Raman spectra on *gD* in single lipid bilayer at water/silica interface.

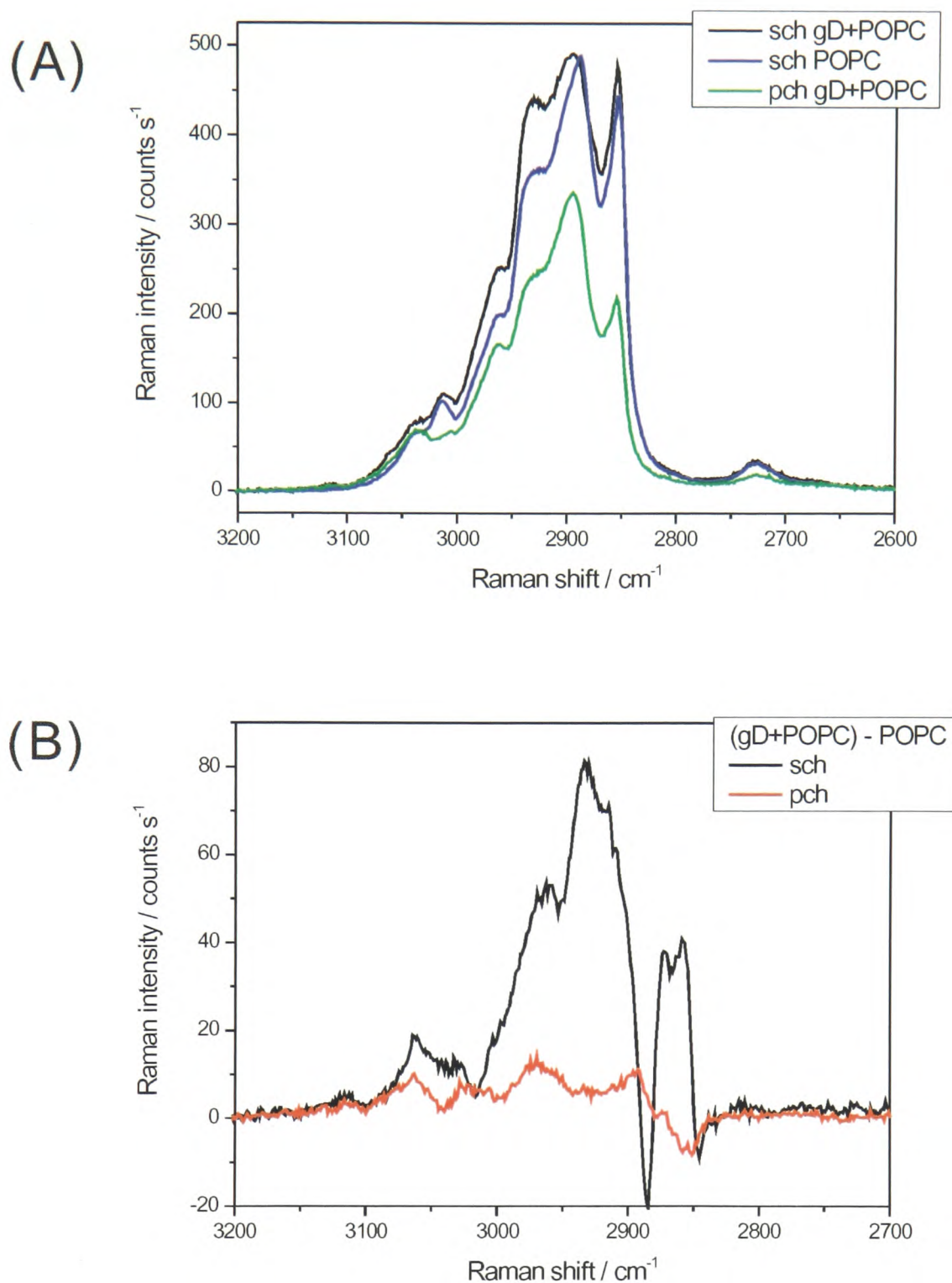


Figure 9.3 Raman spectra of *gD* with *POPC* in C-H stretching region, 532 nm, 0.6 W, 10×20 s in *s-pol* (10×40 s in *p-pol*), 100 μm slit width, 5 pixels, room temperature (21.5 °C), Olympus 50×, *gD:POPC* = 1:25 (mol/mol): (A) *gD* with *POPC* by *s-pol* (in black), *POPC* by *s-pol* (in blue), *gD* with *POPC* by *s-pol* (in green); (B) subtraction of *gD* with *POPC* by *POPC* by *s-pol* (in black) and *p-pol* (in red). All of spectra were acquired with un-*pol* detection.

Spectra with orthogonal polarisations show peaks at the same frequencies, but with different intensities, as for pure *pslbs* presented in Chapter 7 and 8. Both spectra have a peak at 3062 cm^{-1} indicative of aryl C-H stretching of the aromatic amino acids, proving the existence of *gD* in the *POPC* multistack film. Fig. 9.3 (B) shows the spectra after subtraction of the spectrum of pure *POPC*. The presence of *gD* in the lipid changes the lipid spectra so peaks remain in the subtracted spectra at 2934 cm^{-1} , 2960 cm^{-1} and 2873 cm^{-1} from methylene/methyl stretching modes that do not subtract completely. Although features due to *gD* are detectable in the C-H stretching region, the presence of strong lipid peaks that do not cleanly subtract out makes the interpretation of *gD* peaks in this region of the Raman spectrum difficult.

Polarised spectra of cast films of *gD* + *POPC* in the fingerprint region are shown in Figure 9.4. The spectrum of pure *POPC* was then subtracted from the spectra of *gD* + *POPC*, choosing a subtraction factor that nulled out the carbonyl stretch. These subtracted spectra are shown in Figure 9.5.

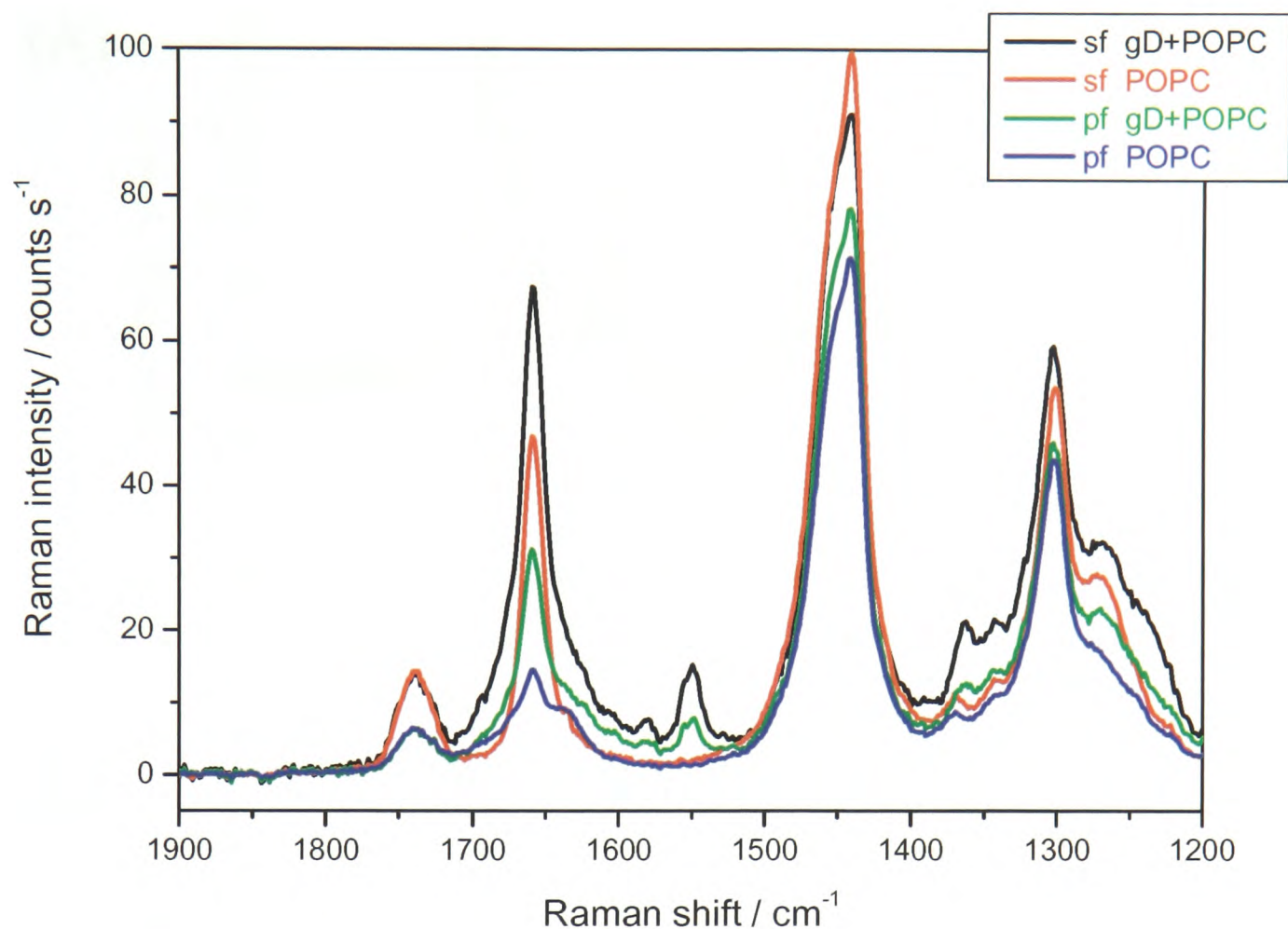


Figure 9.4 Comparison of polarised *TIR*-Raman spectra of *gD* cast with *POPC* and *POPC* alone in fingerprint region, 532 nm, 0.6 W by *s-pol* in *gD+POPC* (0.9 W by *p-pol*), 0.6 W by *s-pol* in *POPC* alone (0.6 W by *p-pol*), 10×60 s in *s-pol* and *p-pol*, 100 μm slit width, 5 pixels, room temperature, Olympus ×50, *gD:POPC* = 1:25 (mol/mol): *s-pol* spectra of *gD* with *POPC* (in black), *s-pol* spectra of *POPC* (in red), *p-pol* spectra of *gD* with *POPC* (in light green), and *p-pol* spectra of *POPC* (in blue).

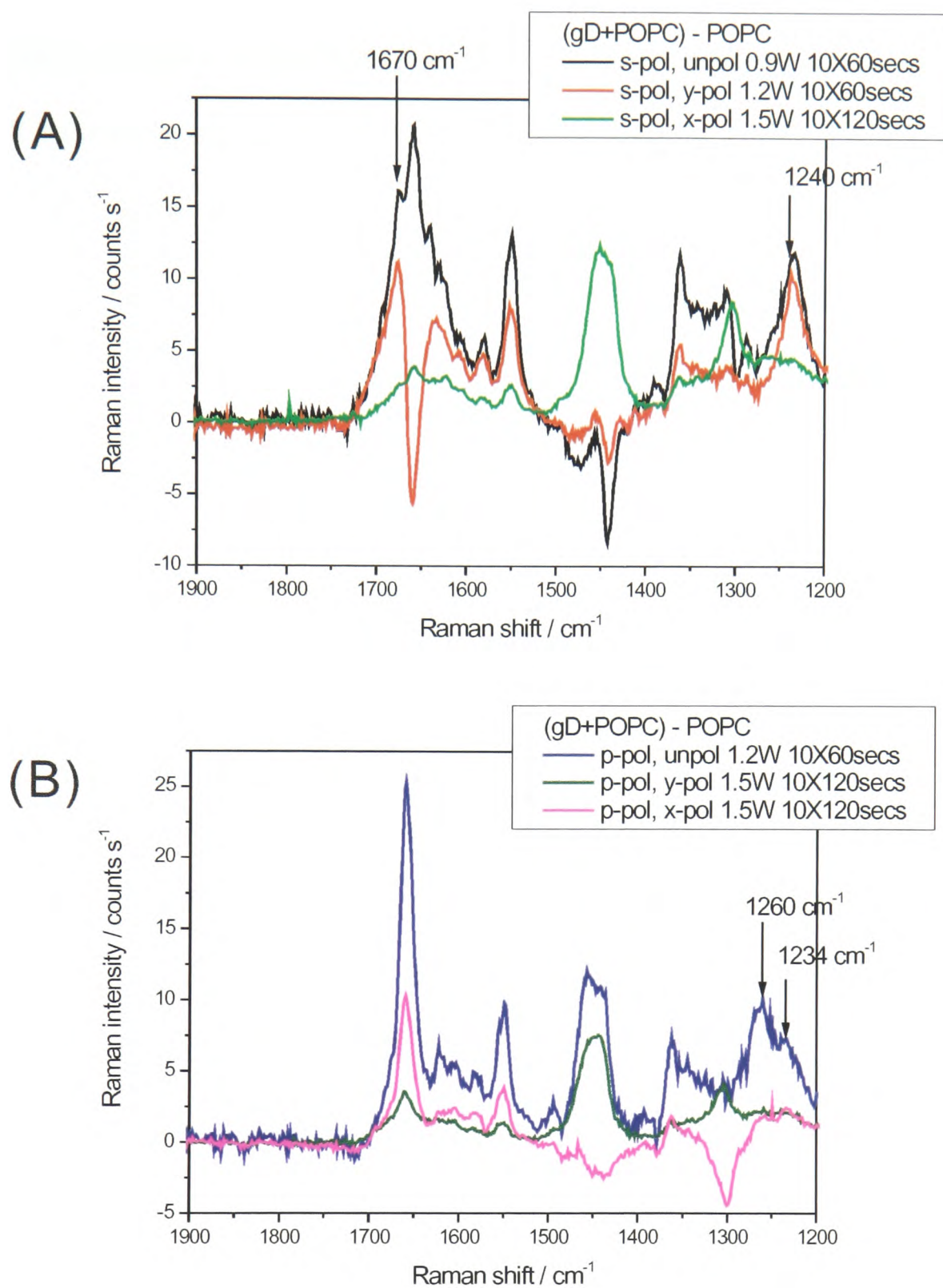


Figure 9.5 Subtracted spectra of *gD* cast with *POPC* by *POPC* in the fingerprint region, 532 nm, 100 μm slit width, 5 pixels, room temperature, Olympus 50 $\times$, *gD:POPC* = 1:25 (mol/mol): (A) *s-pol* (in black), *sy-pol* (in red), *sx-pol* (in light green); (B) *p-pol* (in blue), *py-pol* (in green), and *px-pol* (in pink)

From the vinyl residue in the subtracted spectra of Figure 9.5 (B), it is clear that *gD* induces changes in spectra of the lipid in the fingerprint region as well as in the C-H stretching region, which has been ascribed to a reduction of lipid order (Dunker, *et al*, 1979; Short, *et al*, 1987). The overlap of lipid and peptide bands, particularly the C=C stretch and the Amide I, and the CH₂ twisting mode and the Amide III, complicate the interpretation of peptide spectra since it is difficult to distinguish peptide and lipid peaks unambiguously (without isotopic substitutions). The arbitrary choice of subtraction factor also leads to large variations in the residual lipid peaks in the subtracted spectra in different polarization combinations. Choosing a subtraction factor to null out the vinyl peak does not improve the clarity of subtracted spectra (Fig. 9.6), which are dominated by large negative CH₂ resonances. By moving to a saturated lipid (such as *DMPC*), the overlap of the Amide I with the vinyl group is eliminated, and this is the strategy we adopted (see next section). It will turn out that the lipid subtraction also works better for the single lipid bilayer than for the multistack film, perhaps because of the defined orientation of the lipid chains.

Raman spectra in the 600-1100 cm^{-1} region are shown in Figure 9.7. Although there is a strong and broad silica peak centered at 1090 cm^{-1} , we could subtract the silica background and identify the *Trp* at 764, 876, 1013, and 1127 cm^{-1} (Aslanian, *et al*, 1986; Iconomidou, *et al*, 2000, 2001; Picquart, *et al*, 2000). The strong peak at 1078 cm^{-1} is the C-N stretching (Aslanian, *et al*, 1986; Iconomidou, *et al*, 2000, 2001; Picquart, *et al*, 2000).

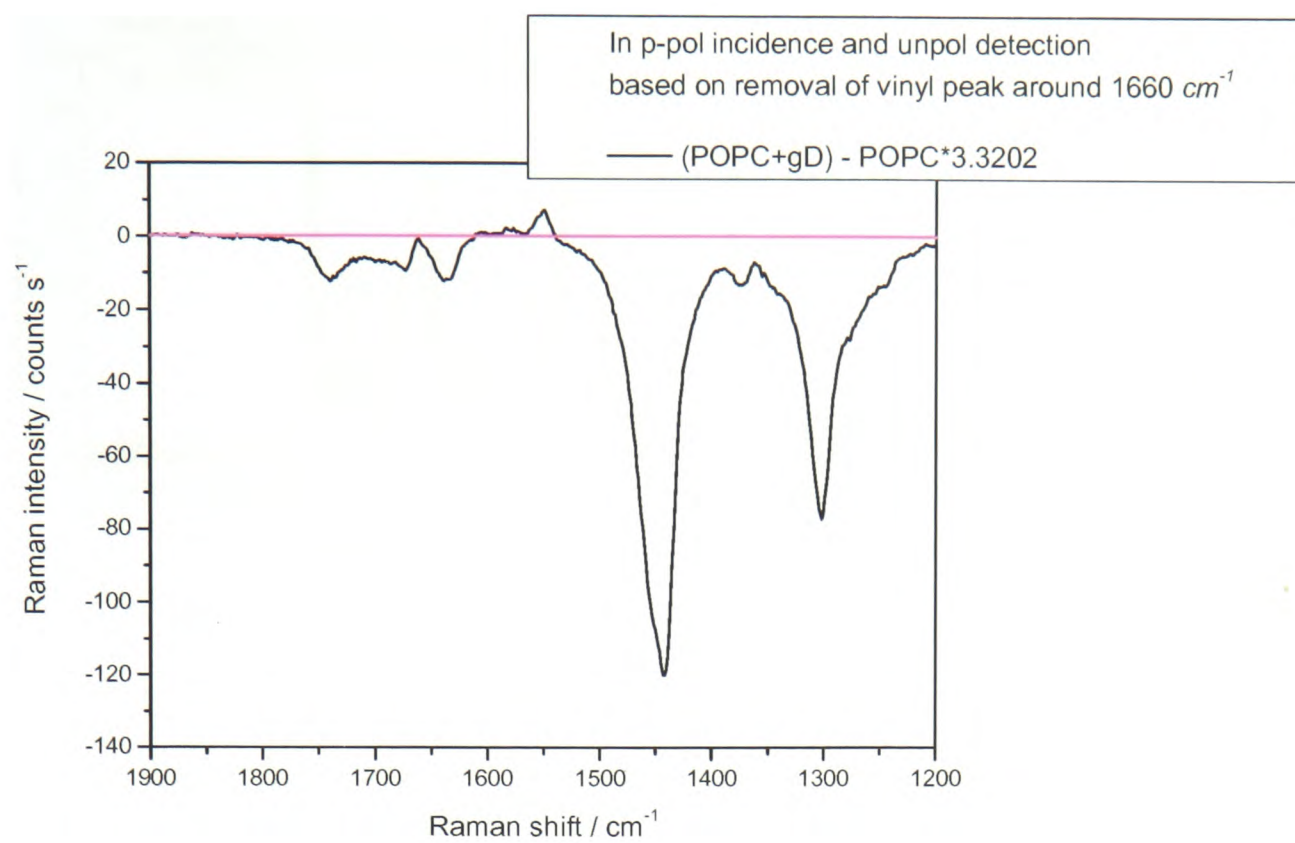


Figure 9.6 *gD*+*POPC* spectrum minus *POPC* with the vinyl peak around 1660 cm^{-1} nulled out. *p-pol* incidence, *un-pol* detection, 532 nm, 0.9 W, 10×60 s, 100 μm slit width, 5 pixels, room temperature, *gD*:*POPC* = 1:25 (mol/mol).

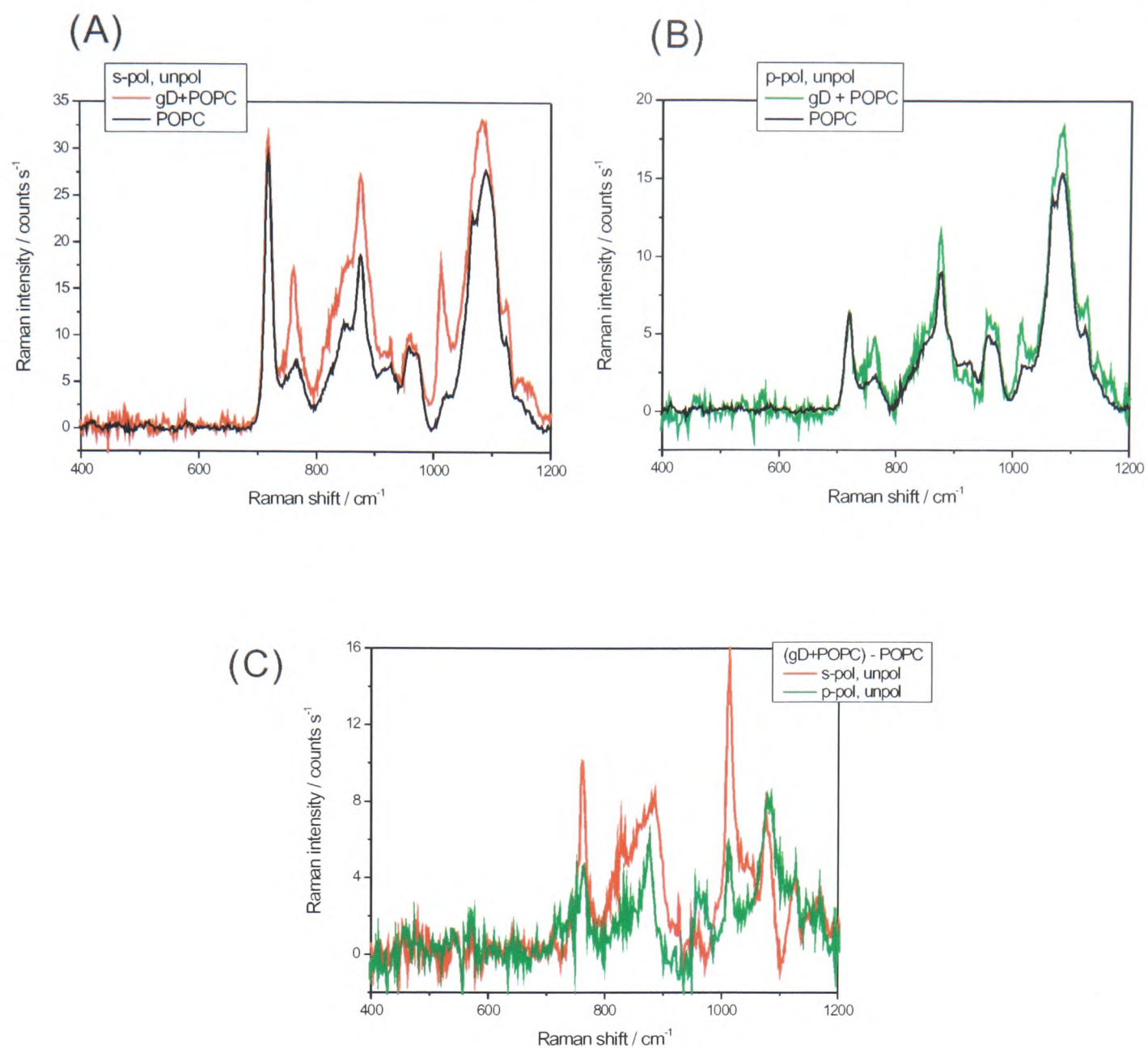


Figure 9.7 Subtracted spectra of *gD* cast with *POPC* in 800 cm^{-1} centered region (C), 532 nm , 0.9 W by *s-pol* (1.2 W by *p-pol*), $10\times 60\text{ s}$, $100\text{ }\mu\text{m}$ slit width, 5 pixels, room temperature, Olympus $50\times$: (A) *s-pol* *gD+POPC* (in red) and *POPC* (in black), (B) *p-pol* *gD+POPC* (in light green) and *POPC* (in black). Axis of detection was un-pol.

We also investigated the dependence of the Raman spectra on the initial ratio gD to lipid (Figure 9.8). While the peaks assignable to gramicidin increase with decreasing ratio of lipid to gD , the increase is not linear, showing that the final composition of the mixed $gD/POPC$ bilayer does not correspond to the initial composition of the system.

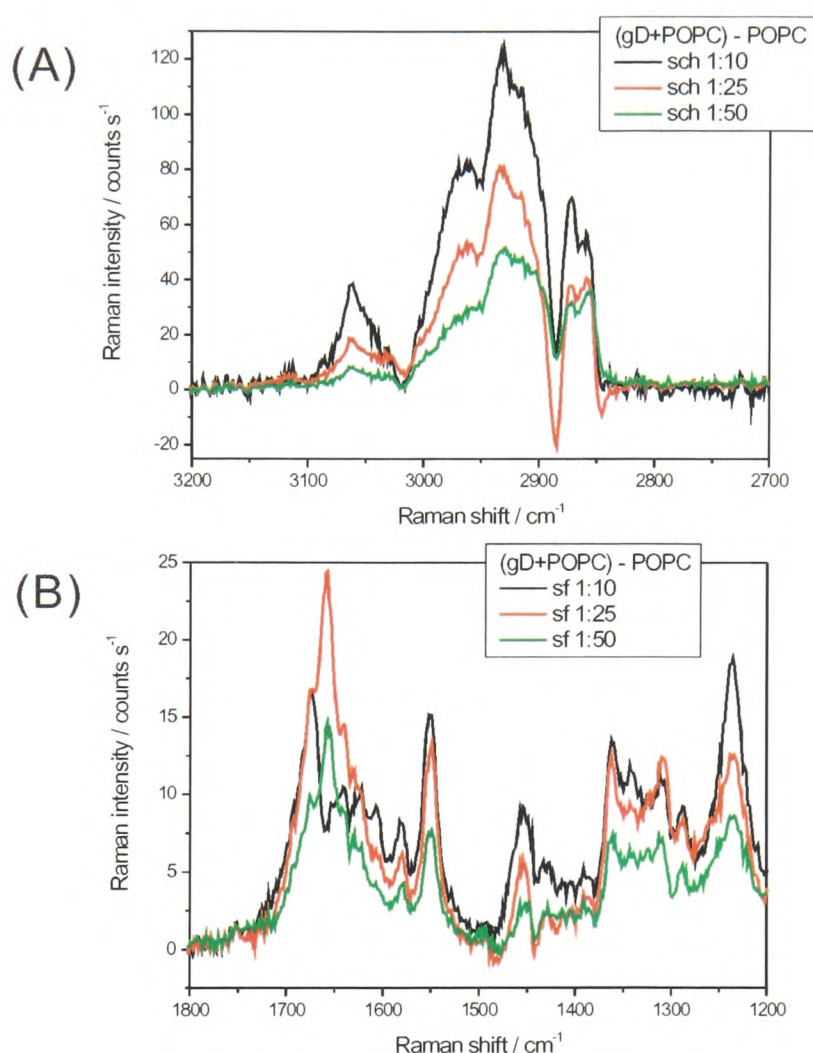


Figure 9.8 Subtracted spectra of gD cast with $POPC$ minus $POPC$ for different initial concentration ratios of gD to $POPC$, 532 nm, s -pol in excitation, un-pol in detection, 0.6 W in C-H stretching region (0.9 W in fingerprint region), 10×20 s in C-H stretching and 10×60 s in fingerprint region), 100 μm slit width, 5 pixels, room temperature (21.5 °C), Olympus 50 $\times$: (A) in C-H stretching region, $gD:POPC$ (mol/mol) = 1:10 (in black), 1:25 (in red), and 1:50 (in green); (B) in fingerprint region, $gD:POPC$ (mol/mol) = 1:10 (in black), 1:25 (in red), and 1:50 (in green).

9.1.3 Photodamage in mixed *gD*/lipid supported bilayers

The first issue that we addressed with the *TIR*-Raman spectra of single supported lipid bilayers was that of photodamage. Figure 9.9 shows Raman spectra of *gD* in a *POPC* *pslb* for different acquisition times of 3, 6 and 15 *min*.

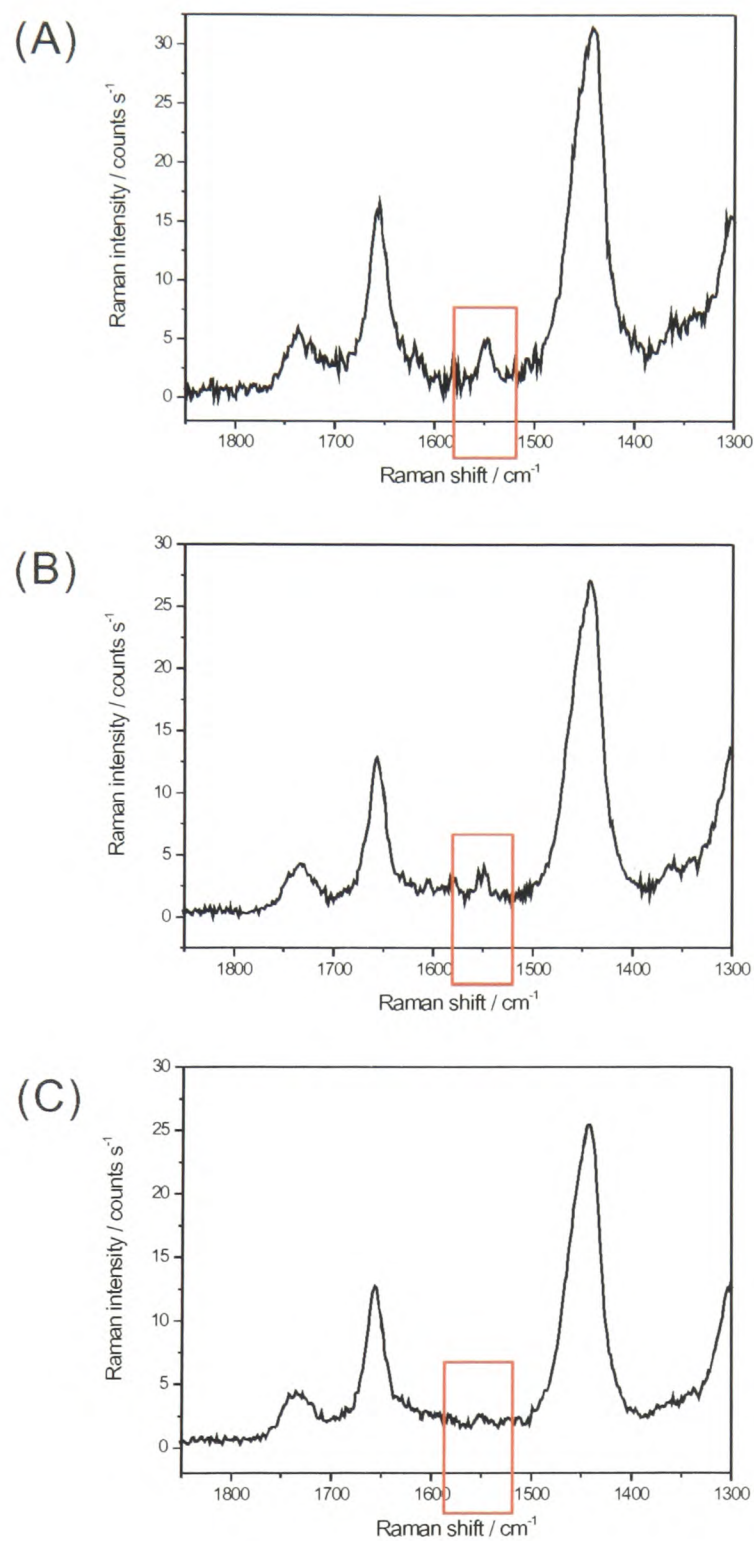


Figure 9.9 Raman spectra of *gD* in *POPC* *pslb* in different acquisition time of 532 *nm*, 0.9 *W*, *s-pol* in excitation, *un-pol* in detection, 200 μm slit width, 11 pixels, room temperature, Zeiss 40 $\times$: (A) 1 $\times$ 180 *s*; (B) 2 $\times$ 180 *s*; (C) 5 $\times$ 180 *s*. *gD:POPC* (*mol/mol*) = 1:11.6, The red box in the spectra indicate *Trp* ω 3 region.

The longer the acquisition time, the weaker is the *gD* signal (as measured by the *Trp* ω_3 mode at 1549 cm^{-1}) relative to the lipid background. This provides prima facie evidence for photoinduced damage of the *gD*, though we have previously established no damage to the lipid under the same irradiation conditions. If photodamage is a linear effect, there is nothing to be gained by decreasing the laser power and increasing the acquisition time, the effect of photodamage on the spectrum will be the same.

An alternative strategy to improve *gD* spectra is to move the sample stage during the acquisition of the spectra so that an individual area of the surface is not exposed to the laser beam for sufficiently long to cause an unacceptable level of photodamage. The initial 3 *min* spectrum was acquired during 3 *min* from the first spot and then the laser spot was moved at a rate of $1\text{ }\mu\text{m s}^{-1}$ in the *y*-direction (which has been defined as parallel to the electrical field of *s*-polarised incidence in the plane of water-silica interface, see Chapter 5.8) with a computer controlled translation stage. Figure 9.10 shows three spectra taken for 3, 6 and 15 *min*, within slow scanning after the first 3 *min*. The progressive loss of the *gD* peaks seen without translation of the spot (Figure 9.9) is no longer observed. The signal level from the lipid decreased with time, probably because the sample was slightly out of focus towards the end of the scan. Whilst scanning the sample is not the most elegant way of eliminating photodamage, it does the job and represents a significant milestone in our investigation of the polarised Raman spectra of *gD* in single lipid bilayers at the water/silica interface.

Figure 9.11 shows a comparison of a fixed spot and a moving sample stage for the *gD*/DMPC bilayers used subsequently. The computer-controlled stage had broken down, so the sample was moved manually every 2 *min*.

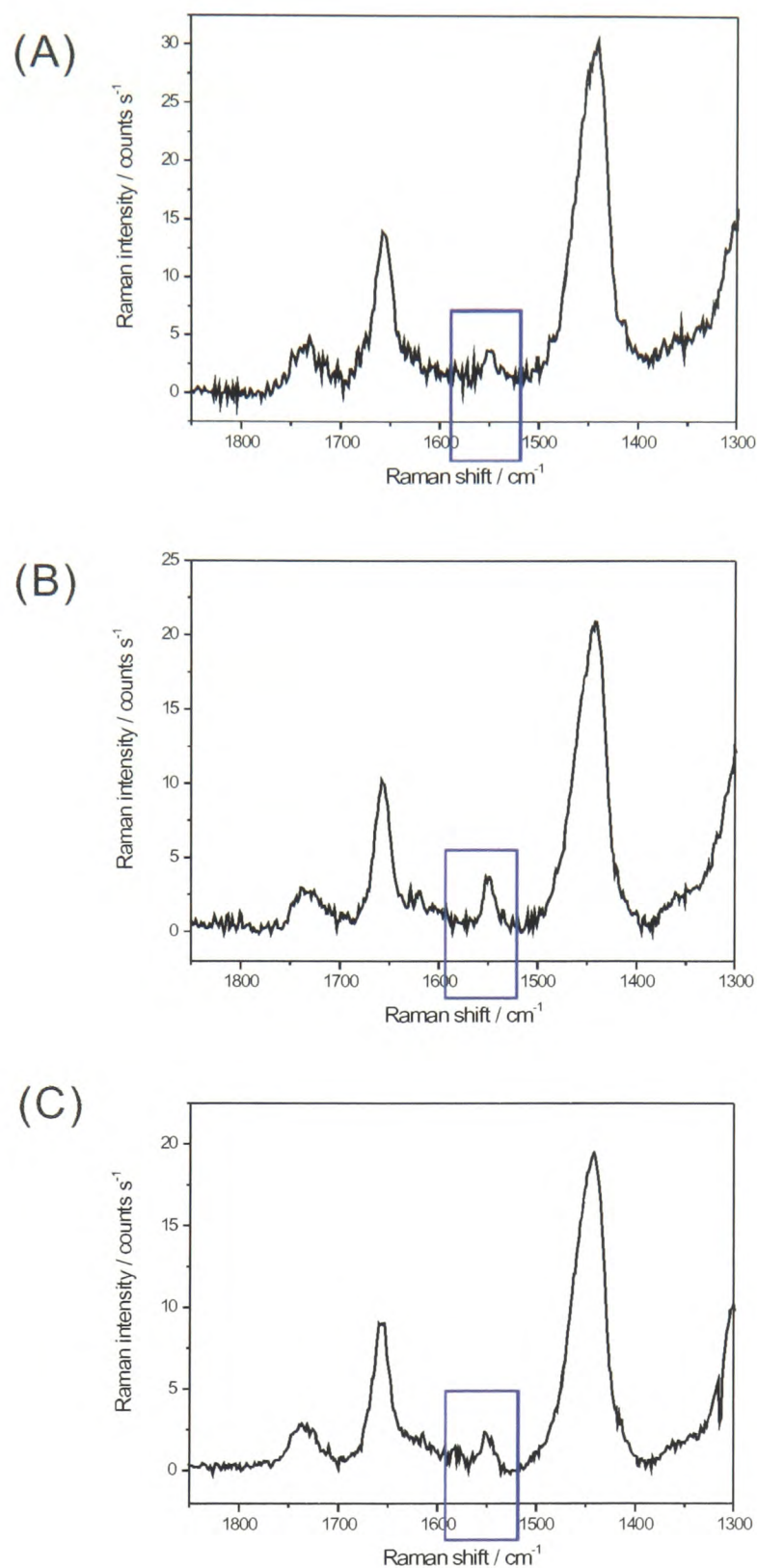


Figure 9.10 Raman spectra of *gD* in *POPC pslb* for different acquisition times with translation of the sample stage, 532 nm, 1.2 W, *s-pol* in excitation, *un-pol* in detection, 200 μm slit width, 11 pixels, room temperature, Zeiss $\times 40$: (A) 1×180 s; (B) 2×180 s (fixed initially for 1×180 s and then moved by $1 \mu\text{m s}^{-1}$); (C) 5×180 s (fixed initially for 1×180 s and then moved by $1 \mu\text{m s}^{-1}$). *gD:POPC* (mol/mol) = 1:11.6, The blue box in the spectra indicate *Trp* $\omega 3$ region.

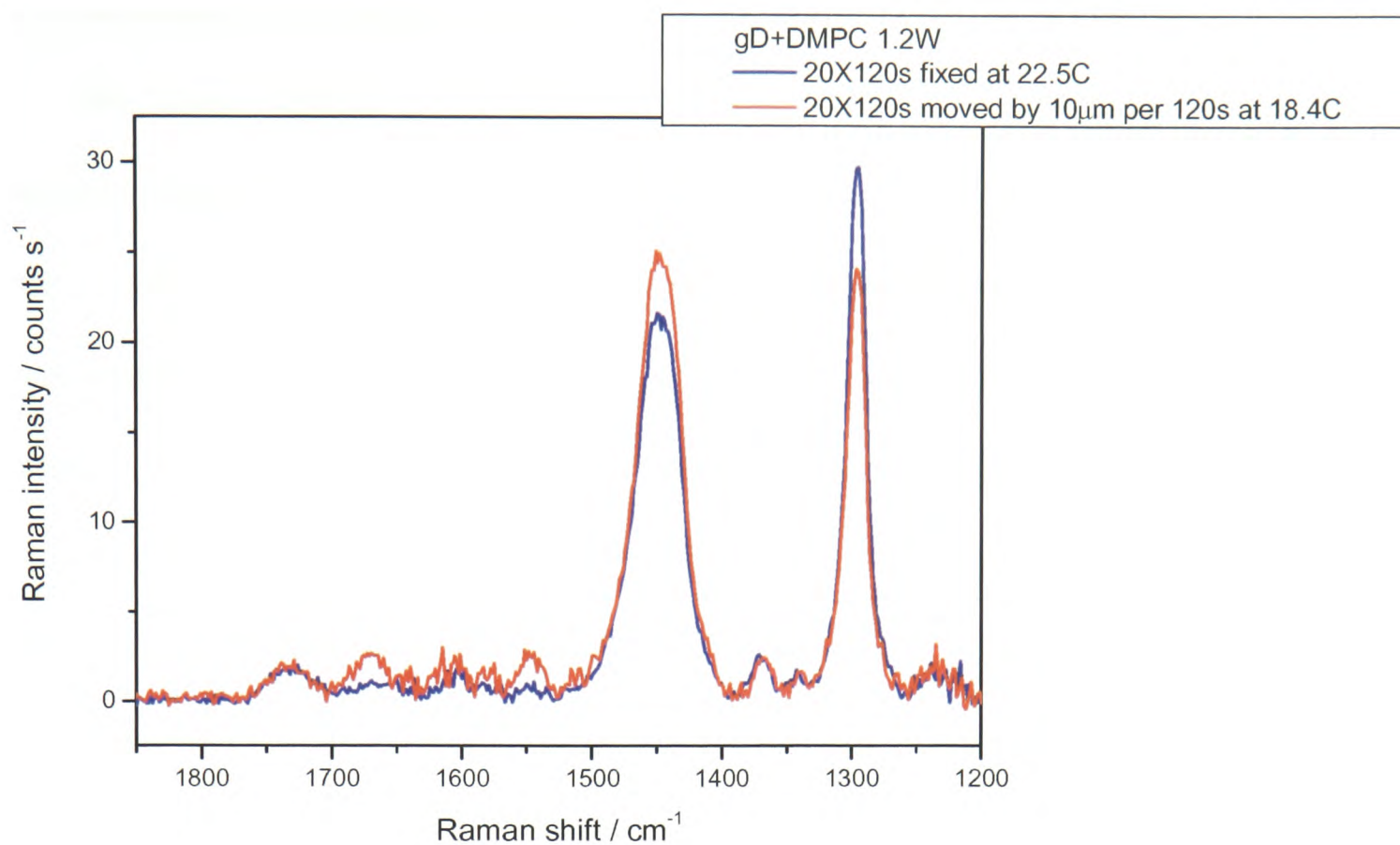


Figure 9.11 Comparison of *TIR*-Raman spectra of *gD* in *DMPC pslb* with a fixed and moving sample stage, 532 *nm*, 1.2 *W*, *s-pol* excitation, *un-pol* detection, 200 μm slit width, 11 pixel: measured spot fixed for 20 $\times$ 120 *s* at 22.5 $^{\circ}\text{C}$ (in blue); measured spot moved by 10 μm every 120 *s* at 18.4 $^{\circ}\text{C}$ (in red) . The change in lipid peaks arises from the slightly different temperatures of the two experiments.

9.1.4 Gramicidin D in a supported DMPC bilayer at the water/silica interface

We chose *DMPC* as a suitable candidate for accommodating *gD* in the hydrophobic region of lipid acyl chains. *DMPC* is a saturated lipid and therefore avoids overlap of amide I with the vinylic stretching mode in the Raman spectra. *DMPC* is one of most popular lipid species in lipid and protein biology due to the frequency of its occurrence in living cells (Koynova & Caffrey, 1998). Additionally it is easy to maintain the temperature either above or below the main melting transition around 24 °C with our temperature-controlled sample stage.

The preparation of a *pslb* of *DMPC* incorporating *gD* required fusion above the main phase transition of *DMPC*. Raman spectra were then acquired at a temperature below the melting transition of the pure lipid, to stabilise the lowest energy structure of the peptide. The dominant peaks in the Raman spectra of the *DMPC/gD* mixtures all arise from the lipid, so to extract the peaks due to the peptide, the Raman spectrum of a pure *DMPC pslb* was subtracted from the spectrum of the mixed bilayer. The relative heights of the various lipid peaks are different in the pure and mixed bilayers, so the pure lipid spectra were scaled so that the carbonyl group subtracted perfectly. As a consequence, some of the other lipid peaks (such as the CH₂ scissoring and twisting modes) remain in the subtracted spectra. Our reference spectra of *gD* by itself and *gD* in a *POPC* multistack film were used to assist the curve-fitting of the *gD* spectra in the *pslb*.

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As we were interested principally in the secondary structure of *gD* in a single lipid bilayer at water-silica interface, we focused our observations on the fingerprint region rather than the C-H stretching region; most structurally sensitive Raman bands are in the region 1200-1700 cm^{-1} . The presence of overtones makes quantitative assignments in the C-H stretching region difficult. The low frequency region (600-1100 cm^{-1}) suffers from increased noise, due to the underlying silica bands, and is less useful for the characterisation of peptide conformation; we did not study this region for the mixed *DMPC/gD* bilayers.

Figure 9.12 shows *s* and *p*-polarised spectra of *DMPC* and of *gD-DMPC* in the fingerprint region. In each case we collected all the scattered light since there was insufficient signal to permit the separate collection of *x* and *y*-polarised spectra.

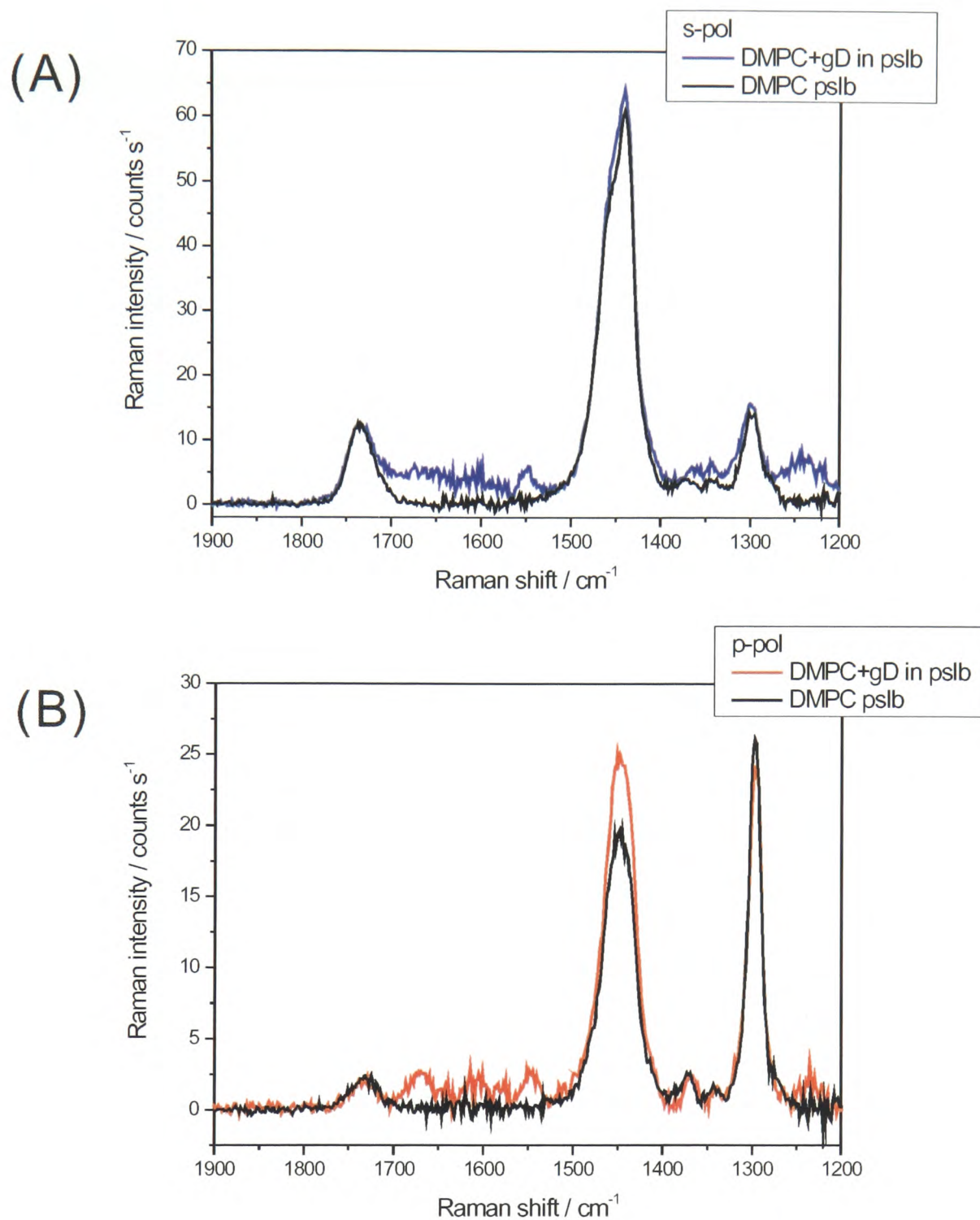


Figure 9.12 Polarised *TIR*-Raman spectra of *gD* in *DMPC pslb*, 532 nm, 1.2 W, 200 μm slit width, 11 pixels, Zeiss 40 $\times$, 18.4 $^{\circ}\text{C}$: (A) *s-pol* spectra of *gD* with *DMPC* (in blue) and only *DMPC* (in black) in *pslb*; (B) *p-pol* spectra of *gD* with *DMPC* (in red) and only *DMPC* (in black) in *pslb*. Axis of detection was unpolarised.

9.1.4.1 Manipulation of spectra

To achieve curve-fits of raw spectra, I introduced the procedure for manipulation of spectra shown in Figure 9.13.

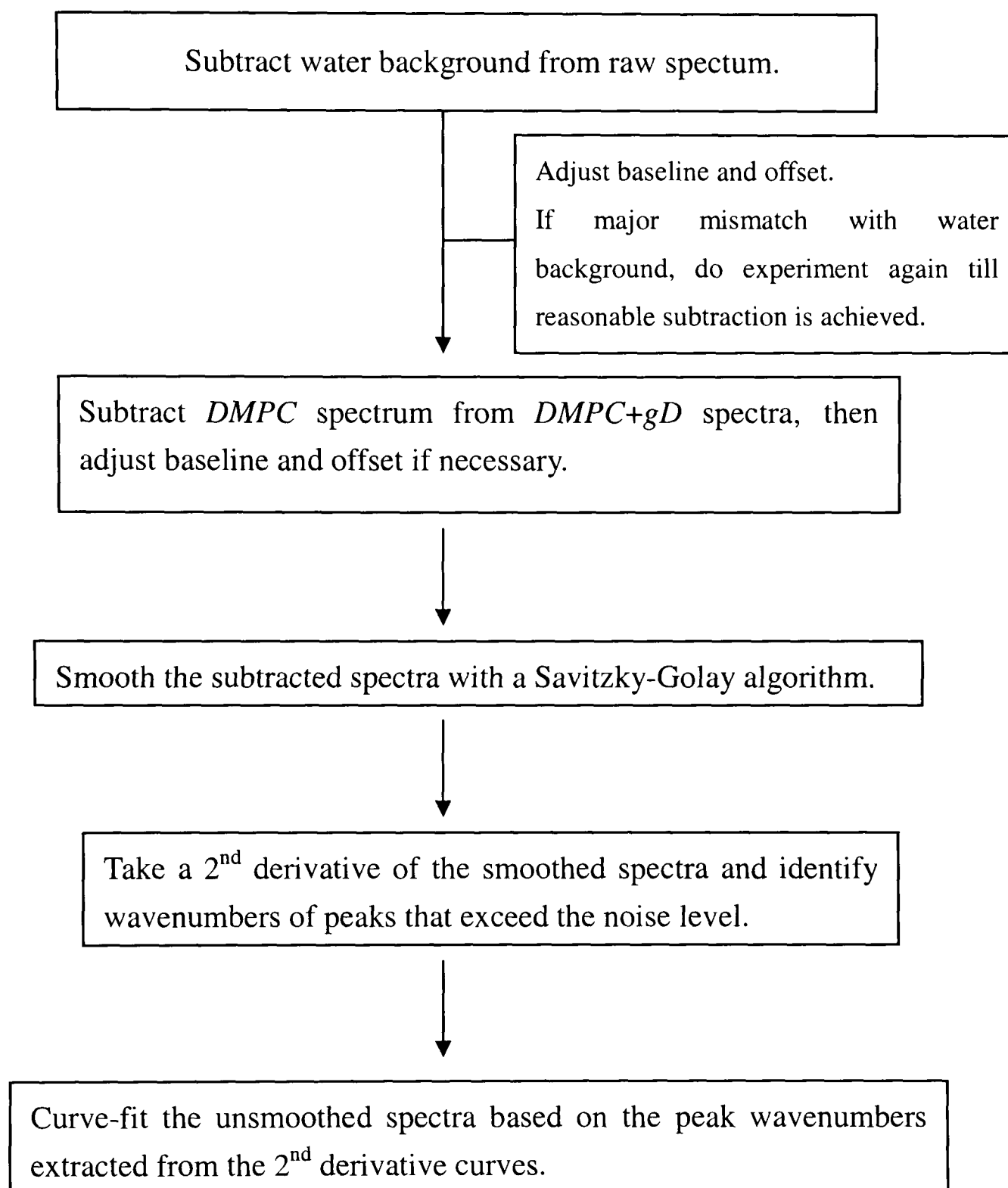


Figure 9.13 Flow diagram of procedure for spectra manipulation.

The raw spectra were fitted mainly using a Gaussian function, guided by the 2nd derivative curve of the smoothed curve. The correct choice of parameters for the smoothing and derivative are very important, since the wrong choice may obscure real peaks or lead to the identification of noise as peaks. The Savitzky-Golay smoothing filter is a popular least-squares digital polynomial smoothing routine. The recommended polynomial of the smoothing filter is the 4th order (degree 4) (Ziegler, 1981), though I experimented with other orders. The filter width (that is, the number of points over which the moving polynomial is fitted) for Gaussian or Lorentzian peaks is optimised relative to the full width at half maximum (*fwhm*) of the peak. The larger the filter width, the greater is the enhancement in the signal-to-noise ratio but at the cost of resolution if the filter width is too great compared to the *fwhm*. The polynomial filtering becomes approximately optimal when the filter width of the degree 4 Savitzky-Golay filter is between 1 and 2 times the *fwhm* of desired features in the line; Bromba and Ziegler (1981) recommend a width of 1.72 *fwhm*. I started by smoothing the spectra of a pure *DMPC pslb* using a quartic filter. The choice of filter width is complicated by the fact that the spectra contain peaks of different *fwhm*. Figure 9.14 depicts the original spectrum of *DMPC pslb* together with two smoothed curves (degree 4) with filter widths of 47 and 21 points. Since the separation between data points is 1.4 cm^{-1} , the filter width of 47 is optimal for a *fwhm* of $\sim 38 \text{ cm}^{-1}$, which is the width evaluated for the peak of C-H scissoring mode centered at 1449 cm^{-1} . The smoothed curve (red) is an accurate representation of the 1449 cm^{-1} mode (and the carbonyl mode at 1732 cm^{-1}), but severely distorts the much narrower peak from the C-H twisting mode at 1297 cm^{-1} . The optimal filter width for this peak is 21, since the C-H twisting mode has an *fwhm* of $\sim 17 \text{ cm}^{-1}$. The result of S-G smoothing with a filter width of 21 point was drawn in light blue in Figure 9.14. This filter preserves the heights and widths of all the peaks while still effectively removing the noise.

In the same way, we examined the optimal filter width for smoothing the *DMPC+gD pslb*, shown in Figure 9.15. The filter width of 35 (light blue) was chosen with reference to features with a *fwhm* of $\sim 20\text{ cm}^{-1}$. This filter gave better results than the filter width 45 (red), but still smoothed out the small but sharp peak at 1340 cm^{-1} . Decreasing the filter width to 15 (Figure 9.16) captures this peak, but the residual noise in the spectrum is now too great to take a second derivative (Fig. 9.17 (A)). Therefore we need a compromise to find an appropriate filter width that permits the taking of second derivatives without too great a loss of resolution. The filter width 35 represented a reasonable balance between retaining the maximum amount of information in the *DMPC+gD pslb* spectra while still yielding a sensible 2nd derivative curve, Fig. 9.17 (B). In the vicinity of the amide I mode, where perfect water subtraction is always problematic due to the strong water bending modes, the smoothed curves with a filter width of 35 (Figure 9.15 and 9.18) are good enough for analysis.

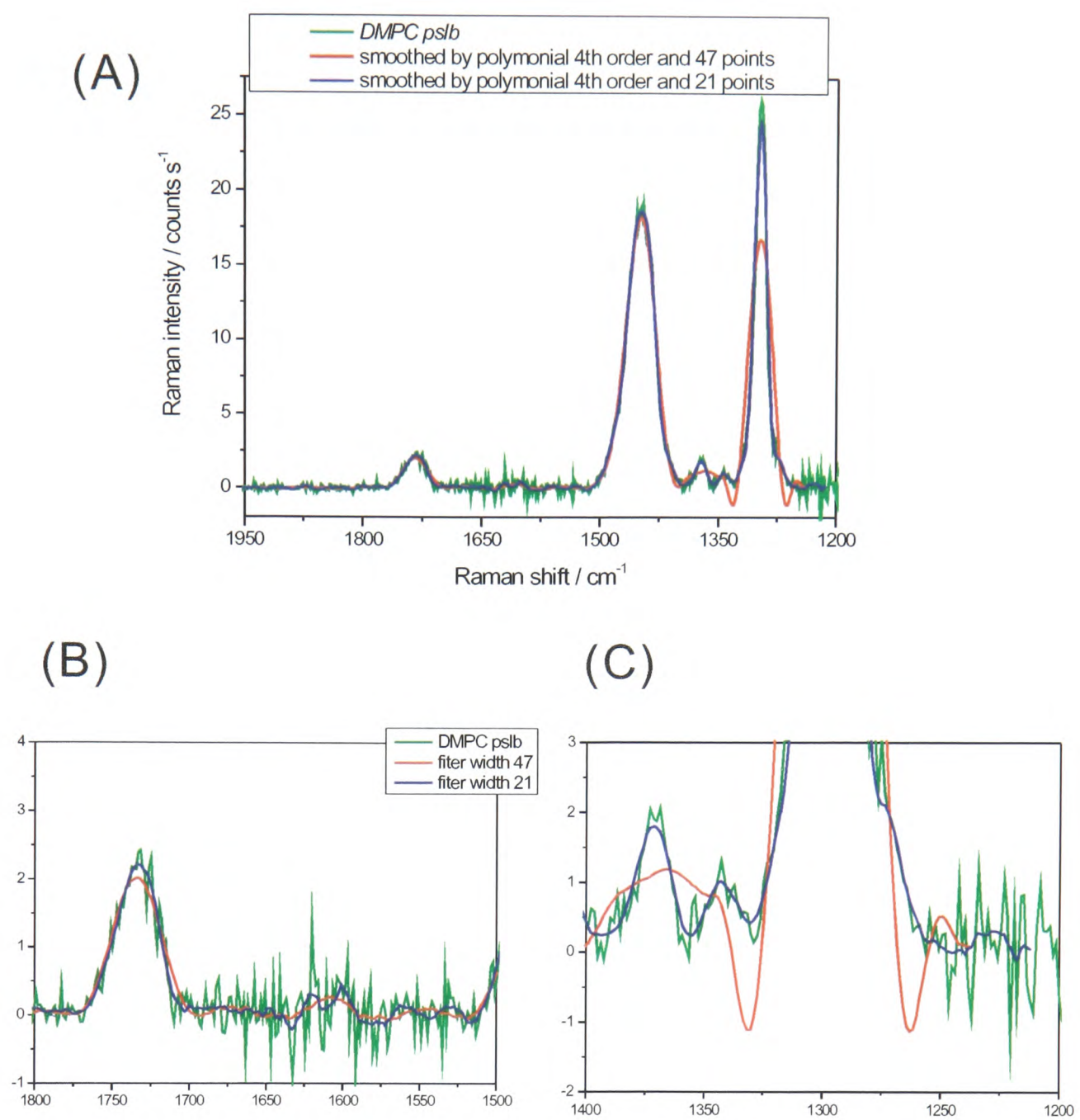


Figure 9.14 Smoothing of *p-pol* DMPC *pslb* spectra, after subtraction of the water background (in light green), degree 4, filter width 47 (in red) and 21 (in blue): (A) full spectral region of fingerprint. Expansion of (B) carbonyl and amide I region, (C) amide III region and surroundings.

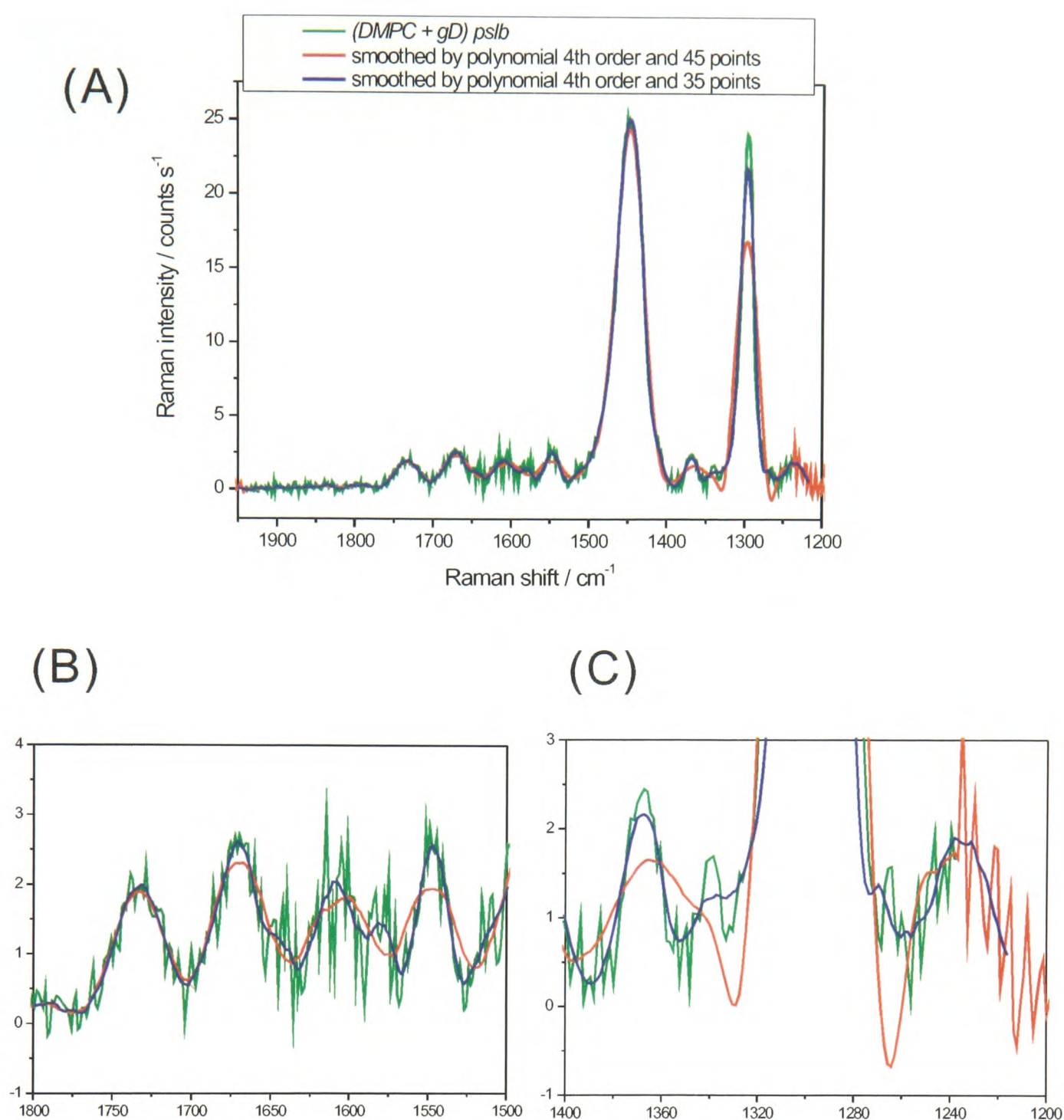


Figure 9.15 Smoothing of *p-pol* DMPC+gD *pslb* spectra, after subtraction of the water background (in light green), degree 4, filter width 45 (in red) and 35 (in blue): (A) full spectral region of fingerprint. Expansion of (B) carbonyl and amide I region, (C) amide III region and surroundings.

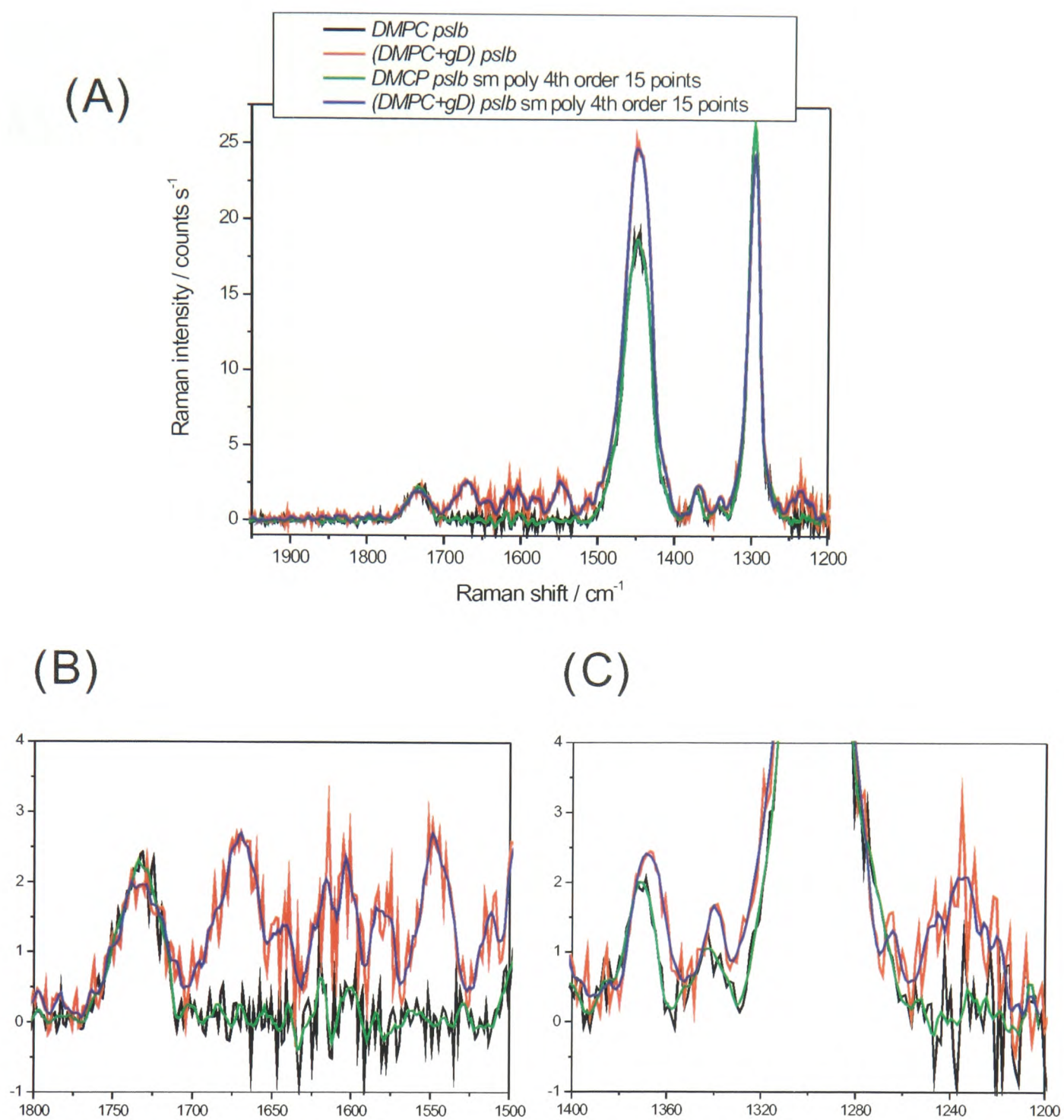


Figure 9.16 Smoothing of *p-pol* *DMPC* (in black) and *DMPC+gD pslb* (in red) spectra, after subtraction of the water background, degree 4, filter width 15 (in light green and blue for *DMPC* and *DMPC+gD pslbs* respectively): (A) full spectral region of fingerprint. Expansion of (B) carbonyl and amide I region, (C) amide III region and surroundings.

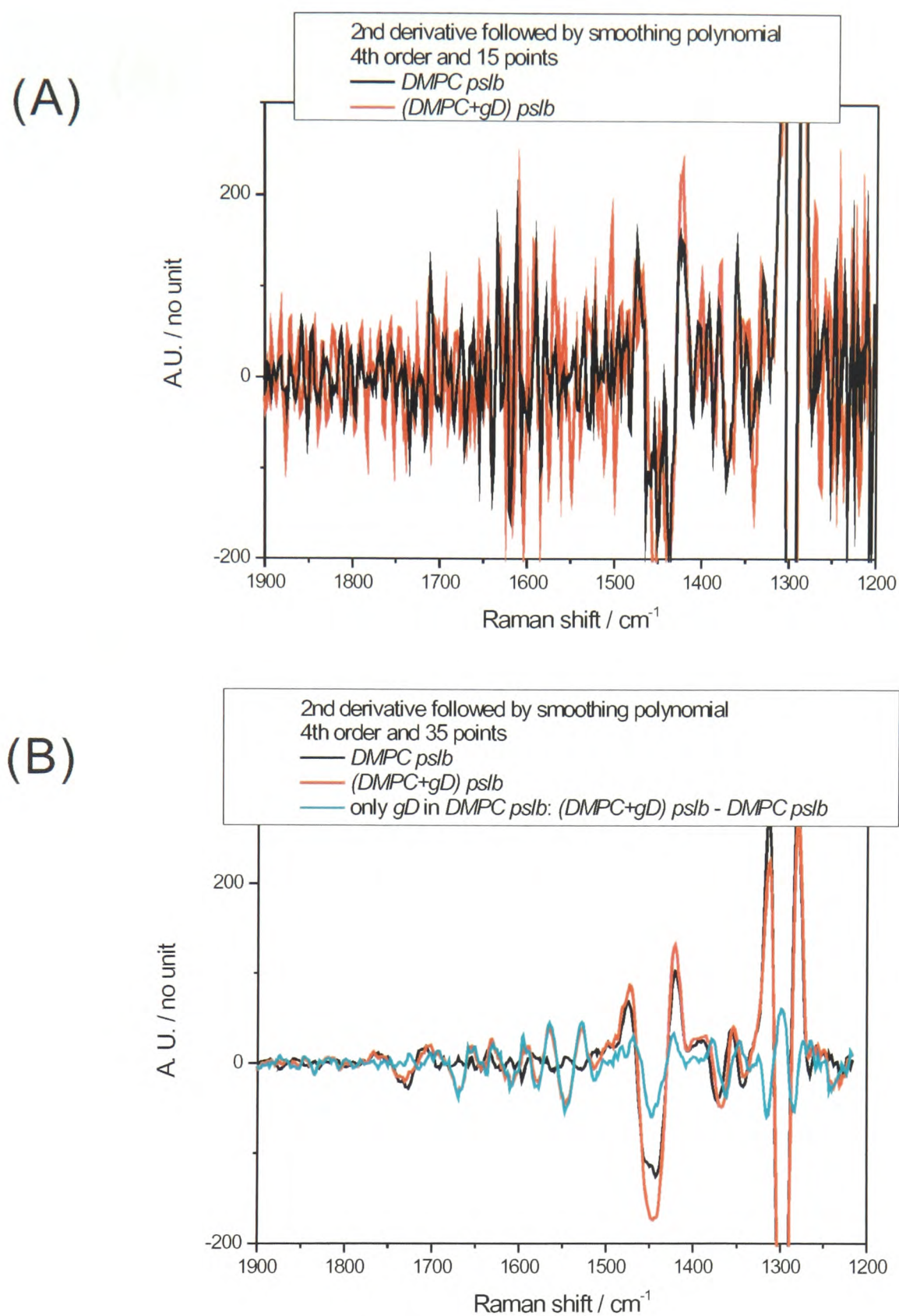


Figure 9.17 Second derivatives of *p-pol DMPC pslb* (in black) and *DMPC+gD pslb* (in red) accompanied by a smoothing filter of degree 4 with (A) filter width 15 and (B) filter width 35. The second derivative of the subtracted spectrum, $(\text{gD} + \text{DMPC}) - \text{DMPC}$, is plotted in light blue.

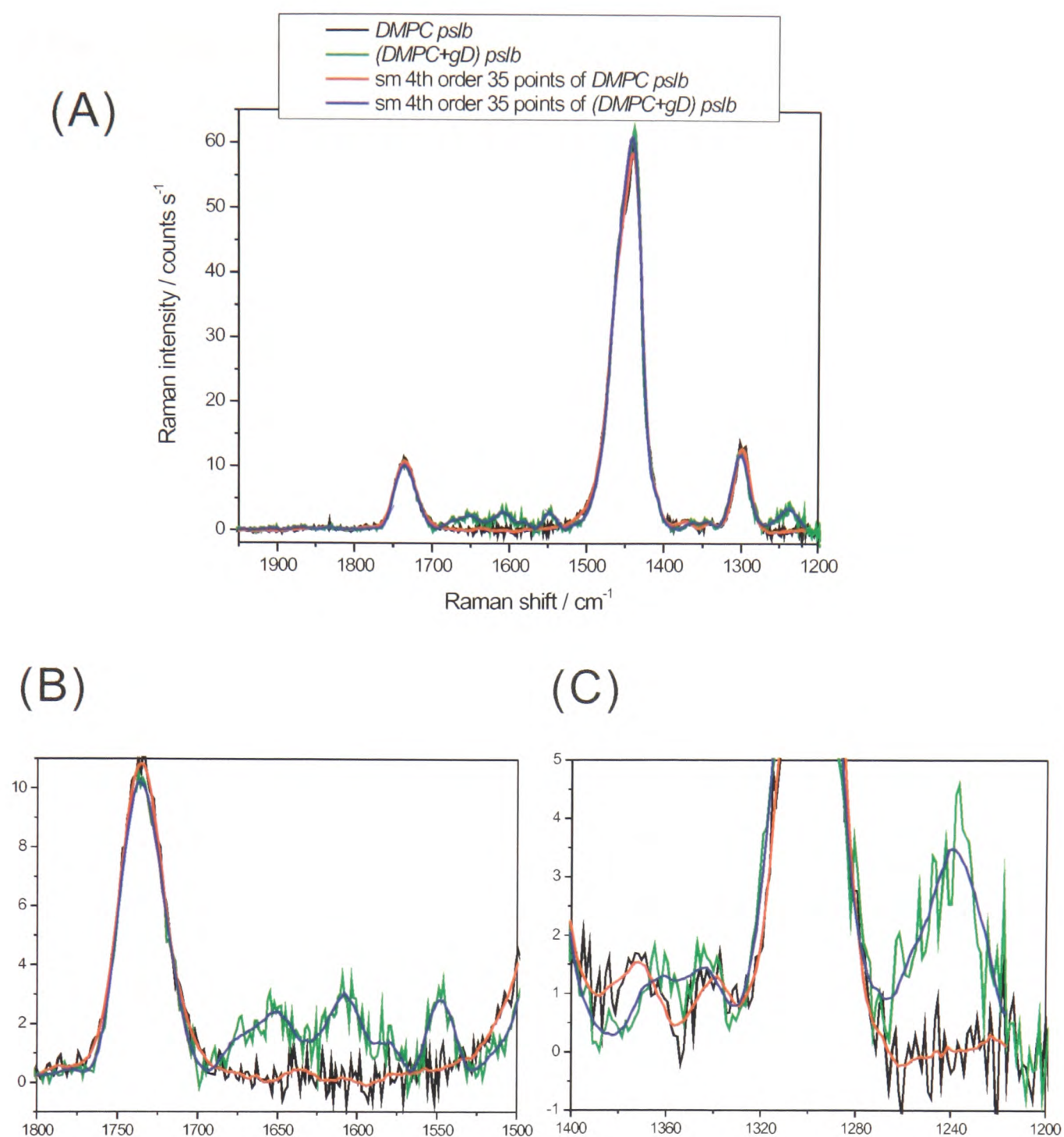


Figure 9.18 Smoothing of *s-pol* *DMPC* (in black) and *DMPC+gD pslb* (in light green) spectra, after subtraction of the water background, degree 4, filter width 35 (in red and blue for *DMPC* and *DMPC+gD pslbs* respectively): (A) full spectral region of fingerprint. Expansion of (B) carbonyl and amide I region, (C) amide III region and surroundings.

9.1.4.2 Curve-fitting of polarised TIR-Raman spectra of *gD* in *DMPC pslb*

Figure 9.19 presents the smoothed curves of the polarised TIR-Raman spectra of the *gD* in *DMPC pslb* after subtraction of the pure *DMPC* spectra, with the optimal parameters of the smoothing filter (degree 4 and width 35). In order to determine the underlying positions of the constituent peaks prior to performing the curve-fit, the 2nd derivative of the Raman spectra of the *gD* in *DMPC pslb* was evaluated from the smoothed spectra. However, we should be careful in selecting the minimum number of peak positions from the 2nd derivative curve because the poor signal-to-noise ratio of the spectra can make it difficult to distinguish the real peaks from noise artifacts. So we used the 2nd derivative curve of the *DMPC pslb* spectra to help us establish the size of ‘peaks’ that arise purely from smoothing of random noise in the regions of the amide I mode, 1500-1600 cm^{-1} , tryptophan bands from 1300-1400 cm^{-1} , and amide III mode. Our assessment is based on the fact that the minima in the 2nd derivative curve of the lipid spectra do not represent real peaks except in the vicinity of the carbonyl band at $\sim 1730 \text{ cm}^{-1}$, the broad CH_2 scissoring mode in 1400-1500 cm^{-1} , the CH_2 twisting mode in 1280-1320 cm^{-1} . The criterion of our selection was that the minima of the derivative curve of the subtracted *gD* spectra which could be fairly well discriminated from the *DMPC* spectra in the region. The peaks in the subtracted *gD* spectra, assignable to the peptide, that I believe are real are indicated as the pink arrows in Figure 9.20 for the *p*-polarised Raman spectra. (Figure 9.21 for *s*-polarised spectra) Using these peak positions, we could then proceed with curve-fitting as shown in Figure 9.22 (for *p*-polarised) and 9.23 (for *s*-polarised spectra). Note that the raw spectra, not the smoothed spectra, are used for curve-fitting: this approach is considered good practice.

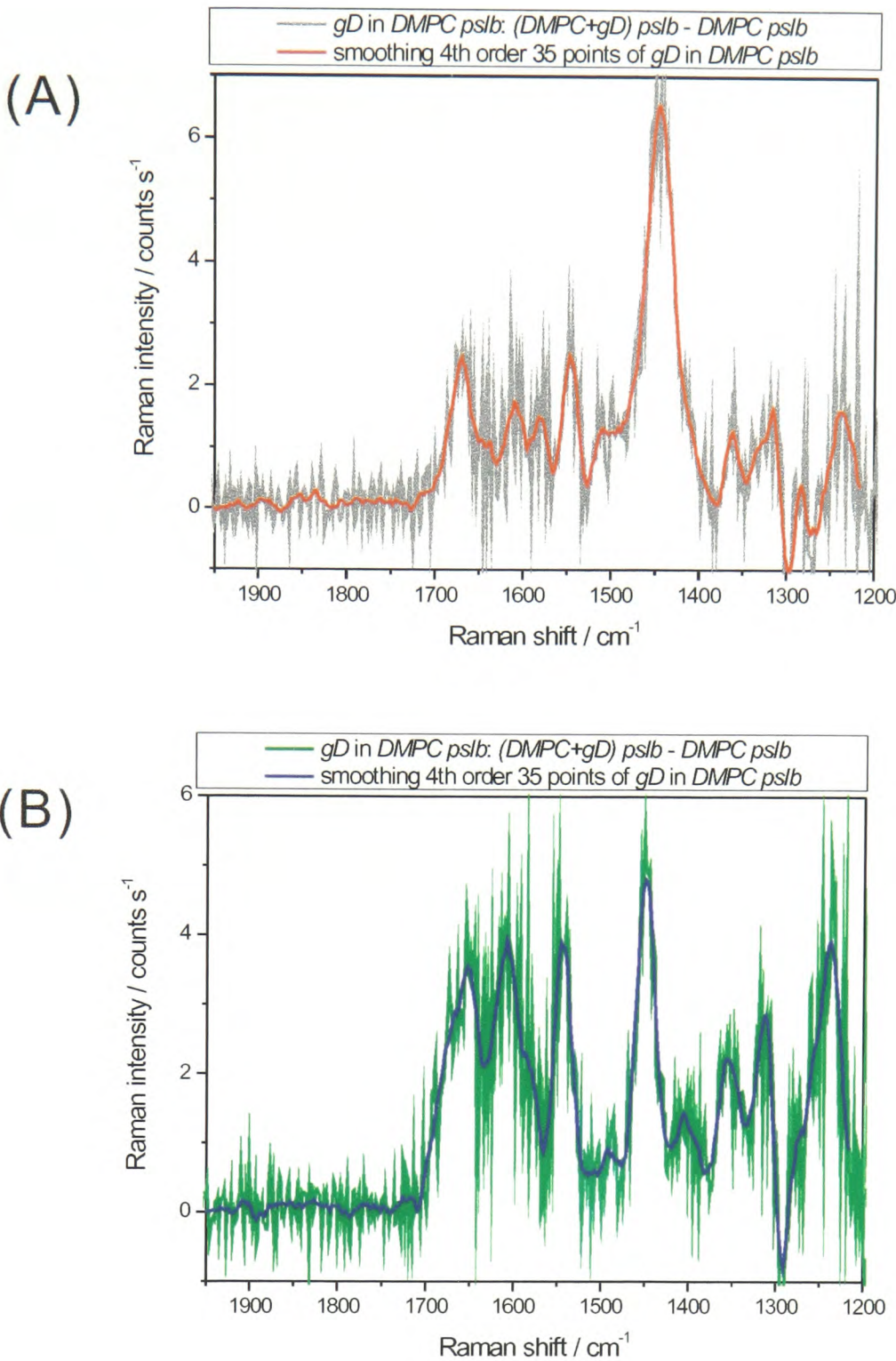


Figure 9.19 Polarised *TIR*-Raman spectra of *gD* in *DMPC pslb* (in gray and light green) and smoothed curves by quartic filter of Savitzky-Golay algorithm with filter width 35 (in red and blue), 532 *nm*, 1.2 *W*, 200 μ m slit width, 11 pixels, 18.4 °C: (A) *p*-polarised spectra; (B) *s*-polarised spectra. Axis of detection was unpolarised.

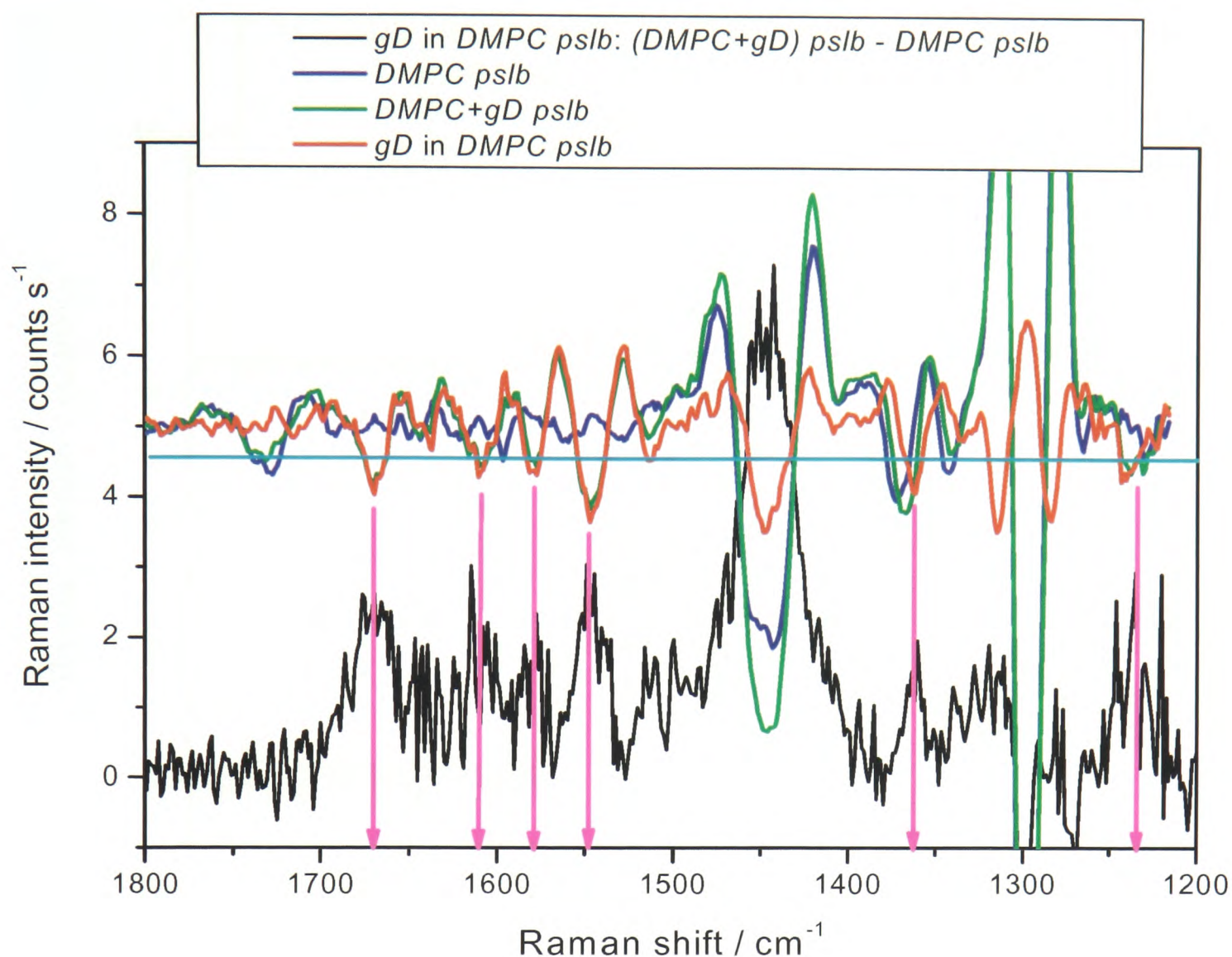


Figure 9.20 *P*-polarised spectra of *gD* in *DMPC pslb* with its 2nd derivatives, 532 nm, 1.2 W, 18.4 °C. Subtracted spectra of *DMPC+gD pslb* – *DMPC pslb* (in black); 2nd derivative of the raw spectra of *DMPC pslb* (in blue), 2nd derivative of the raw spectra of *DMPC+gD pslb* (in light green), 2nd derivative of the raw spectra of *gD* in *DMPC pslb* ((*DMPC+gD*) – *DMPC*, in red). The axis of detection was unpolarised.

The 2nd derivative spectra were treated by Savitzky-Golay smoothing algorithm of quartic filter with the filter width of 35. The minimum position of the 2nd derivative curve of the *DMPC pslb* raw spectra in the region 1600-1700 cm^{-1} (light blue line) is used as a threshold to determine which of the minima in the 2nd derivative curve of the *gD* in *DMPC pslb* are real: the peak positions in *gD* are indicated by pink.

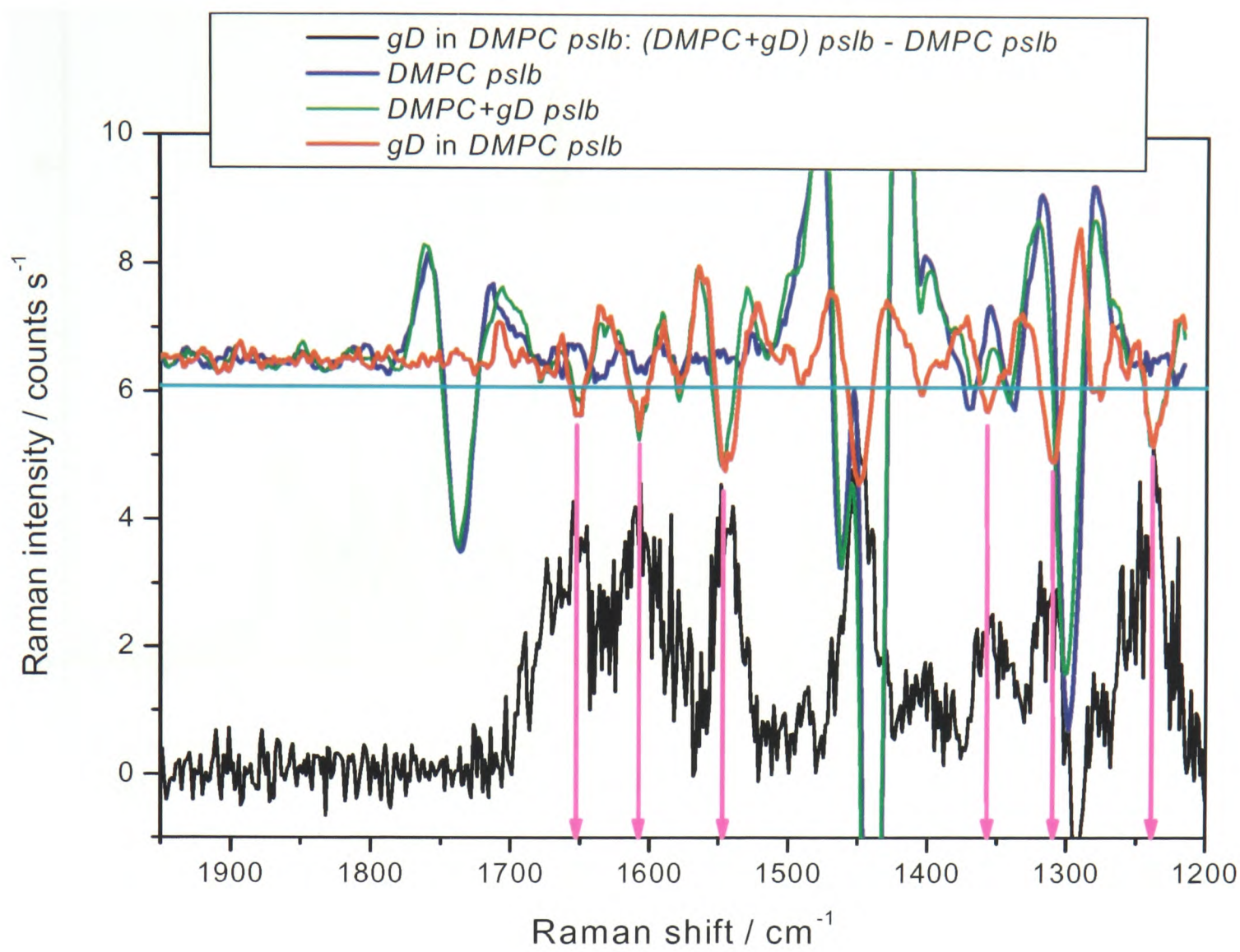


Figure 9.21 S-polarised spectra of *gD* in *DMPC pslb* with its 2nd derivatives. Conditions as in Figure 9.20.

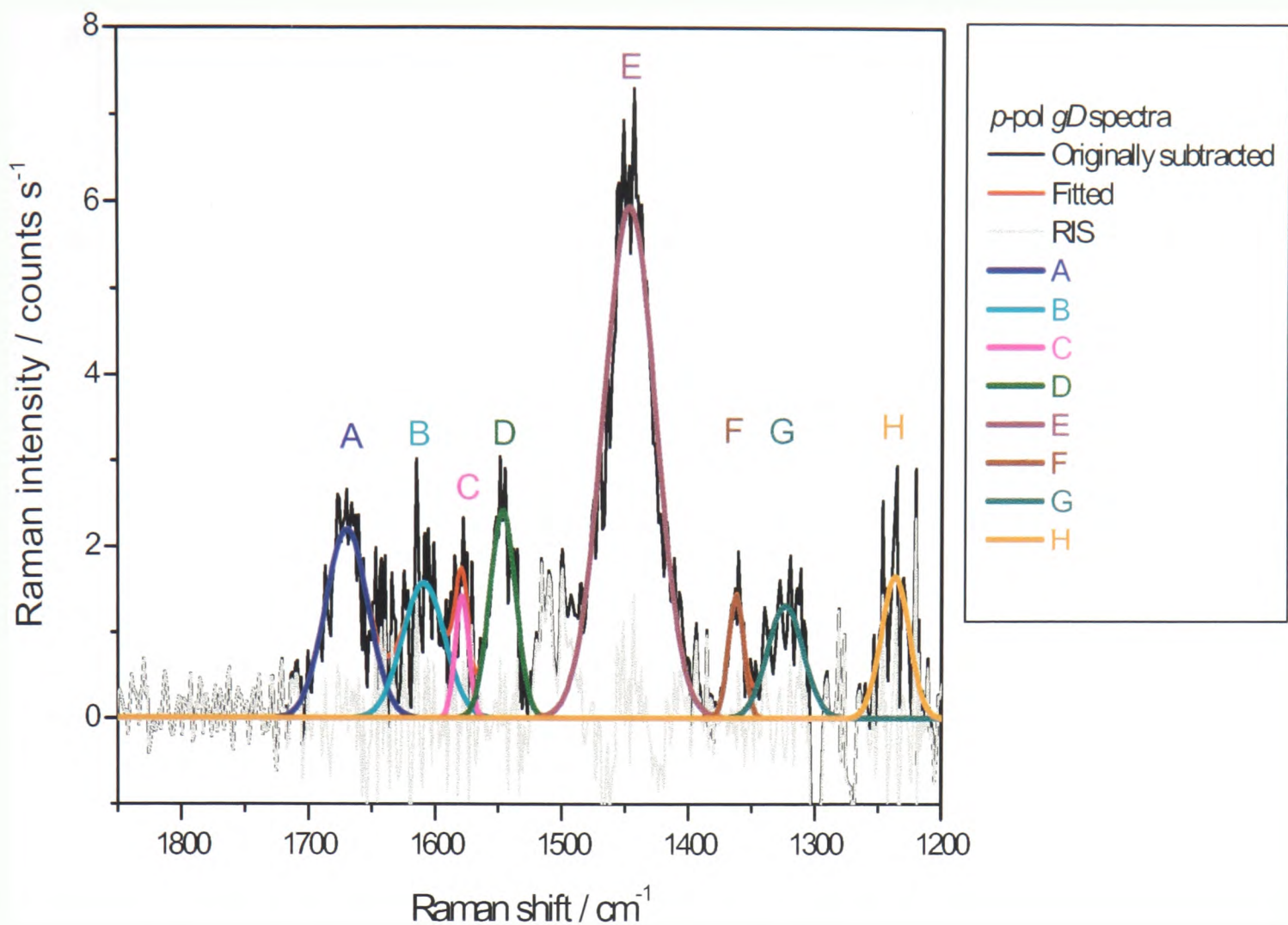


Figure 9.22 Curve-fits of *p-pol* TIR-Raman spectra of *gD* in a *DMPC* *pslb* $\langle((DMPC+gD) - \text{water background}) - (DMPC - \text{water background})\rangle$ (in black), fitted curve (in red); residuals after curve-fits (in light gray). Conditions as in Fig. 9.20. Suggested peak assignments are given as follows: **A**: amide I mode (1670 cm^{-1}); **B**: 1609 cm^{-1} ; **C**: *Trp* ω 2 (1579 cm^{-1}); **D**: *Trp* ω 3 (1547 cm^{-1}); **E**: CH_2 scissoring mode (1447 cm^{-1}); **F**: *Trp* ω 7 (1362 cm^{-1}); **G**: 1323 cm^{-1} ; **H**: amide III mode (1237 cm^{-1}).

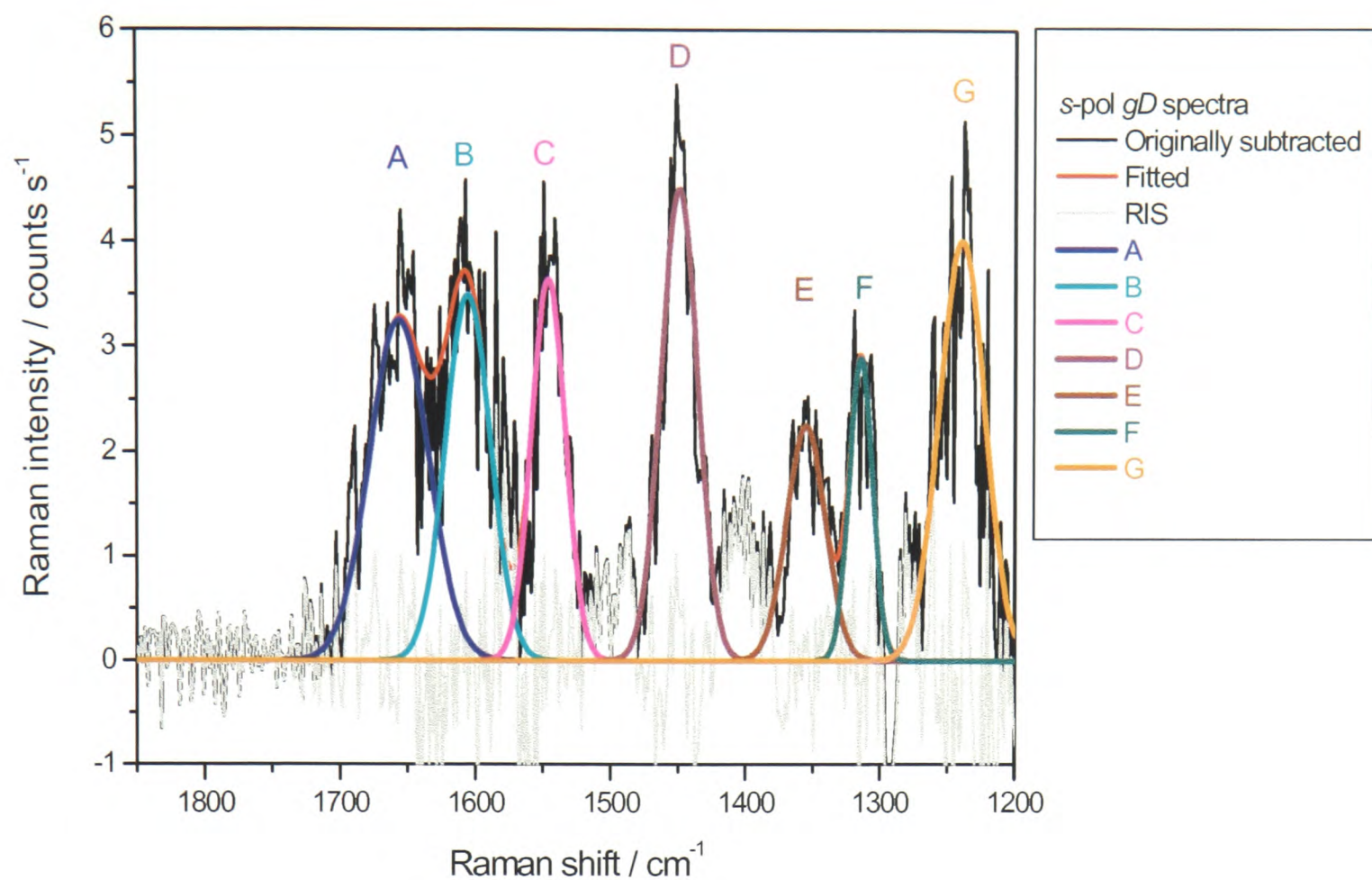


Figure 9.23 Curve-fits of *s-pol* TIR-Raman spectra of *gD* in *DMPC pslb*: $\langle((DMPC+gD) - \text{water background}) - (DMPC - \text{water background})\rangle$ (in black); fitted curve (in red); residuals after curve-fits (in light gray). Conditions as in Fig. 9.20. Suggested peak assignments are given as follows: A: amide I mode (1656 cm^{-1}); B: 1606 cm^{-1} ; C: *Trp* $\omega 3$ (1546 cm^{-1}); D: CH_2 scissoring mode (1449 cm^{-1}); E: 1355 cm^{-1} ; F: 1315 cm^{-1} ; G: amide III mode (1240 cm^{-1}).

Followed the identification of the likely positions of real vibrational bands, the unsmoothed subtracted spectra were curve fitted with the minimum number of bands identified as being above noise level. Gaussian peaks were used throughout. Though this choice may lead to larger residuals in the dips between strong peaks, where Lorentzian tails are unaccounted for, it is desirable to minimize the number of fitting parameters for noisy spectra. The curve fits and the residuals are shown in Figures 9.22 and 9.23. The peak positions extracted from these fits are presented in Table 9.1.

	<i>s</i> -pol (<i>cm</i> ⁻¹)	<i>p</i> -pol (<i>cm</i> ⁻¹)
Amide I	1656	1670
Trp ω1	1606	1609
Trp ω2	-	1579
Trp ω3	1546	1547
CH ₂ bending (scissoring mode)	1449	1447
Trp ω7	1355	1362
Amide III	1240	1237

Table 9.1 Assignments of peaks in subtracted spectra of *gD* in *DMPC pslb*, based on the curve-fits in Figures 9.22 and 9.23.

The peak of the intensity of the amide I was at 1670 cm^{-1} in the *p*-polarised spectrum and at 1656 cm^{-1} in the *s*-polarised spectrum. According to the literature, a strong and broad amide I peak centered at 1670 cm^{-1} is characteristic of antiparallel β -sheet conformation with β -hydrogen bonding of the carbonyl group (Benaki, *et al*, 1998; Koenig, 1972; Krimm & Abe, 1972; Spiro & Gaber, 1977). Gramicidin D has a very unusual sequence, however, and does not form the same structures as a pure L-amino acids, so the question arises as to which of the plausible helical structures most closely approximates in its hydrogen bonding pattern to the β -antiparallel structure. For completeness, we should note early theoretically calculations of *IR* absorption frequencies in single-stranded parallel π -helix of model peptide systems related to gramicidin A, reported by Chirgadze & Nevskaya (Chirgadze & Nevskaya, 1976) at 1656 (strong) and 1682 (weak) cm^{-1} . In the following section, I propose that the most likely helical model can be considered as either a single-stranded parallel π_{LD} or a double-stranded antiparallel π_{LD} (Iqbal & Weidekamm, 1980).

Trp vibrations were assigned to the sharp minima at 1547 cm^{-1} in both the *s* and *p*-polarised the 2nd derivative spectra. The *p*-polarised spectrum shows a second band at $\sim 1580\text{ cm}^{-1}$, which is only a shoulder in the *s*-polarised spectrum. The peak positions of indole ring vibrations are reported to be at 1620 , 1579 , and 1552 cm^{-1} for $\omega 1$, $\omega 2$, and $\omega 3$ in the literature (Maryuama & Takeuchi, 1995; Miura, *et al*, 1989). The two *gD* peaks can be confidently assigned to the $\omega 2$, and $\omega 3$ modes. The broad peak around $1605\text{-}1610\text{ cm}^{-1}$ in the *gD* spectra (strongest in *s*-polarised) is less easy to assign, since it is shifted some distance from the literature values for *Trp* $\omega 1$. In the Raman spectrum of cast films (Fig 9.2), the $\omega 1$ mode at 1622 cm^{-1} is of comparable intensity to the $\omega 2$ mode, so one might expect to observe it in the *pslb* also. The reason why the $\omega 1$ mode shows a much greater shift than the $\omega 2$ and $\omega 3$ modes in a lipid environment is unclear.

The wavenumber of the amide III mode is consistent with the antiparallel β -

sheet conformation type structure inferred from the Amide I region: the 2nd derivatives in both *s* and *p*-polarised spectra show a minimum at 1240 cm^{-1} . The subtracted spectra in Figure 9.19 show a peak at $1315\text{--}1325\text{ cm}^{-1}$ in both polarizations. This feature might arise from a frequency shift in the relatively strong lipid mode at 1300 cm^{-1} , but could alternatively arise from the Amide III mode, in which case it would be associated with helical character (Chen & Lord, 1974; Spiro & Gaber, 1977). The α -helix is characterised by the absence of intensity below 1275 cm^{-1} (Small, *et al*, 1970; Yu, *et al*, 1972) and by the presence of a strong band around 1310 cm^{-1} (Frushour & Koenig, 1974; Lippert, *et al*, 1976; Yu, *et al*, 1973).

Finally, the bands at around 1360 cm^{-1} in the second derivatives of the *s* and *p*-polarised spectra may correspond to the $\omega 7$ mode of *Trp* ‘buried’ in a hydrophobic regions (Aslanian, *et al*, 1986; Yu, 1974).

9.1.5 Discussion of *gD* in *DMPC pslb*

In our Raman spectra of *gD* cast alone, cast film with *POPC*, and *gD* in *DMPC* single lipid bilayer at water-silica interface, *gD* could be identified by well-reported characteristic Raman bands: an antiparallel β -sheet centered at around 1670 cm^{-1} in amide I mode, which is correlated with a peak at around 1238 cm^{-1} in amide III mode, *Trp* fingerprints at 1580 cm^{-1} , $1554\text{--}1546\text{ cm}^{-1}$ for $\omega 2$, and $\omega 3$ mode, “buried” state of *Trp* to hydrophobic environment of $\omega 7$ mode at 1360 cm^{-1} . These peaks were also accompanied with the residues of CH_2 bending modes such as scissoring mode centered at around 1446 cm^{-1} and twisting mode at around 1305 cm^{-1} .

But there were some dissimilarity between the *DMPC pslb* and the cast films in certain peak positions: $1606\text{--}9\text{ cm}^{-1}$ and $1315\text{--}23\text{ cm}^{-1}$. The peak at 1620 cm^{-1} was a distinguishing feature as Raman vibration of *Trp* $\omega 1$ mode in the *gD* cast film, but it

was not clearly observed in the cast film with *POPC* because of the presence of a strong and broad vinylic band at 1660 cm^{-1} (Figure 9.5). (It was the one of obvious reasons why we replaced *POPC* with *DMPC*.) But in *DMPC pslb*, *gD* had peaks at 1606-1609 cm^{-1} (Figure 9.22-23), which was a large red shift compared to the literature frequencies of the *Trp* $\omega 1$ mode (Maryuama & Takeuchi, 1995; Miura, *et al*, 1989). Another possibility might be a new amide I mode of π -helix conformation which has not previously been experimentally observed by any vibrational spectroscopic technique. But if we were to adopt the second speculation, we still have to explain the absence of *Trp* $\omega 1$ mode in our Raman spectra of *gD* in *DMPC pslb*.

The peak at 1315-1323 cm^{-1} is problematic because, even after subtraction of the pure *DMPC* lipid spectrum, this region might be profoundly influenced by lipid contribution since we know that the intense broad band of CH_2 twisting mode centered at 1305 cm^{-1} is conformationally sensitive (Figure 8.5 and 8.6 in Chapter 8.2). Nevertheless, there is a band at 1307 cm^{-1} in our *gD* cast film, which was indicative of α -helical conformation. So we might anticipate some Amide III signal in this region.

It has been generally accepted that the secondary structure of *gD* in lipid bilayers comprise dimeric single-stranded helices with 6.5 residues per turn forming a 4-Å diameter pore (single-stranded $\beta^{6.3}$ π -helix model) (Kovacs, *et al*, 1999), although there are still contradictory articles claiming that the conducting conformation is a right-handed antiparallel intertwined double-stranded $\pi\pi^{7.2}$ β -helices studied in gramicidin A in lipid bilayer by x-ray and multidimensional *NMR* spectroscopy (Comment, 1999; Burkhardt, *et al*, 1998). The *CD* data on the *DMPC-gD SUV* showed that the major part of the sample comprised single-stranded $\beta^{6.3}$ π -helical dimers as discussed in Chapter 6. While it is possible that the conformation could change during the fusion process or that a minor component of the mixture, containing a different conformation, could preferentially adsorb, it is reasonable to interpret our Raman spectra of the *pslb* in terms

of the accepted channel structure.

Our Raman spectra of *gD* spanning the *DMPC pslb* were clearly based on the *CD* data showing major constitution of single-stranded $\beta^{6.3}$ π -helix dimers in the *DMPC-gD SUV* as discussed in Chapter 6. As mentioned previously, *CD* spectra well characterised the single-stranded $\beta^{6.3}$ π -helix dimer structure of a channel form (Masotti *et al*, 1980; Urry *et al*, 1983 which differed from various conformations in organic solution, although *gD* shows polymorphism in various solvents but we used trifluoroethanol to preferentially form helical and channel structures like a panacea. Gramidicin dissolved in organic solution expresses multiplex conformations of typically double-stranded dimer which may be parallel or antiparallel, left-handed or right-handed with a range of residues per turn from 5.6 to 6.4 (Kovacs, *et al*, 1999; Bystrov & Arseniev, 1988). But in the heterogeneous anisotropic lipid environment, it is almost exclusively single-stranded (Kovacs, *et al*, 1999). Nevertheless it is accompanied with a fraction of other conformations depending on a cosolvent (Bano, *et al*, 1991; Killian *et al*, 1988), environment (Cox, *et al*, 1992; Salom, *et al*, 1998), and heat (Masotti, *et al*, 1980) effects during preparation. (It was explained in Chapter 6.) The *CD* spectra could clearly confirm the single-stranded dimeric structure only, nothing more about detailed conformations.) Therefore our *gD* in *DMPC pslb* was believed to a single-stranded dimeric structure with the assumption that there was no conformational change during the fusion and incubation of *SUV* solution to form *pslb* onto silica window over main phase transition temperature.

A right-handed double-stranded helical model with 7.2 residues/turn (as referred to Figure 4.3) proposed by x-ray and *NMR* spectroscopy of Cs^+ and H^+ complex *gD* which has ever been issued to take controversy of gramicidin structure as conducting, channel form in lipid bilayer (Burkhard *et al*, 1998), because this proposed model could be simply excluded by our *CD* spectra implying single-stranded dimeric

structure.

The Amide I band appeared at 1656 cm^{-1} in the *s*-polarised spectrum and 1670 cm^{-1} in the *p*-polarised spectrum. As far as I know, there has been only one theoretical calculation of a parallel π -helix for a model peptide related to gramicidin A, which yielded *IR* absorption frequencies of 1656 (strong) and 1682 (weak) cm^{-1} for the amide I mode (Chirgadze & Nevskaya, 1976). The antiparallel β -sheet is characterised by a peak at 1670 cm^{-1} and the parallel β -sheet by a peak at 1660 cm^{-1} . Thus, while the Amide I frequency is consistent with the proposed channel structure (with and antiparallel β -sheet-like bonding) the Amide I region does not rule out alternative structures. The antiparallel beta-sheet structure is supported by the peak at around 1240 cm^{-1} in amide III mode. However, the problem in assigning with certainty the residual intensity (after subtraction) around 1300 cm^{-1} makes it difficult to unequivocally rule out other structures. For example, the *s*-polarised spectra would be consistent with some α -helical character (1656 cm^{-1} in amide I mode and $1315\text{--}1325\text{ cm}^{-1}$ in Amide III). There are no literature data for the Raman frequencies of other helical structures such as 3^{10} α -helix, π -helix, and γ -helix.

The positions of the amide I and amide III bands are not the only structural marker for membrane-bound peptides. For peptides containing tryptophan, one can make use of a correlation established by *T. Maruyama*, *T. Miura*, and *H. Takeuchi* (Maruyama & Takeuchi, 1995; Miura, *et al*, 1989; Takeuchi, 2003) for the wavenumber of the ω_3 mode in crystals of tryptophan derivatives. They found that the frequency of the ω_3 mode was correlated to the torsion angle $\chi^{2,1}$ defined by the three bonds $\text{C}_2=\text{C}_3\text{--}\text{C}_\beta\text{--}\text{C}_\alpha$ in peptide, illustrated in Figure 9.24. The correlation is given by

$$\nu(\omega_3) = 1542 + 6.7 \left(\cos 3 \left| \chi^{2,1} \right| + 1 \right)^{1.2} \text{ cm}^{-1}$$

From known or proposed structures of a peptide, one can evaluate the torsional

angles, $\chi^{2,1}$, and compare with experimental values determined by vibrational spectroscopy. The relationship is limited to the $|\chi^{2,1}|$ value within a range of 60-120° because the equation was derived to use angles in this region.

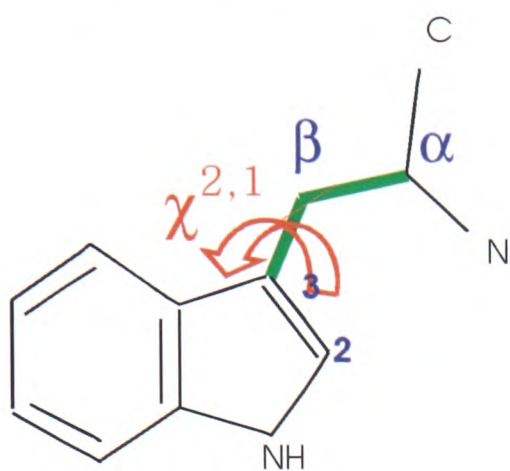


Figure 9.24 Schematic of defining torsion angle $\chi^{2,1}$ in *Trp* residue. The zero of $\chi^{2,1}$ corresponds to the eclipsed conformation of $C_2=C_3-C_\beta-C_\alpha$. A torsion angle is considered positive or negative according as when the system is viewed along the central bond in the direction $C_3 \rightarrow C_\beta$, the bond to the front atom C_2 requires a rotation to the right or left, respectively, in order that it may eclipse the bond to the rear atom C_α (Blanch, *et al*, 2001).

Figure 9.25 shows the 3-D structure typical of the active channel form, deposited in the Brookhaven Protein Data Bank (*PDB*) with file names of 1MAG.

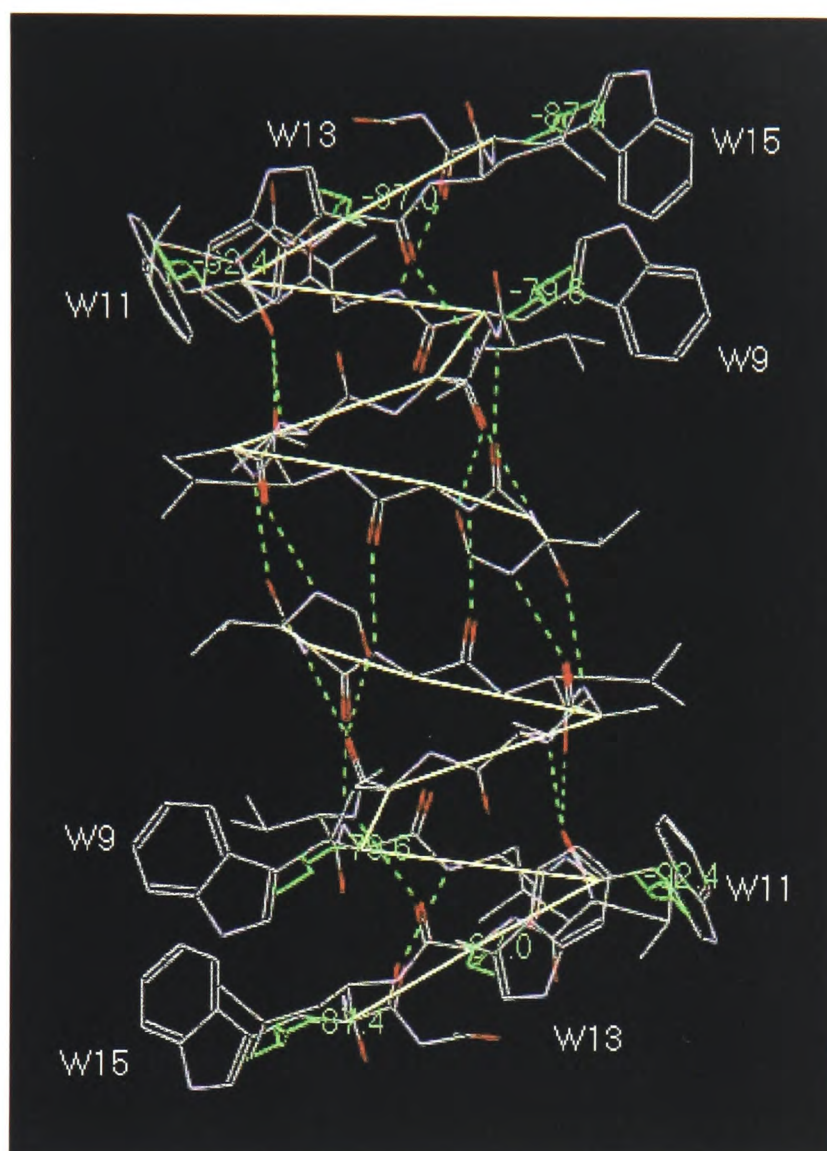


Figure 9.25 A possible channel models of *gD* in lipid bilayer: single-stranded dimeric N-terminal-to-N-terminal helices (redrawn from *pdb* 1MAG, solid-state *NMR* data of *gA* in *DMPC*).

From the *NMR* structure we can evaluate the torsional angles of each of the four distinct *Trp* groups and calculate the vibrational frequency of the ω_3 mode predicted by the correlation of Maruyama (See Table 9.2). The average vibrational frequency is found to be 1547.4 cm^{-1} , which is in excellent agreement with our observed Raman shifts of 1546 cm^{-1} in the *s*-polarised spectra and 1547 cm^{-1} in the *p*-polarised spectra (the latter value being more accurate). As reference, we had an observed peak at 1553

cm^{-1} in the cast film of *gD* alone and 1549 cm^{-1} in the multistacked cast film of *gD+POPC*. The difference between the *pslb* and the multistack cast film may not be significant, but there is a distinct difference from the cast film of pure *gD*, confirming that the peptide is in a different conformation in the lipid bilayer.

Out of interest, I also carried out the calculation of the *Trp* $\omega 3$ wavenumber for the right-handed double-stranded helical model with 7.2 residues/turn proposed by Burkhard *et al* (Burkhard *et al*, 1998). It gave an averaged value of 1546.1 cm^{-1} (for Cs^+ binding *gD*) or 1548.7 cm^{-1} (for H^+ binding *gD*) for the two helices with all the torsional angles within $60\text{-}120^\circ$. This wavenumber is sufficiently close to that of the channel form that the *Trp* $\omega 3$ mode could not on its own distinguish the two possibilities.

	$\chi^{2,1}$	Calculated $\nu_{\omega 3}$
Upper π helix	-79.6°	$\sim 1544.8\text{ cm}^{-1}$
W9		
W11	-92.4°	$\sim 1549.7\text{ cm}^{-1}$
W13	-87.0°	$\sim 1547.5\text{ cm}^{-1}$
W15	-87.4°	$\sim 1547.6\text{ cm}^{-1}$
Lower π helix	-79.6°	$\sim 1544.8\text{ cm}^{-1}$
W9		
W11	-92.4°	$\sim 1549.7\text{ cm}^{-1}$
W13	-87.0°	$\sim 1547.5\text{ cm}^{-1}$
W15	-87.4°	$\sim 1547.6\text{ cm}^{-1}$
		Average = $\sim 1547.4\text{ cm}^{-1}$

Table 9.2 $\chi^{2,1}$ and calculated $\nu_{\omega 3}$ of channel model (from *pdb* 1MAG).

To end this discussion, I consider some of the limitations of *TIR*-Raman scattering revealed by this study. The first is that the signal to noise (which is limited

in part by photodamage) is insufficiently good to locate weak peaks with confidence or to carry out a fully polarization-resolved study. Polarised spectra would help to determine the orientation of orientation of specific vibrational bands, such as the Amide I band which consists mostly of C=O stretching.

Second, we still had difficulty in the background subtraction of the strong and broad water in O-H bending region, which is very sensitive to changes in, for example, penetration depth and may be intrinsically different in samples with different surface chemistry. This region includes one of the most useful Raman bands for identifying secondary structure, the amide I mode. Mismatched background can cause distortion and addition of artifacts in subtracted spectra. Even with perfect subtraction, the water background contributes increased noise. To suppress the background level, the penetration depth can be minimised by increasing the angle of incidence, but this solution sacrifices Raman signal. So there is always an optimised condition to compensate water background with Raman intensity. Alternatively we can replace H₂O with D₂O to make contrast better in the amide I region, but it is still hard to get good subtraction due to H-D exchange. With better signal to noise, we could introduce isotopically substituted amino acids (like deuterated form) to observe the orientation of specific vibrations in windows in the *IR* spectrum

Third, Raman spectroscopy has a poor database in the literature. To assign peaks that have not yet been studied in samples with a well-characterised secondary structure (such as the π -helix in *gD*), we may need theoretical calculation to assist identification.

Fourth, Raman spectroscopy can never hope to provide as complete a picture of the membrane peptide/protein structure as high resolution data from *x*-ray or *NMR* spectra. Raman spectroscopy has the advantage of accessing the aqueous environment, but it is still digging at a low resolution level.

9.2 Interaction of indolicidin with a planar supported lipid bilayer

In the previous sections, I have described the detection of membrane-bound peptide, gramicidin D, which was incorporated into *SUVs* before fusion with the silica surface. In this section, I show that we can also follow the interaction of a soluble peptide, indolicidin, with a preformed *pslb* of pure lipid. This work was carried out in collaboration with Ruth Smith, who has also been studying indolicidin by *FT-IR*. We address the time-dependent changes in the Raman spectra of a *DMPC pslb* exposed to an aqueous solution of indolicidin, below the reported critical aggregation concentration of $\sim 50 \mu\text{g/mL}$ ($\sim 26 \mu\text{M}$) (Ahmad, *et al*, 1995). These are the first reported Raman spectra of indolicidin in a lipid environment.

Figures 9.26 (A) and 9.27 (A) show Raman spectra in the C-H stretching region and fingerprint region, respectively, of a *DMPC pslb* exposed to indolicidin, at a temperature (30°C) above the main chain melting transition. Figure 9.26 (B) and Figure 9.27 (B) show the spectra after subtraction of the initial spectrum of pure *DMPC* with a subtraction factor of 1. The indolicidin solution was injected at $t = -3 \text{ min}$, (3 minutes being the minimum time for setting up the acquisition parameters and internal focusing) and replaced with pure water at $t = 150 \text{ min}$. The pronounced peak at 3060 cm^{-1} arises from the symmetric C-H stretching vibration of the indole ring of *Trp*. Until the bulk solution was replaced by *UHQ* distilled water (from 10 min to 138 min as noted in the legend of Figure 9.26), the peak intensities at 3060 cm^{-1} fluctuated. Fluctuations in the scattering intensities were also observed in the fingerprint region of Figure 9.27. The peak intensities of *Trp* $\omega 3$ mode (noted as an arrow in green in Figure 9.27) indicating how many indolicidin molecules are attached to the *DMPC pslb*, also varied randomly until the indolicidin solution was replaced by fresh water. After the reservoir of

indolicidin was rinsed out with fresh water, the intensities of peaks assignable to indolicidin decreased, both at 3060 cm^{-1} in the C-H stretching region and in the fingerprint regions. After rinsing, the spectra were stable, but still contained peaks assignable to indolicidin.

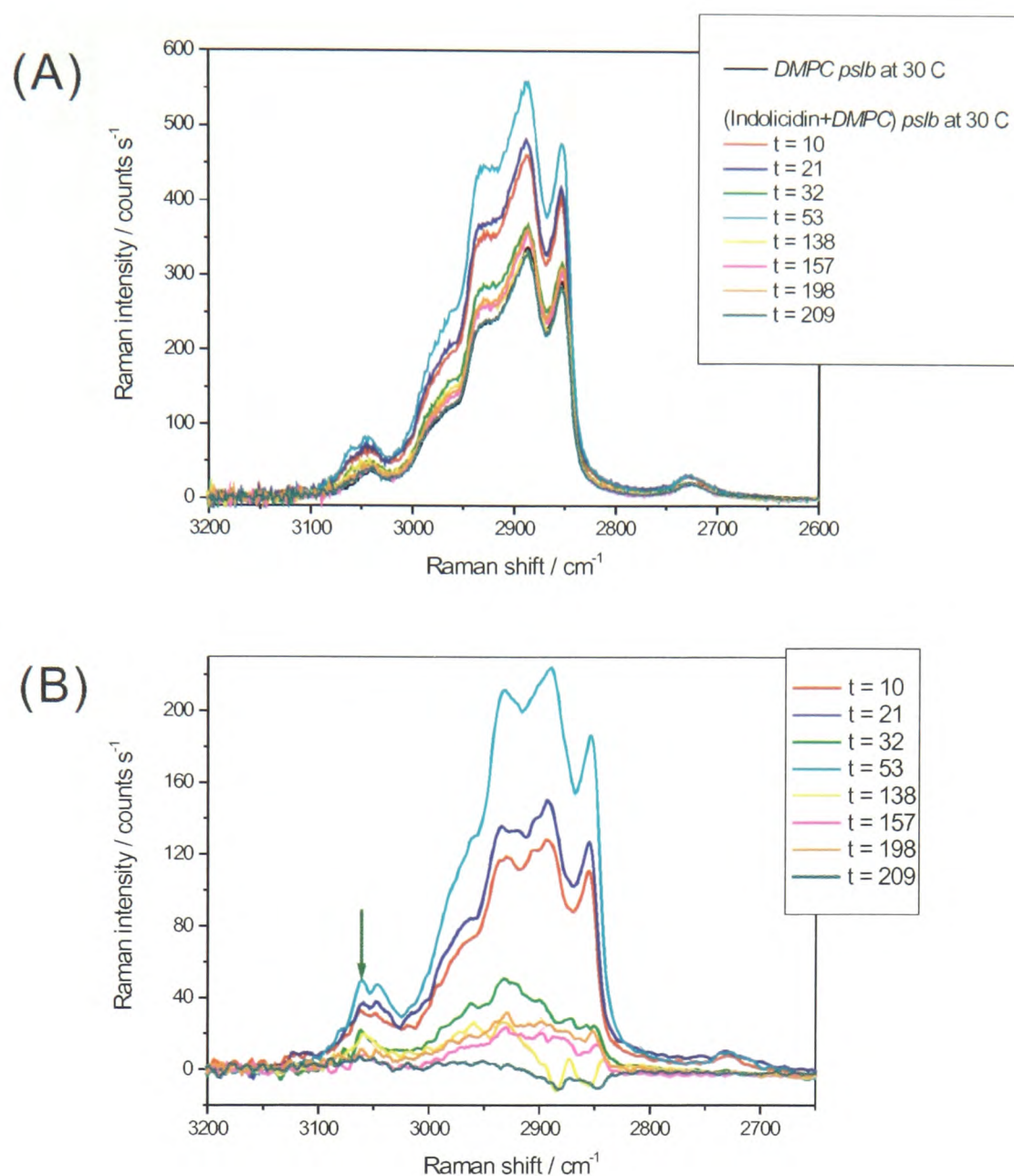


Figure 9.26 The time-dependent Raman spectra of a *DMPC* lipid bilayer exposed to indolicidin at pH 7 in 10 *mM* Tris buffer solution with 150 *mM* NaCl., (A) the spectra with *DMPC* (compared with *DMPC* only in red), (B) the spectra subtracted by *DMPC*, 12 μM ($\sim 22.84 \mu\text{g/mL}$) of indolicidin solution was loaded onto preformed *DMPC ps/b* at 30.0 °C, 532 nm, 1.2W, *s-pol* excitation, unpolarised detection, 200 μm slit width, 11 pixels, Zeiss 40 $\times$, 1 $\times$ 45 s of acquisition. The indolicidin solution was replaced with *UHQ* water after 150 min. The green arrow designates the characteristic peak of C-H vibration in indole ring of *Trp*. For convenience of observing the intensity change at 3060 cm^{-1} , the subtracted spectra was smoothed by Savitzky-Golay method in a way of quartic order and filter width 18.

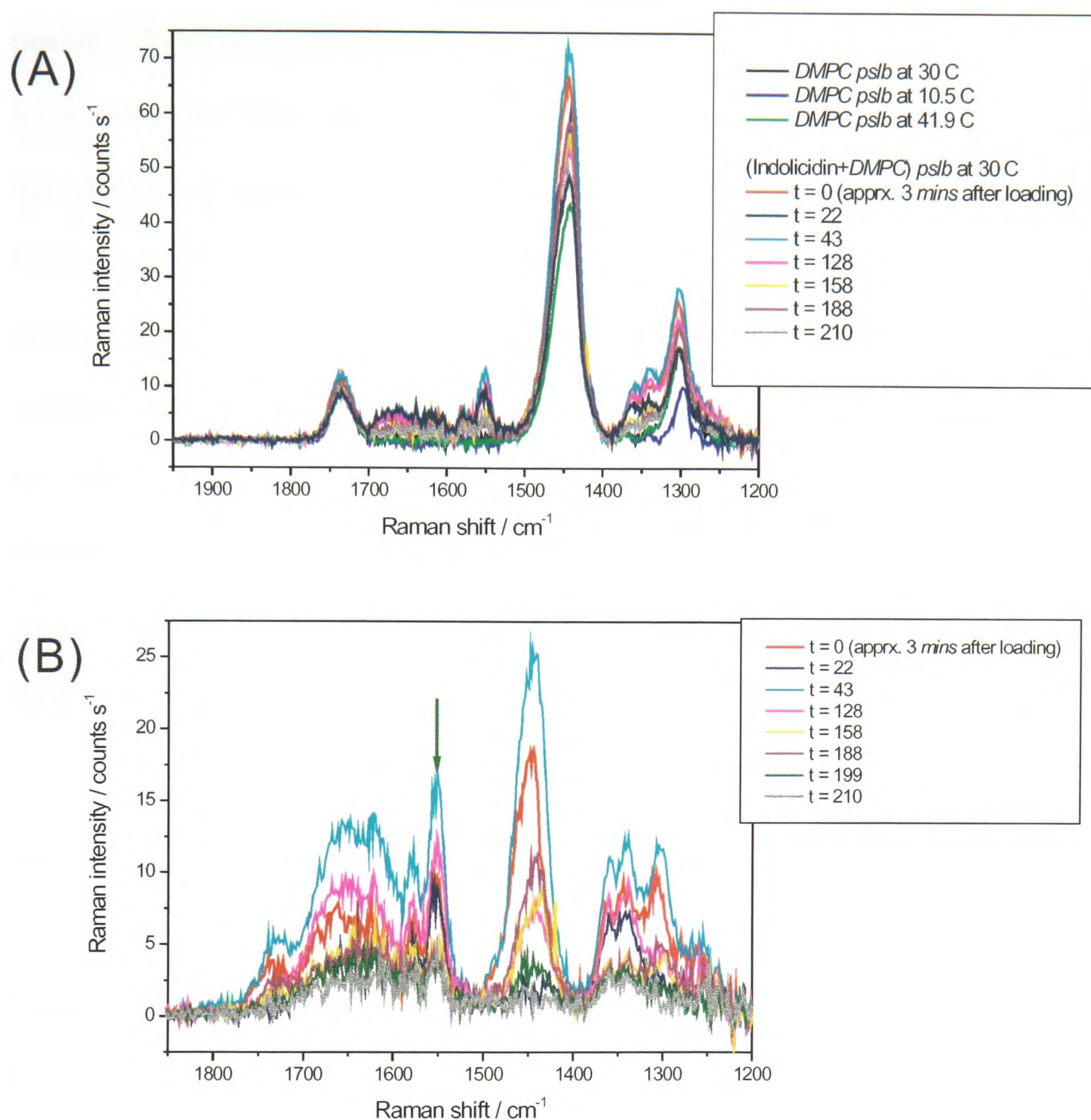


Figure 9.27 The time-dependent Raman spectra of a *DMPC* lipid bilayer exposed to indolicidin, in the fingerprint region: (A) (*DMPC* + indolicidin) spectra after water subtraction, compared with *DMPC pslb*. $T = 30\text{ }^{\circ}\text{C}$ (B) $\langle((\text{DMPC} + \text{indolicidin}) - \text{water background}) - (\text{DMPC} - \text{water background})\rangle$, $12\text{ }\mu\text{M}$ ($\sim 22.84\text{ }\mu\text{g/mL}$) of indolicidin solution was loaded onto preformed *DMPC pslb*. Acquisition time = 600 s. Other conditions as in Fig. 9.26. The green arrow indicates the peak position of *Trp* ω_3 mode.

Two features are worthy of comment: first, the fluctuation of the spectra before rinsing. These fluctuations are confined not just to the indolicidin peaks but also to the lipid peaks. Yet after rinsing the lipid spectrum returns very nearly to its original level. The presence of aggregates on the surface (see below) could explain fluctuations in the indolicidin peaks, but it is difficult to explain fluctuations in the lipid peaks unless the peptide is interacting with the lipid bilayer itself. Three mechanisms have been proposed for how holes may be generated by the insertion of indolicidin into a bilayer: the barrel-stave, toroidal-pore, and carpet models (Brogden, 2005). However, these insertion events are very localised and involve only small numbers of peptide molecules. Any fluctuations in the number of such holes might be expected to average out over the laser spot, which is very large on a molecular length scale.

The fluctuations in the Raman spectrum in the presence of indolicidin may arise at least in part from the presence of aggregates on or near the surface of the prism. Even though we worked below the concentration of indolicidin at which aggregation is reported in the literature, images of the laser spot on the silica prism showed an increase in scattering in the presence of indolicidin, and a subsequent decrease when the indolicidin was rinsed out (Fig. 9.28). These images suggest the presence of loosely-bound aggregates of indolicidin on the surface. It is possible that indolicidin interacting with the surface may induce aggregate formation, or simply that small aggregates are present at concentrations below that which has been reported in the literature.

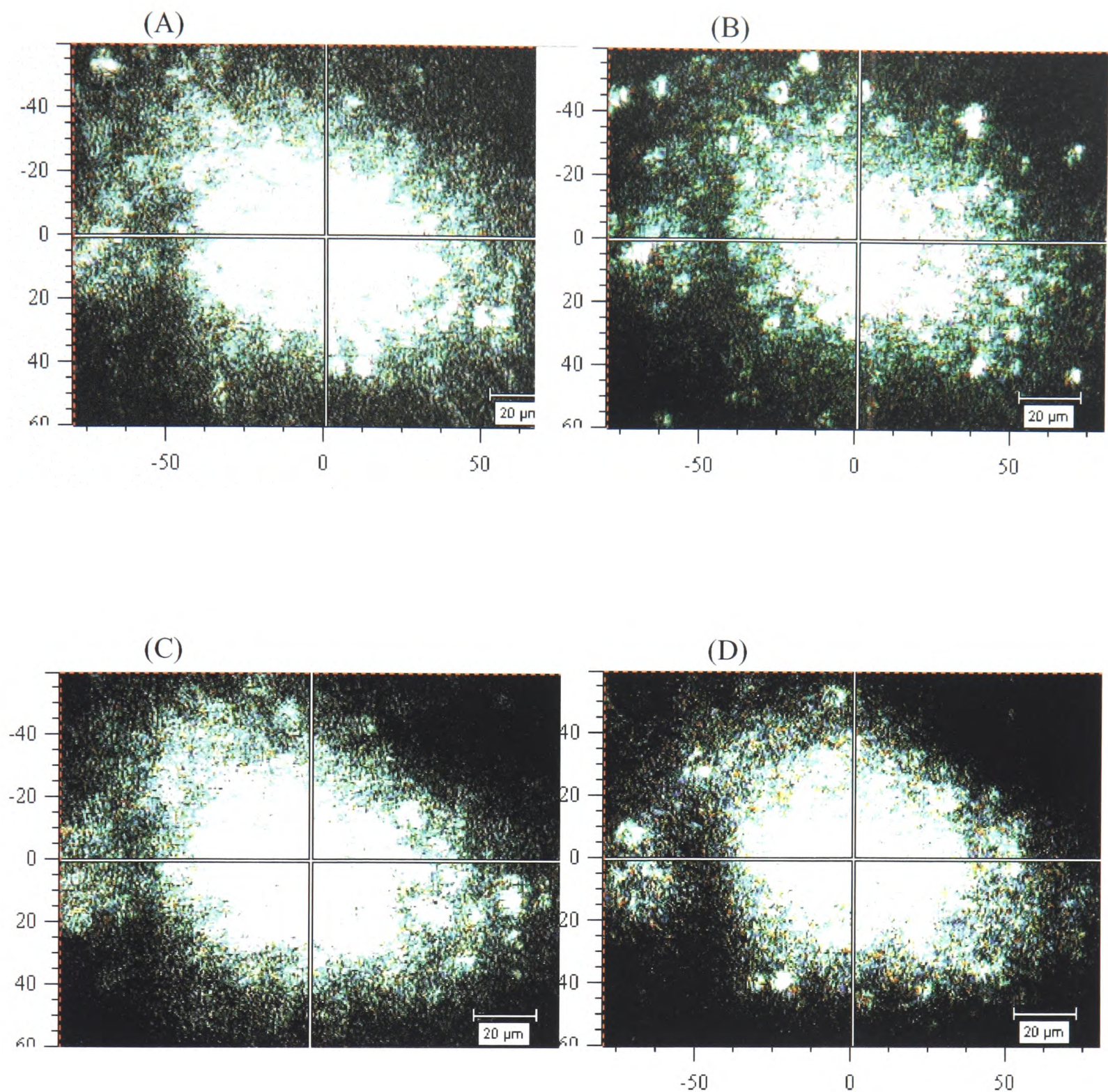


Figure 9.28 CCD images of the laser spot at different stages of the exposure of the pslb to indolicidin. (A) Just after indolicidin solution added to the preformed *DMPC* pslb, (B) 90 min after indolicidin was added. (C) 90 min after the indolicidin solution was rinsed out with pure water, (D) *DMPC* pslb only as a reference image.

The second noteworthy point is that upon replacement of the indolicidin solution by pure water, the intensity of the peaks assignable to the peptide decreased

substantially, but did not disappear. This residue is particularly noticeable in the Trp $\omega 3$ band. The fluctuations in the intensity that characterized the incubation period also ceased.

Although the peptide bands in the Raman spectra of indolicidin interacting with a *pslb* are at least as strong as those of *gD*, a detailed interpretation of the indolicidin spectra is complicated by the limited literature on Raman bands of β -turn peptides. In particular, there are no reports for the β -turn type VI structure by Raman spectroscopy that has been proposed as a possible conformation of indolicidin in a lipid bilayer. We can, however, attempt to identify the principal Raman bands from the adsorbed indolicidin. Proceeding as before, I smoothed the subtracted spectra with a quartic S-G function with 18 points (Fig. 9.29) and then took a second derivative to identify the positions of peaks (Fig. 9.30). These peaks were then used to guide the curve-fitting of the unsmoothed data (Figures 9.31, 9.32, and 9.34.).

The peak positions to be selected for curve-fitting were considered by the comparison of the second derivatives of each subtracted spectra with the lipid spectra as treated in case of *gD* in *DMPC pslb* and they were very cautiously inspected based on the relevant reference Raman bands prior to curve-fitting. Figure 9.29 shows the Raman spectra of [(indolicidin-associated *DMPC pslb*) – *DMPC pslb*] measured at onset stage of interaction for 5×120 s (noted as “*f0*”) treated by the smoothing filter of degree 4 with different filter widths. And the figure 9.30 presents the 2nd derivative curves of the Raman spectra of “*f0*” and only *DMPC pslb* in order to determine peak positioning for curve-fitting. In Figure 9.29, the filter widths in the range between 18 and 33 when the ratio of filter width to *fwhm* in quartic order is selected as 1.72 according to the literature (Bromba & Ziegler, 1981) could be considered because spectra have the various constituent features of different band width and the filter width can be evaluated. The filter width of 33 was not good enough smoothing the original spectra whereas the

filter width of 18 did smoothing work well, especially for the possibly important peaks presented in the raw spectra in the regions of the amide I and III modes (Figure 9.29 (B) and (C)).

With the established parameter of quartic order and filter width of 18, the smoothed spectrum of $t = 0$ was processed by the 2nd derivative as shown in Figure 9.30. In the amide I modes we could allocate two constituent peaks based on the second derivative curve as shown in Figure 9.30. However the amide III modes made us difficult to position the peaks due to the broadness and low signal-to-noise ratio. When the peak at 1258 cm^{-1} fitted into the raw spectra according to the recommendation of the 2nd derivative curve, the relatively big residue in the region of between 1250 and 1300 cm^{-1} remained. It might compel the shift of our allocating of the peak to higher frequency.

Any expectation to a preferred conformation was thoroughly excluded for justifice of peak assignment.

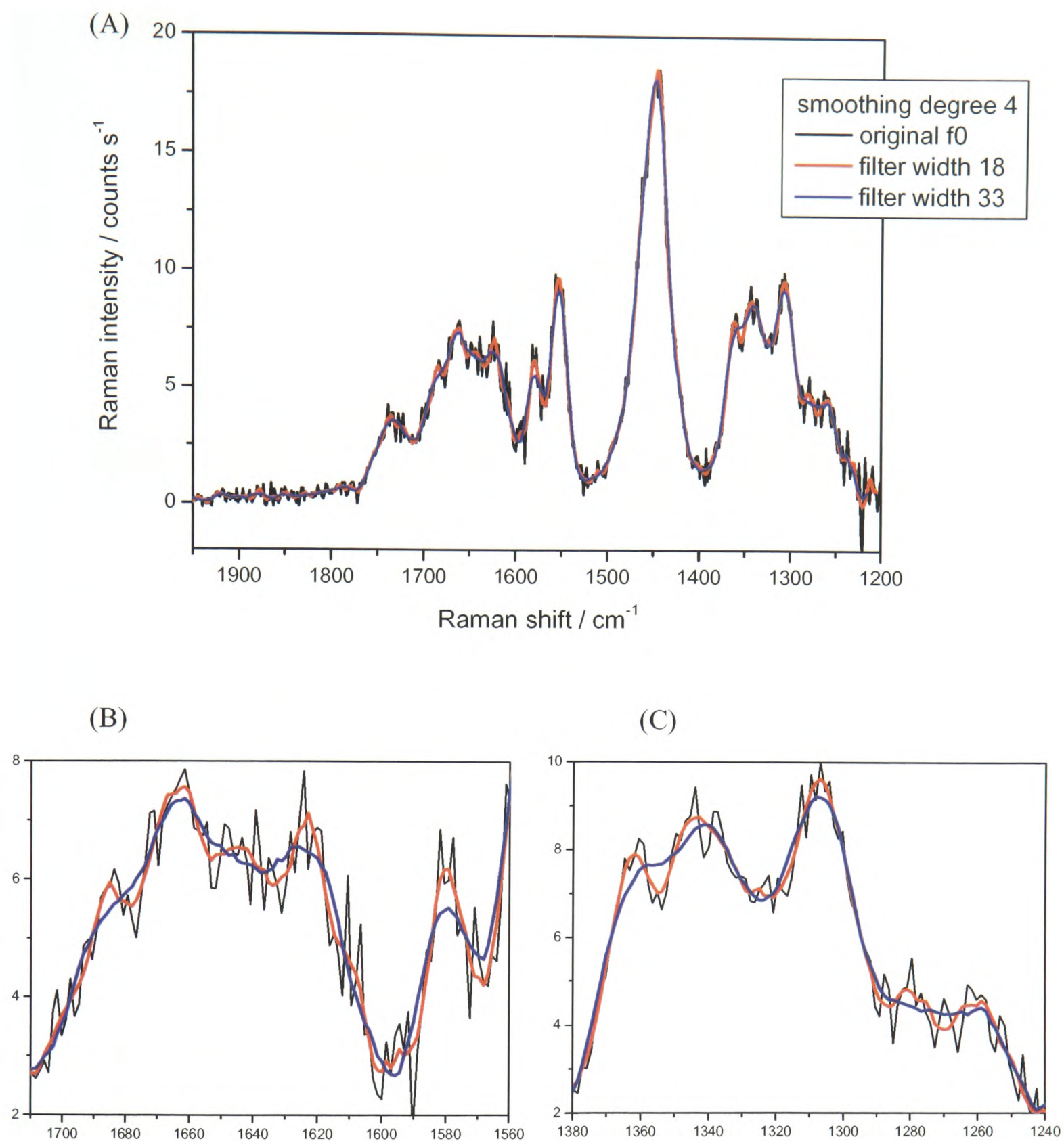


Figure 9.29 Smoothed curves of indolicidin-reacted *DMPC pslb* measured at onset of interaction with indolicidin ($t = 0$), acquisition time of 5×120 s) with different filter widths (18 in red, 33 in blue) for the quartic Savitzky-Golay algorithm: (A) in all fingerprint region, (B) in the region of amide I mode, and (C) in the region of amide III mode.

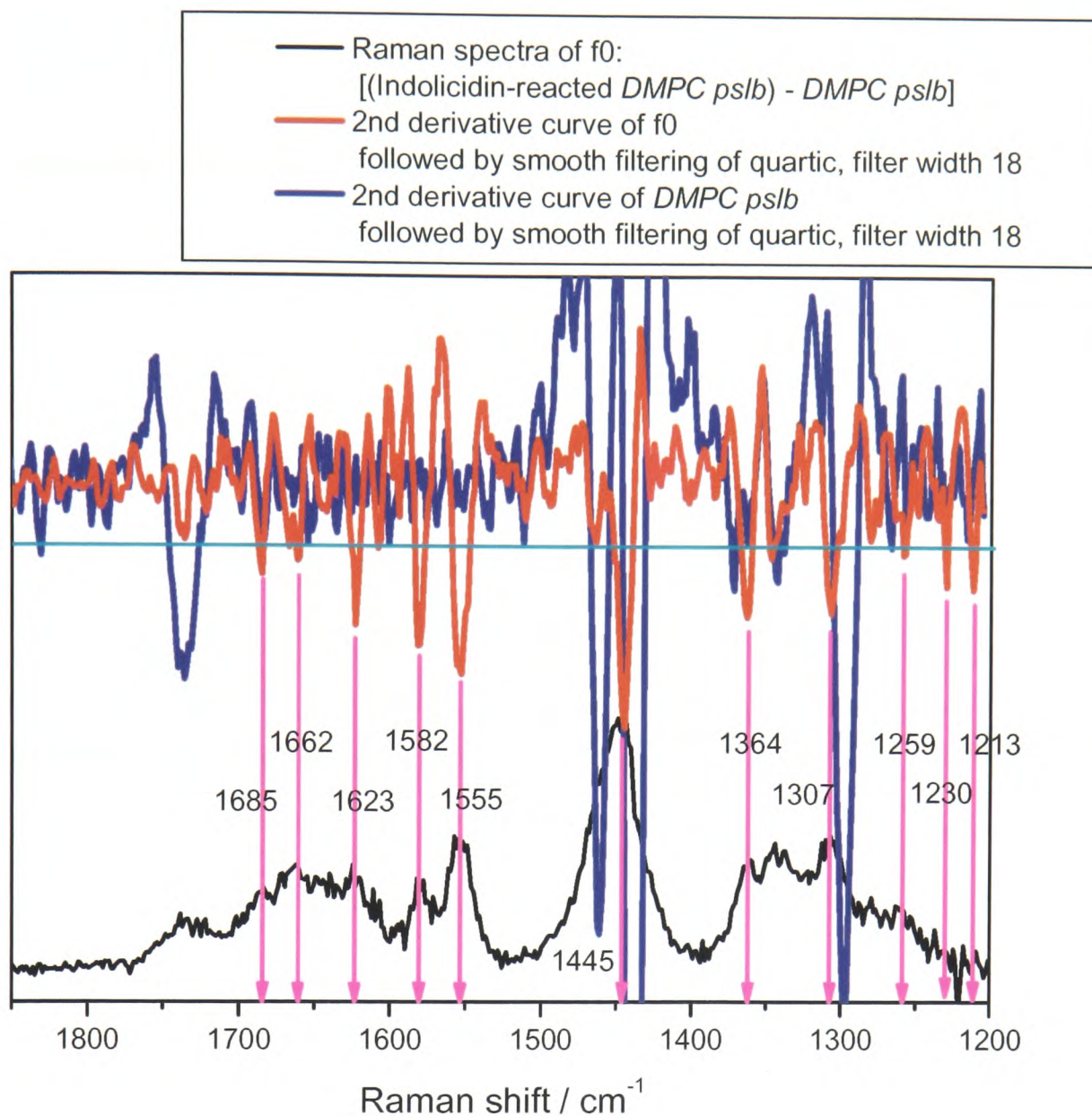


Figure 9.30 The 2nd derivative curves of the Raman spectra of [(indolicidin-reacted *DMPC pslb*) - *DMPC pslb*] at onset of interaction with indolicidin ($t = 0$), compared with only *DMPC pslb*. The minimum position of the 2nd derivative curve of the *DMPC pslb* raw spectra in the region of 1600-1700 cm^{-1} limits reading of minimal in the 2nd derivative curve of the *gD* in *DMPC pslb* (by a line in light blue). The peak positions in consideration are indicated by arrows in pink. And these 2nd derivative curves are plotted together with the spectra of $t = 0$ in convenience of peak positioning.

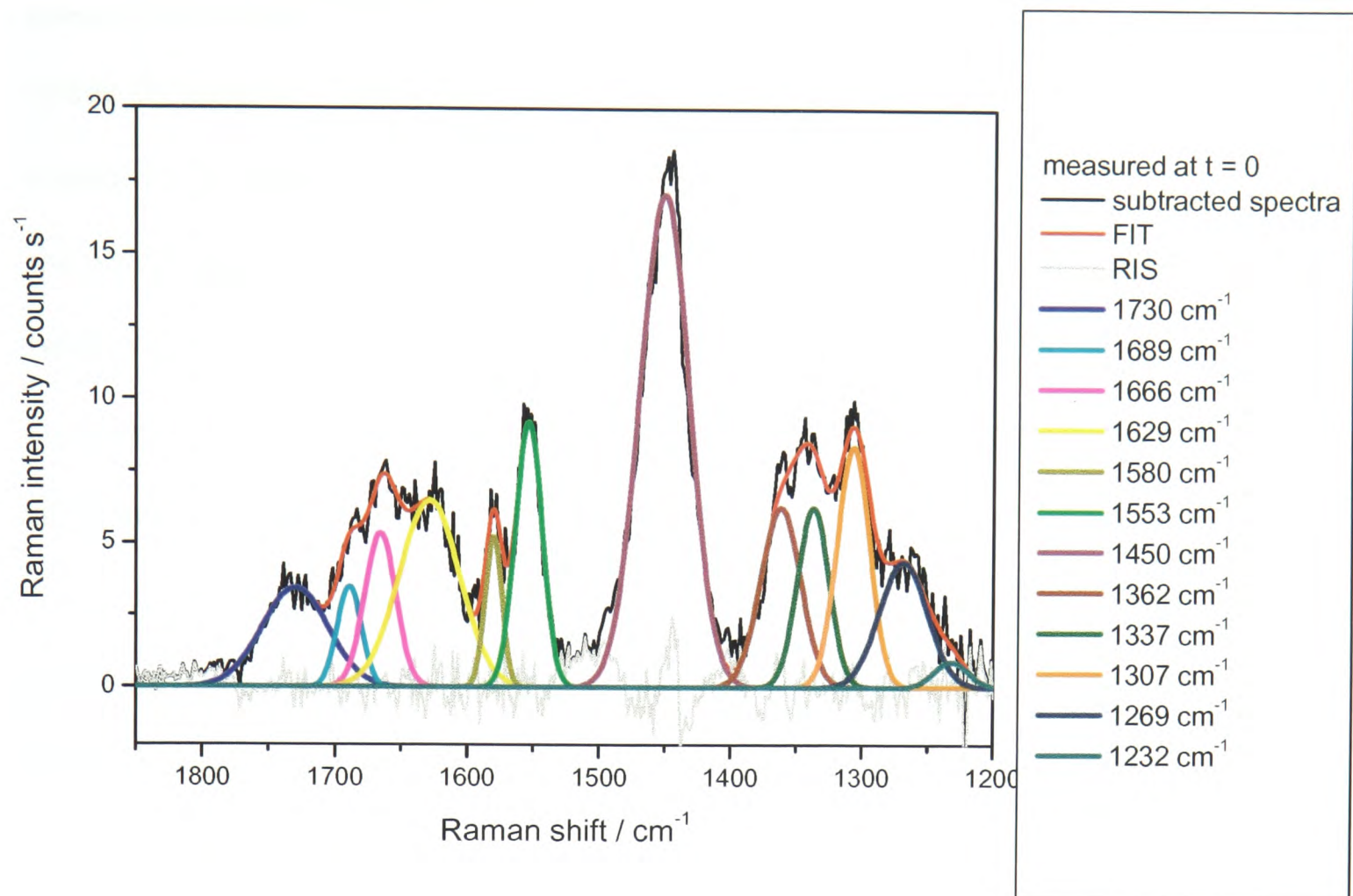


Figure 9.31 Tentative peak assignment of the subtracted Raman spectra of $12\text{ }\mu\text{M}$ ($\sim 22.84\text{ }\mu\text{g/mL}$) indolicidin: $\langle ((\text{DMPC} + \text{indolicidin}) - \text{water background}) - (\text{DMPC} - \text{water background}) \rangle$. The spectra was measured just after solution added onto DMPC pslb, 532 nm , 1.2 W , $s\text{-pol}$ in excitation, unpolarisation in detection, $200\text{ }\mu\text{m}$ slit width, 11 pixels, $30.0\text{ }^{\circ}\text{C}$, Zeiss $\times 40$, $5 \times 120\text{ s}$ of acquisition.

Figure 9.31 shows the spectrum of *DMPC*+ indolicidin minus the *DMPC* spectrum in the fingerprint region measured immediately (ca. 3 min) after the *pslb* was placed into contact with the indolicidin solution. The amide I (1600 to 1700 cm^{-1}) and amide III regions (1200 to 1400 cm^{-1}) each contain a number of broad features. There was still a remnant of the carbonyl of the ester group of *DMPC* at 1733 cm^{-1} , although the water background of *DMPC*-indolicidin spectra was well matched with the *DMPC*. I believe that the residual carbonyl signal is not the result of an artifact or wrong subtraction but arises from a conformational change in the lipid head group as a result of its interaction with indolicidin.

As expected of such a *Trp*-rich peptide, the spectra present well-defined peak positions of ω_1 , ω_2 , ω_3 at 1622, 1579, 1554 cm^{-1} , respectively. The amide I region was fitted with two peaks at 1689 and 1666 cm^{-1} , though one could also fit a single broad peak representing a range of different conformations. The higher frequency part of the Amide I peak is consistent with a β -turn conformation stabilised by the intramolecular hydrogen bond (Etori, *et al*, 1997; Iconomidou, *et al*, 2000; Krimm & Bandekar, 1980, 1986; Maxfield, *et al*, 1981), while the lower frequency region has been ascribed to irregular conformations, i.e. turns and random coils (Etori, *et al*, 1997; Picquart, *et al*, 2000).

All of the amide III modes of the β -turn types I-III have been predicted in the region of 1290-1330 cm^{-1} , but these β -turns are probably not distinguishable from one another in Raman spectrum, (Krimm & Bandekar, 1980), nor can we distinguish them from changes in the strong lipid peaks that also fall in this region. However the amide III bands from β -turns fall at a significantly higher frequency than those associated with β -sheet and α -helix structures, (the former typically ranged in 1245-1225 cm^{-1} and the latter in 1320-1280 cm^{-1} in the literature (Fu *et al*, 1994; Singh *et al*, 1990)). The intensity of the Amide III band in the low frequency region (< 1250 cm^{-1}) of indolicidin

is relatively weaker than in *gD* (Figure 9.23), relative to the tryptophan $\omega 1$ and $\omega 2$ modes.

The 2nd derivative curve of the lipid spectrum contains two peaks between 1320 and 1400 cm^{-1} . The subtracted indolicidin spectrum also has two peaks, but at different frequencies. It is difficult to know how much of the peaks in the subtracted spectrum are due to indolicidin and how much to lipid, but my best estimates are that the indolicidin peaks are at 1361 and 1337 cm^{-1} .

The peak at 1361 cm^{-1} is assignable to the $\omega 7$ mode of *Trp* ring vibration. It is expected because indolicidin has 5 *Trp* residues - the highest fraction of *Trp* (39 %) among antimicrobial peptides. Although other possibility of the *PP II* helix (Mikhonin, *et al*, 2004) has also been reported at this position, there is no correlated peak in the amide I mode nor the 1321 cm^{-1} mode which is mainly contributable to *PP II* helix (Blanch, *et al*, 2000; Syme, *et al*, 2002). Therefore we do accept most probable candidate of the peak as the *Trp* $\omega 7$ mode.

The peak around 1308 cm^{-1} is arguable due to the distortion by subtraction of influencing peak of CH_2 twisting mode. It most likely reflects the sensitivity of this mode to the structure of the bilayer, rather than a peptide peak, although a mode from the *PP II* helix has been reported here (Mikhonin, *et al*, 2004).

The peak at 1338 cm^{-1} could be either β -turn or *PP II* helix (Brandekar & Krimm, 1979; Mikhonin, *et al*, 2004). The former is more likely supported by other peak positions representing it (1687 cm^{-1} in amide I mode and 1269 cm^{-1} in amide III mode (Benaki, *et al*, 1998; Iconomidou, *et al*, 2000)) and with no evidence of *PP II* helix at other positions.

The region of between 1200 and 1300 cm^{-1} is generally catagorised into 1240-1260 cm^{-1} for disordered conformation (Pande, *et al*, 1986) and 1235-1245 cm^{-1} for β -pleated sheet (Etori, *et al*, 1997; Spiro & Garber, 1977). This region was difficult to

curve fit since it contains a relatively broad and featureless peak in the Raman spectrum. The main maximum at 1269 cm^{-1} can be assigned for either to β -turn or random structure (Benaki, *et al*, 1998; Chi, *et al*, 1998; Iconomidou, *et al*, 2000). The former is consistent with the Amide I band at 1689 cm^{-1} and the latter with the Amide I band at 1666 cm^{-1} . The breadth of the Amide III band makes it likely that more than one conformation is contributing to the spectrum. (Jin, *et al*, 2004; Tu, 1982).

The assignments are summarised in Table 9.3.

	S-polarised spectra
Carbonyl of ester in <i>DMPC</i> head group	1730 cm^{-1}
Amide I (β -turn)	1689 cm^{-1}
Amide I (random coil)	1666 cm^{-1}
<i>Trp</i> $\omega 1$	1629 cm^{-1}
<i>Trp</i> $\omega 2$	1580 cm^{-1}
<i>Trp</i> $\omega 3$	1553 cm^{-1}
CH ₂ bending (scissoring mode)	1450 cm^{-1}
<i>Trp</i> $\omega 7$	1362 cm^{-1}
Amide III (β -turn?)	1337 cm^{-1}
CH ₂ bending (twisting mode)	1307 cm^{-1}
Amide III (β -turn, random coil)	1269 cm^{-1}

Table 9.3 Assignments of subtracted spectra of *indolicidin* interacting with *DMPC* *pslb* at early stage based on the curve-fits in Figure 9.31.

Our assignments favor β -turns and random coil as the prevalent secondary structures.

Figure 9.32 and 9.34 show the deconvoluted spectra of indolicidin treated in the same way as the subtracted spectra in Figure 9.31. The former was measured in the late stage of reaction ($t = 128$, 22 *min* before solution rinsed-out) before the indolicidin solution was replaced with pure water, and the latter ($t = 210$) 1 *h* after replacement. For $t = 128$ *min* a filter width of 18 was used. For $t = 210$ *min* the spectra are much weaker and hence have poorer signal to noise. A larger filter width of 27 was used for this spectrum.

Compared with the curve-fitted spectra at the initial contact ($t = 0$) in Figure 9.31, most of constituent peaks for the spectra of the late stage of indolicidin interaction ($t = 128$) in Figure 9.32 remain unchanged except several points: firstly an increased intensity of *Trp* features, secondly the absence of carbonyl peak in ester group of lipid head (2^{nd} derivative didn't show the minima for it), and finally the more intense characteristic of random coil structure (which is indicative of both the curve-fit at 1667 cm^{-1} in the amide I mode and at 1255 cm^{-1} in the amide III mode). Figure 9.34 shows clearly reduced spectral intensities after replacement of the indolicidin solution with water.

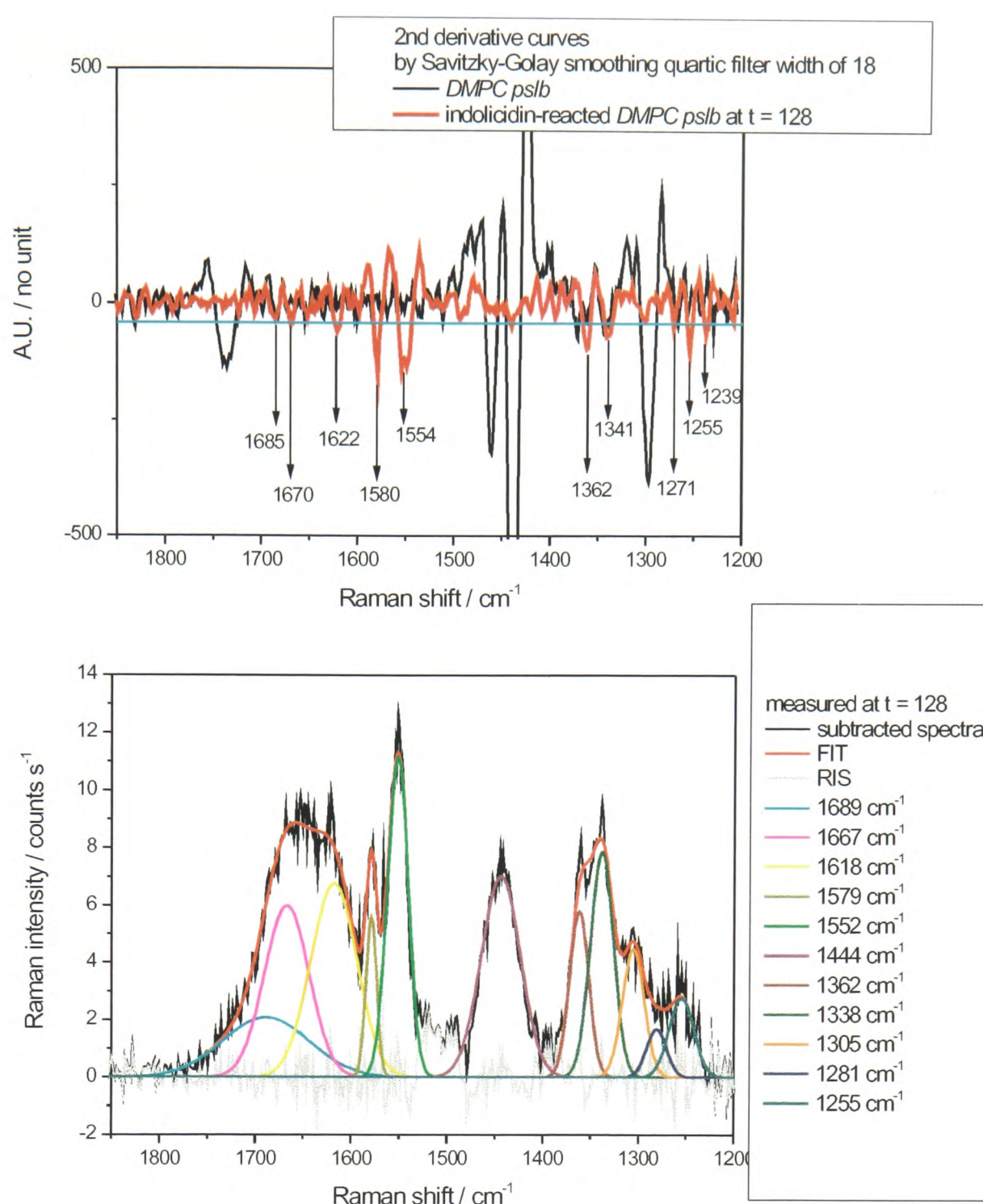


Figure 9.32 Tentative peak assignment of the subtracted Raman spectra of 12 μM ($\sim 22.84 \mu\text{g/mL}$) indolicidin with possible peak positioning based on the 2nd derivative (followed by Savitzky-Golay smoothing filtering quartic and filter width of 18): $\langle ((\text{DMPC} + \text{indolicidin}) - \text{water background}) - (\text{DMPC} - \text{water background}) \rangle$. The spectra was measured at $t = 128$ after solution added onto *DMPC pslb* (just before rinsing by UHQ water). Rinsing by UHQ water was done 2 h 20 min after solution loaded., 532 nm, 1.2W, *s-pol* in excitation, *un-pol* in detection, 200 μm slit width, 11 pixels, 30.0 °C, Zeiss 40 $\times$, 5 $\times$ 120 s of acquisition.

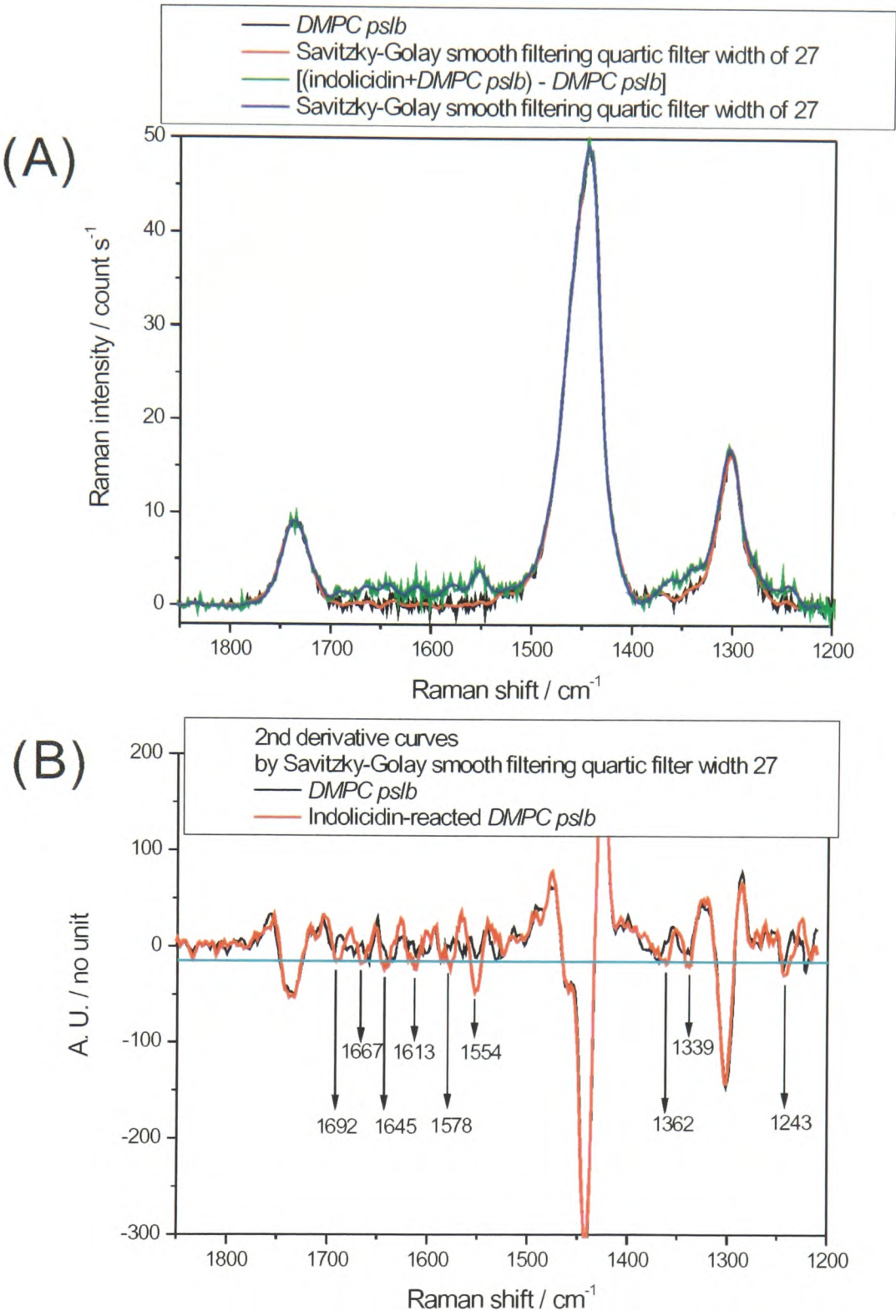


Figure 9.33 (A) Smooth filtering of Savitzky-Golay algorithm by quartic order and filter width of 27 for *DMPC pslb* and (indolicidin + *DMPC pslb*) spectra and (B) their 2nd derivative curves.

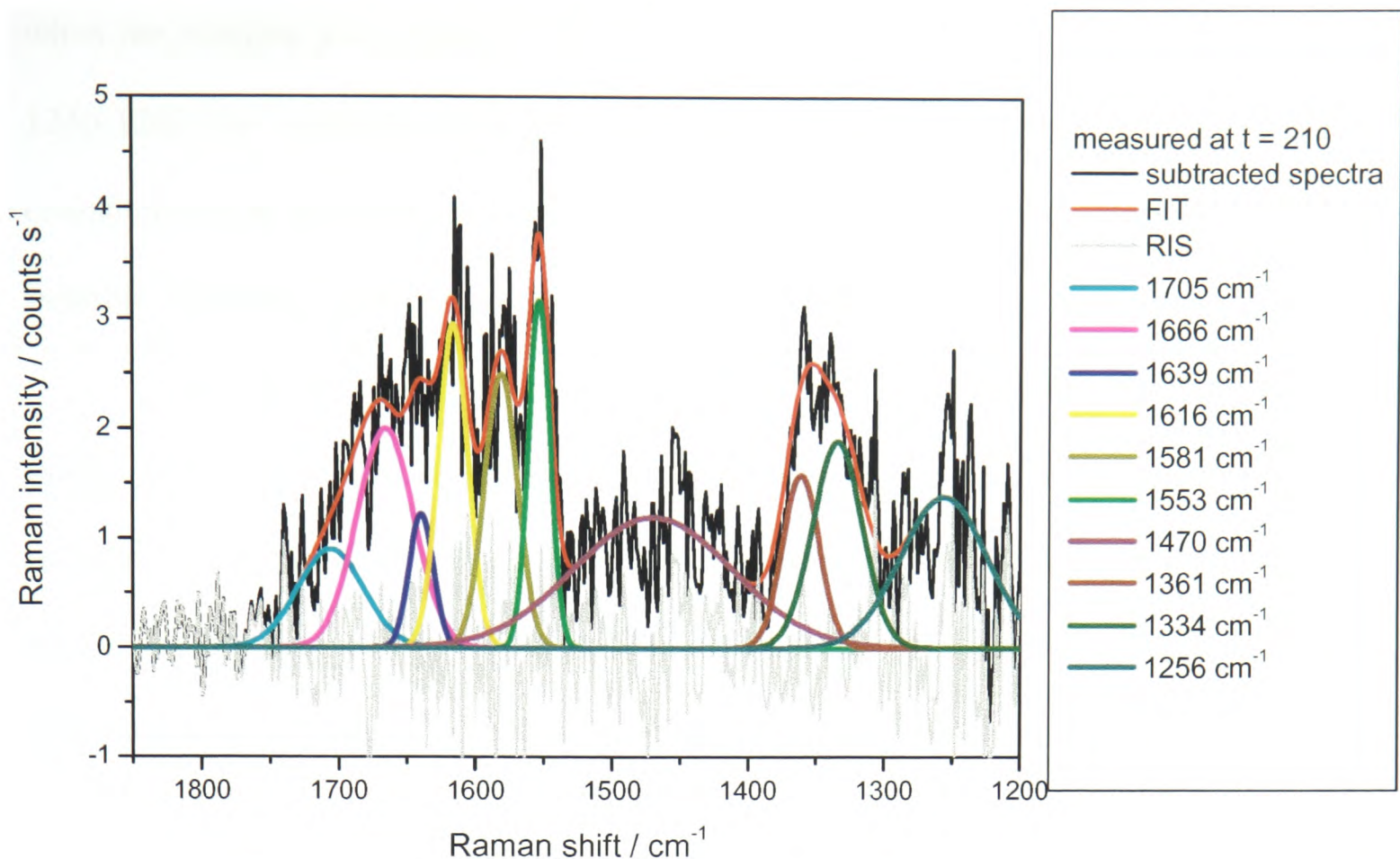


Figure 9.34 Tentative peak assignment of the subtracted Raman spectra of 12 μM ($\sim 22.84 \mu\text{g/mL}$) indolicidin: $\langle((\text{DMPC} + \text{indolicidin}) - \text{water background}) - (\text{DMPC} - \text{water background})\rangle$. The spectra was measured at $t = 210$ after the solution was added onto the *DMPC pslb* (70 min after rinsing by UHQ water), rinsing by UHQ water was done 2 h 20 min after solution loaded, 532 nm, 1.2 W, *s-pol* in excitation, *un-pol* in detection, 200 μm slit width, 11 pixels, 30.0 $^{\circ}\text{C}$, Zeiss 40 $\times$, 5 $\times$ 120 s acquisition.

The spectra of Figure 9.33 and 9.34 are more interesting because they present characteristics of remaining indolicidin after physical rinsing and they are more vigorously interacting with the lipid bilayer. In the resolved spectra of the washed-out indolicidin-*DMPC pslb* (Fig 9.34), a new constituent peak was fitted at 1639 cm^{-1} (minima of 1645 cm^{-1} in Fig 9.33), which might be assignable to random coil conformation. And there was no peak representative of carbonyl peak in the curve-fits. The amide III mode was hard to fit due to the poor resolution, but vigorously positioned based on the 2nd derivative curve (although 1256 cm^{-1} is higher shifted than we thought

when the minima was pointed at 1243 cm^{-1} , roughly the region of the band between $1240\text{--}1260\text{ cm}^{-1}$ typically belong to the characteristics of random coil.) The β -turn is characterized as the peak at 1705 cm^{-1} in the amide I mode and correlated with the possible additional peak between 1300 and 1250 cm^{-1} .

The CH_2 twisting mode at the spectra of $t = 210$ (after solution rinsed-out) had nearly vanished in the subtracted spectra, whereas it remained comparably strong relation to the amide I and III modes at the spectra of $t = 0$ and $t = 128$ with the indolicidin solution before it was rinsed-out. Also the CH_2 scissoring mode decreased significantly after rinsing, compared with the residues in Figure 9.31 and 9.32.

The residual indolicidin after rinsing-out can be considered as mentioned previously. The spectra measured just after solution was rinsed (the spectra of $t = 157$ in the C-H stretching region of Figure 9.26 (B) and the spectra of $t = 158$ in the fingerprint region of Figure 9.27) show nearly half intensity of the initial one which was acquired on loading of indolicidin onto *DMPC pslb* (the “10m” in Figure 9.26 (B) and the “0m” in Figure 9.27). And the spectra in the late stage 1 h after rinsing (the spectra of $t = 198$ in Figure 9.26 (B) and the spectra of $t = 199$ in the figure 9.27) decreased to approximately half the intensity of the previous spectra ($t = 157$, $t = 158$ in Figure 9.26 (B) and 9.27 respectively) and then the intensity became stable. It implies that although nearly half amount of indolicidin was removed by washing-out of aggregates and redissolution of indolicidin transiently inserted into the bilayer, the rest was still interacting with lipid molecules inside the bilayer, and then equilibrated after half more were smeared out of *DMPC* bilayer. It is conjectured that relatively strong interaction with an inner region of bilayer holds indolicidin attached to lipid molecule during a severe physical rinse. (We can’t specify whether it is hydrophobic (in case of barrel-stave model (Brogden, 2005)) or hydrophilic region (in case of toroidal-pore model (Brogden, 2005)).

9.3 Conclusion

9.3.1 Gramicidin D in DMPC *pslb*

In our Raman spectra, the peaks at 1670 and 1240 cm^{-1} for amide I and III mode supported an antiparallel β -sheet structure. But it was not enough to confirm the single-stranded $\beta^{6.3}$ π -helical dimer, because the double-stranded intertwined structure also has characteristics of β -helices.

For further investigation of our Raman data to relate with a secondary structure, we used the correlation of Raman band of *Trp* ω 3 with torsional angles of 4 *Trps* in 3-*D* coordinates studied by high resolution techniques of x-ray and NMR spectroscopy. First this correlation of our Raman data was applied to discriminate between channel-active and channel-inactive forms using 3-*D* coordinates of *gD* in multistacked phospholipid layers (by solid-state NMR) and in crystal made from methanol solution (by x-ray crystallography), respectively. The structure in organic solution was not fittable for torsional angle calculation because some angles in the 3-*D* coordinates deviated out of the angle range adaptable to the relationship between torsional angle of *Trp* and the wavenumber of *Trp* ω 3 mode. It is possible to say that a model in organic solution can't be explained by this relationship and it is not an appropriate model for our *gD* in DMPC *pslb*. But in the single-stranded $\beta^{6.3}$ π -helix dimer model by solid-state NMR the averaged value of *Trp* ω 3 was perfectly matched with our Raman spectra. So we could say it is a strongly possible model for our *gD* in DMPC *pslb*.

An other possible model of right-handed double-stranded $\beta^{7.2}$ helices in lipid bilayers which was controversially suggested by Buckhart could be considered as well. The angle calculation of their model showing the averaged value of 1546 (Cs^+ binding model) and 1548 cm^{-1} (H^+ binding model) approximated our 1547.3 cm^{-1} . So it seemed to be impossible to discriminate between a single-stranded $\beta^{6.3}$ π -helix dimer and right-

handed double-stranded $\beta^{7.2}$ helices in *DMPC* single lipid bilayers. But we had strong support of *CD* spectra of *gD* in *DMPC SUV* presenting that majority of *gD* forms single-stranded $\beta^{6.3}$ π -helical dimer structure and it was used for our *gD* in *DMPC pslb* by Raman measurement. Therefore we could exclude a possibility of right-handed double-stranded $\beta^{7.2}$ helices in lipid bilayer.

Finally we conclude that our Raman spectra with *CD* data revealed a single-stranded $\beta^{6.3}$ π -helix dimer structure for *gD* in *DMPC pslb*.

9.3.2 Indolicidin-associating *DMPC pslb*

It has been reported that β -turn conformations when bound to membranes or membrane-like environments such as *SDS* micelles are the principal structural motif of indolicidin by the *CD* data (Ladokhin, *et al*, 1997), whereas the typical structure in the absence of membrane environment in the aqueous solution is characterised as an unordered structure (Falla *et al*, 1996; Ladokhin, *et al*, 1997).

Although the β -turn conformations are generally difficult to identify by *CD* spectroscopy alone (Hollósi, *et al*, 1994), a certain form either of β -turns such as β -turn type VIa (which has been proposed model based on the *CD* spectra by Ladokhin (Ladokhin *et al*, 1999) or poly-L-proline type II helix (*PP II* helix) (Falla, *et al*, 1996) in membrane-bound state have been proposed.

Our Raman spectra revealed clear constituent peaks indicative of β -turn as well as unordered random coil through the entire period of indolicidin interaction with *DMPC pslb* in fluidic phase. However we didn't have any clue showing *PP II* helix in our Raman spectra.

Chapter 10 Formation of supported lipid bilayers from micellar solutions of *n*-dodecyl- β -D-maltoside and DPPC

This chapter describes the use of *TIR*-Raman spectroscopy to study the mechanism of formation of the solid-supported lipid bilayer from micellar solutions of a lipid and surfactant. The formation of single lipid bilayer at water/silica interface by the co-adsorption of lipid and surfactant was introduced by *F. Tiberg* (Tiberg, *et al*, 2000). Formation of a mixed micelle of the insoluble lipid with the soluble surfactant provides a means of solubilising the lipid and delivering the lipid to the surface. The nonionic sugar-based surfactant, *n*-dodecyl- β -D-maltoside (*DDM*, Figure 5.2) does not itself adsorb significantly to silica but is a vehicle that can deliver lipid to the surface (Tiberg, *et al*, 2000). The initially formed bilayer contains both lipid and surfactant. A more densely packed surfactant layer is generated by rinsing out the surfactant and then adsorbing more lipid from a mixed lipid/*DDM* solution of progressively lower concentration (Brinck, *et al*, 1998; Tiberg, *et al*, 1994, 2000). Enrichment of the insoluble lipid leads to the formation of lipid bilayer (Tiberg, *et al*, 1994, 2000). This technique has been a useful alternative approach for forming lipid bilayers on silica. It has the advantages of rapid build-up of supported lipid bilayers (over Langmuir-Blodgett method), maintenance of an aqueous film over the lipid bilayer (over spin coating), simple preparation of a mixed micellar solution of lipid and surfactant (over complex procedures for forming liposomes in solution).

The physical characterisation of each stage at all adsorption and rinsing steps was carried out by ellipsometry (Tiberg, *et al*, 2000) and neutron reflectivity (*NR*) experiments (Vacklin, *et al*, 2005a). Ellipsometric measurements of lipid bilayers on oxidized silicon, formed from a micellar solution of L - α -dioleoyllecithin - *DDM*, yielded

a thickness of $43 \pm 3 \text{ \AA}$, refractive index of 1.480 ± 0.004 and mean area per molecule of $62 \pm 3 \text{ \AA}^2$. The thickness was in good agreement with the bilayer thickness of $44 \pm 3 \text{ \AA}$ in the bulk lamellar phase. Tiberg found that the bilayer structure appeared very early in the adsorption process. The re-adsorption/rinsing steps at successively lower concentration of lipid increased the density of the bilayer. Note that ellipsometry cannot distinguish between lipid and *DDM*. The neutron reflection data were fitted to reveal the thickness, volume fraction, area per molecule and surface excess of dioleoylphosphatidylcholine (*DOPC*)/*DDM* bilayer at each stage of adsorption and rinsing. The calculated parameters showed that the thickness and the area per lipid molecule at the final stage agreed well with data from bulk lamellar phases. The amount of *DDM* in the bilayer and the *DOPC* surface coverage were very sensitive to the volume-to-surface ratio of bulk solution used in adsorption and rinsing, possibly due to a combination of depletion effects and differing mass transport in different adsorption cells. With mixed micellar solution of chain-deuterated d_{62} -*DPPC* and hydrogenated-*DDM* (it will be noted as *h-DDM* from now, which can provide contrast to deuterated sample), *NR* can distinguish the composition of volume fraction between lipid and surfactant at each stage of a sequential dilution process by the fitted parameters from the reflectivity profiles (Vacklin, *et al*, 2005b).

Raman spectroscopy can complement the ellipsometry and *NR* studies by revealing the conformational changes in the lipid and surfactant molecules during each successive adsorption and rinsing step (Tiberg, *et al*, 2000; Vacklin, *et al*, 2005a, b). With deuteration, Raman scattering can also distinguish between the two surfactants although *DDM* unfortunately lacks strong peaks distinct from the lipid that could be used for quantification of mixture of hydrogenated materials.

Here we employed *TIR*-Raman spectroscopy to investigate time-dependent conformational changes of both *DPPC* and *DDM* during bilayer formation at

water/silica interface. First, the Raman spectra of hydrogenated *DPPC* (*h-DPPC*) with *h-DDM* are described briefly, followed by experiments with deuterated *DPPC* (*d₇₅-DPPC*).

10.1 Co-adsorption of *h-DPPC* and *h-DDM*

Figure 10.1 presents a time-series of Raman spectra during the co-adsorption of *h-DPPC/h-DDM* micellar solution from the initial exposure of the silica to the solution until the first rinsing with *UHQ* distilled water. The intensity increases linearly with time, reaching a plateau after 150 *min*. This observation contrasts with ellipsometric data on co-adsorption of $L\text{-}\alpha$ -dioleoyllecithin and *h-DDM*, where the surface excess of adsorbed lecithin/*h-DDM* reached a plateau after only 250 *s*. The Raman spectrum reached two-thirds of its final intensity within 5 *min* incubation, suggesting that the bilayer forms at early times. The limiting spectrum after the first adsorption cycle is less intense than for a fully formed *h-DPPC* bilayer at the same temperature (Fig 10.1). Two facts indicate that lipid chains are characteristic of greater conformation disorder than in the *pslb*: the ordering parameter, $I(d^-)/I(d^+)$ is relatively lower than the reference *DPPC* bilayer at the early stage (5 *min*) higher, and peak positions of methylene vibration mode is higher. Figure 10.1 (C) presents the increase of C-H intensity in choline group, which implies more lipids adsorbed onto substrate.

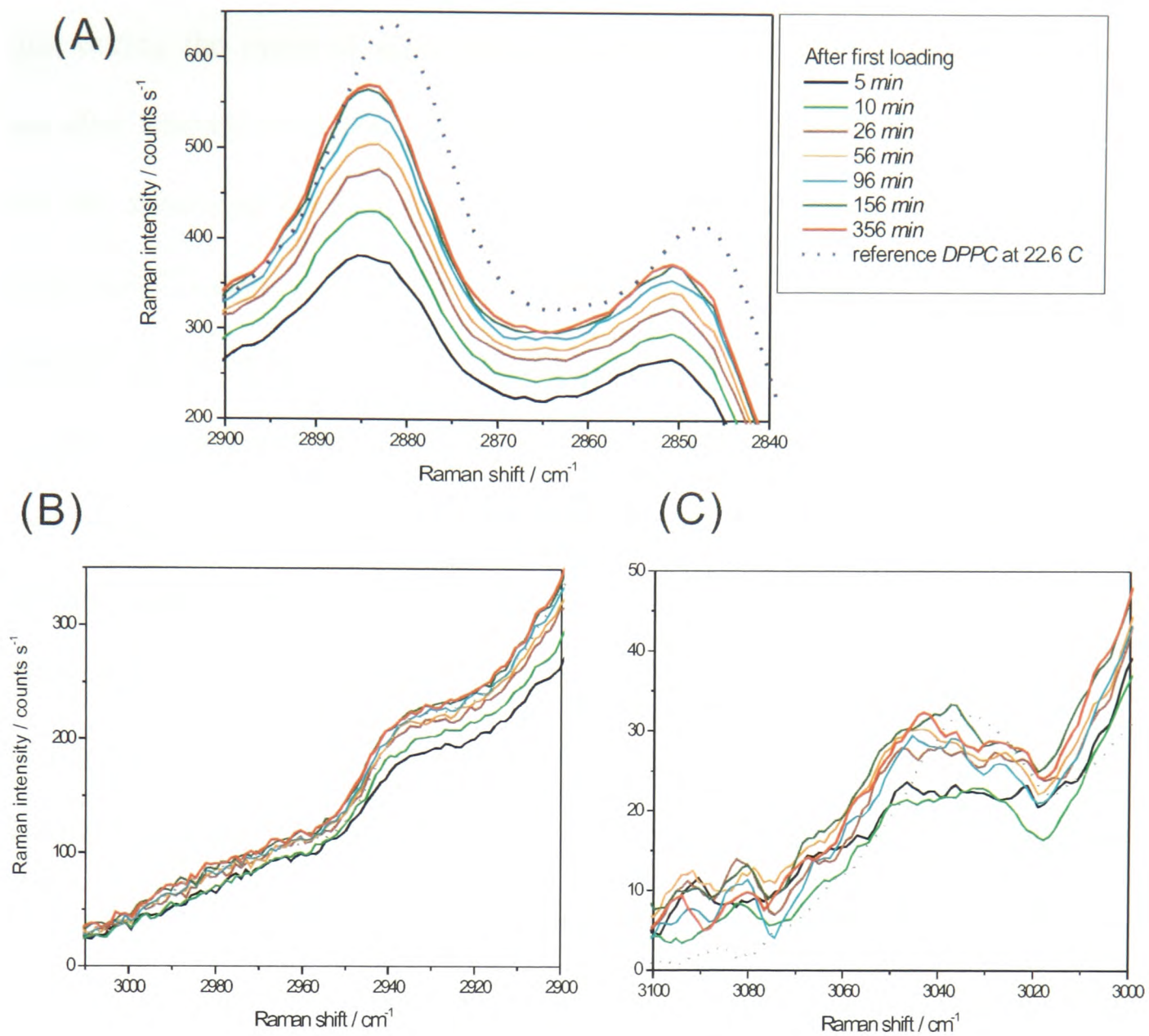


Figure 10.1 Time-dependent *TIR*-Raman spectra of the formation of a bilayer on silica from a micellar solution of *h*-DPPC and *h*-DDM. 532 nm, 1.2 W, *s-pol* excitation, unpolarised detection, 200 μm slit width, 11 pixel width on CCD, at 21.5 °C, Zeiss 40 $\times$ water-immersion objective, 1 $\times$ 45 s. Spectra are labeled according to the number of minutes of incubation of the silica window with the micellar solution (1) of 0.129 mg/mL (*h*-DPPC : *h*-DDM = 1:6, w/w). A reference spectrum of a DPPC bilayer formed from fusion of *SUV*s is shown ($T = 22.6$ °C) A. methylene vibration region, B. methyl vibration region, C. choline vibration region.

Figure 10.2 shows the time-evolution of the Raman spectra in C–H stretching region during the cycle of adsorption and rinsing. The 45-s Raman acquisition was made after interval of at least 30 *min* at each rinsing or readsorption step in order to allow the adsorbing lipid to relax to the thermodynamic equilibrium state. The first rinsing (sub 21) decreases the Raman intensity, due to the removal of loosely adsorbed surfactant on top of the bilayer and/or dissolution of *h*-DDM from the mixed bilayer – we cannot distinguish these two possibilities at this stage. After re-exposure to the mixed solution diluted to 1/10 of its initial concentration (0.0129 *mM*) the intensity increased again (sub 23). This result makes sense in terms of the adsorption of additional lipid molecules. However, after the second rinsing (sub 26) the Raman intensity increased further; subsequent steps had little effect on the spectra. The ellipsometric results always showed an increase in thickness upon exposure to the lipid/surfactant solution and then a decrease on rinsing (Tiberg *et al*, 2000). The Raman spectra do not show any significant peak shifts upon rinsing and the methylene peak frequencies are still blue-shifted from those of pure *DPPC*.

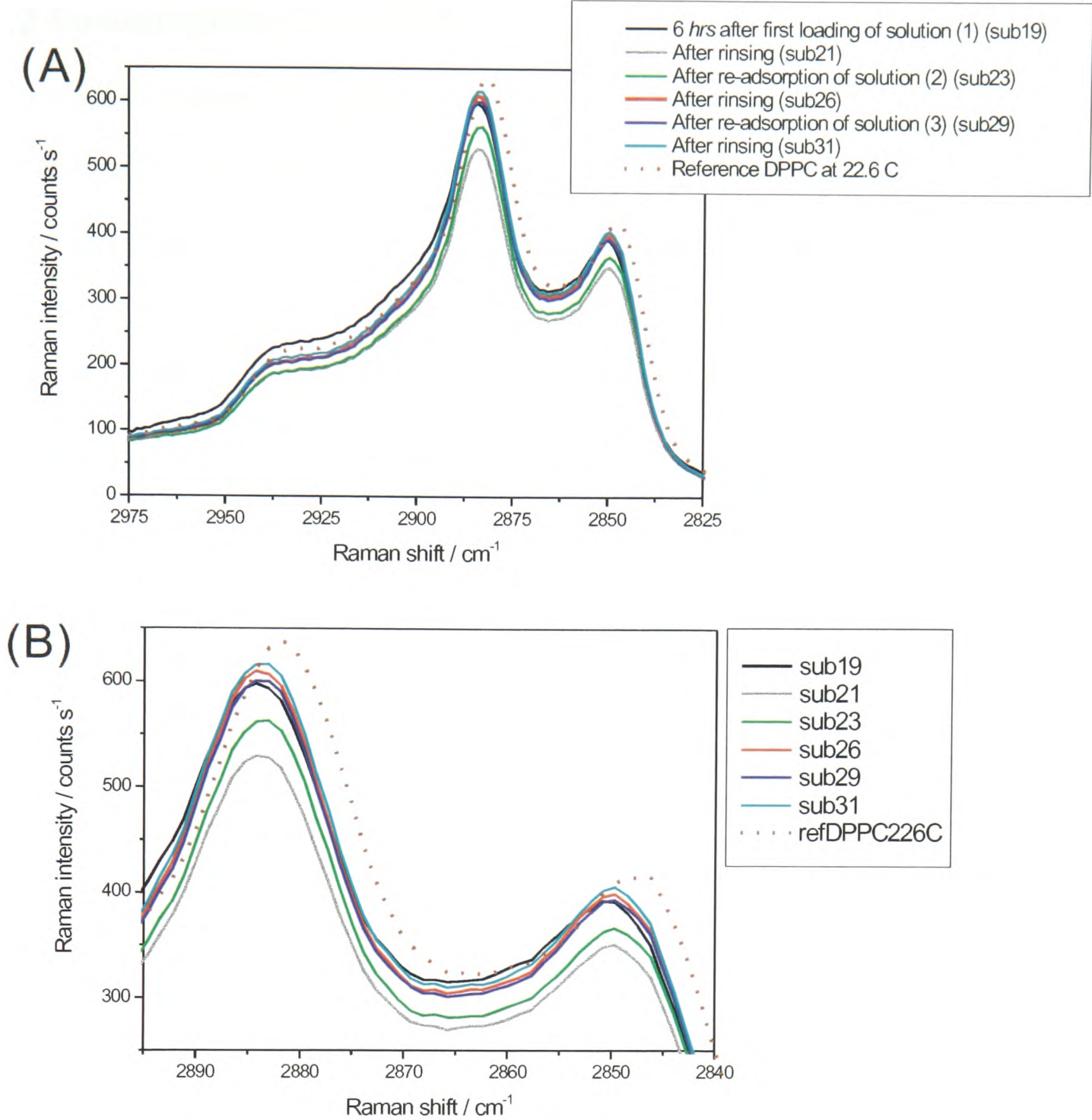


Figure 10.2 Time-dependent *TIR*-Raman spectra of the formation of a bilayer on silica from a micellar solution of *h*-DPPC and *h*-DDM. Conditions as in Fig. 10.1. 6 *h* after initial exposure to micellar solution (1) of 0.1288 *mg/mL* (sub 19, in black), after rinsing (sub 21, in red), after loading solution (2) of 0.01288 *mg/mL* (*h*-DPPC : *h*-DDM = 1:6, w/w) (sub 23, in light green), after rinsing (sub 26, in gray), after loading solution (3) 0.001288 *mg/mL* (*h*-DPPC : *h*-DDM = 1:6, w/w) (sub 29, in light blue), after rinsing (sub 31, in pink), reference DPPC at 22.6 °C (in dotted brown). (The critical micelle concentration of DDM is 0.08527 *mg/mL* (Hines *et al*, 1998).)

9.2 Co-adsorption of d_{75} -DPPC and h -DDM

The experiments with hydrogenated lipid and surfactant do not easily allow us to distinguish the relative amounts of *DDM* and *DPPC* in the adsorbed bilayer. Consequently, we repeated these experiments with the deuterated lipid of d_{75} -DPPC (see Fig 5.2). Note that the glycerol positions are not deuterated, so the lipid will give rise to some peaks in the C–H stretching region. Additionally, the extent of deuteration is not complete, so there will be some small intensity from residual CDH and CD₂H groups in the lipid.

Figures 10.3 and 10.4 show the times series of *s* and *p*-polarised *TIR*-Raman spectra of d_{75} -DPPC and h -DDM during a series of adsorption and rinsing cycles, with decreasing lipid/surfactant concentrations. The peak frequencies and literature assignments are given in Table 10.1.

Observed $\Delta\nu / \text{cm}^{-1}$		Reference $\Delta\nu / \text{cm}^{-1}$	Assignment
<i>s-pol</i>	<i>p-pol</i>		
2280 <i>m</i>	2280 <i>s</i>		CD ₃ <i>as</i> in choline?
2212 <i>w, sh</i>	2217 <i>s</i>	2217 ¹ 2217 ³ 2210 ²	CD ₃ <i>as</i>
2196 <i>m</i>	2198 <i>s</i>	2198 ¹ 2194 ²	CD ₂ <i>as</i>
2183 <i>m</i>	2184 <i>m</i>		
2171 <i>w, sh</i>	2176 <i>w, sh</i>	2178 ¹ 2176 ³	
2104 <i>s</i>	2104 <i>s</i>	2103 ¹ 2101 ² 2099 ³	CD ₂ <i>ss</i>
2129 <i>m, sh</i>	2133 <i>m, sh</i>	2135 ² 2126 ¹ <i>sh</i> 2120 ³ <i>w</i>	CD ₃ <i>ss</i>
2078 <i>m</i>	2074 <i>m</i>	2075 ² 2074 ³ <i>m</i> 2073 ¹	

Table 10.1 Peak assignments of Raman spectra of *d*₇₅-DPPC in the C-D stretching region: strong (*s*), medium (*m*), weak (*w*), shoulder (*sh*), antisymmetric stretch (*as*), symmetric stretch (*ss*). ¹(Bunow & Levin, 1977), crystalline solid, ²(Garber, *et al*, 1978) aqueous dispersion, ³(Sunder, *et al*, 1981), solid

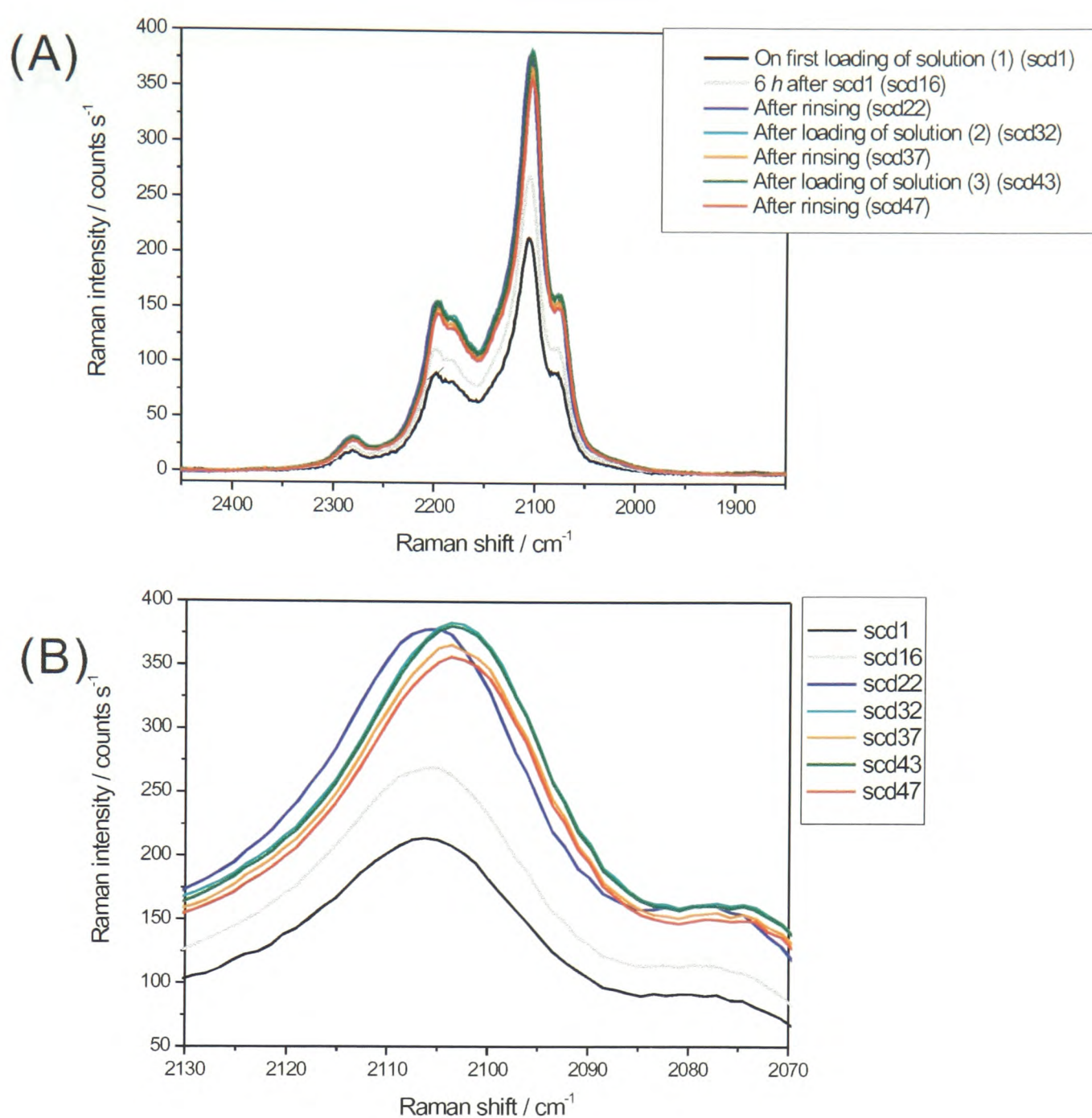


Figure 10.3 Time-dependent *s-pol* TIR-Raman spectra of the formation of a bilayer on silica from a micellar solution of d_{75} -DPPC and *h*-DDM. Other conditions as Fig. 10.1. On first loading of solution (1) of 0.1306 mg/mL (5.07 mg of d_{75} -DPPC and 27.6 mg of *h*-DDM) (scd1, in black), 6 *h* after scd1 (scd 16, in gray), after rinsing (scd 22, in blue), after loading solution (2) of 0.01306 mg/mL (scd 32, in light blue), after rinsing (scd 37, in orange), after loading solution (3) of 0.001306 mg/mL (scd 43, in green), after rinsing (scd 47, in red).

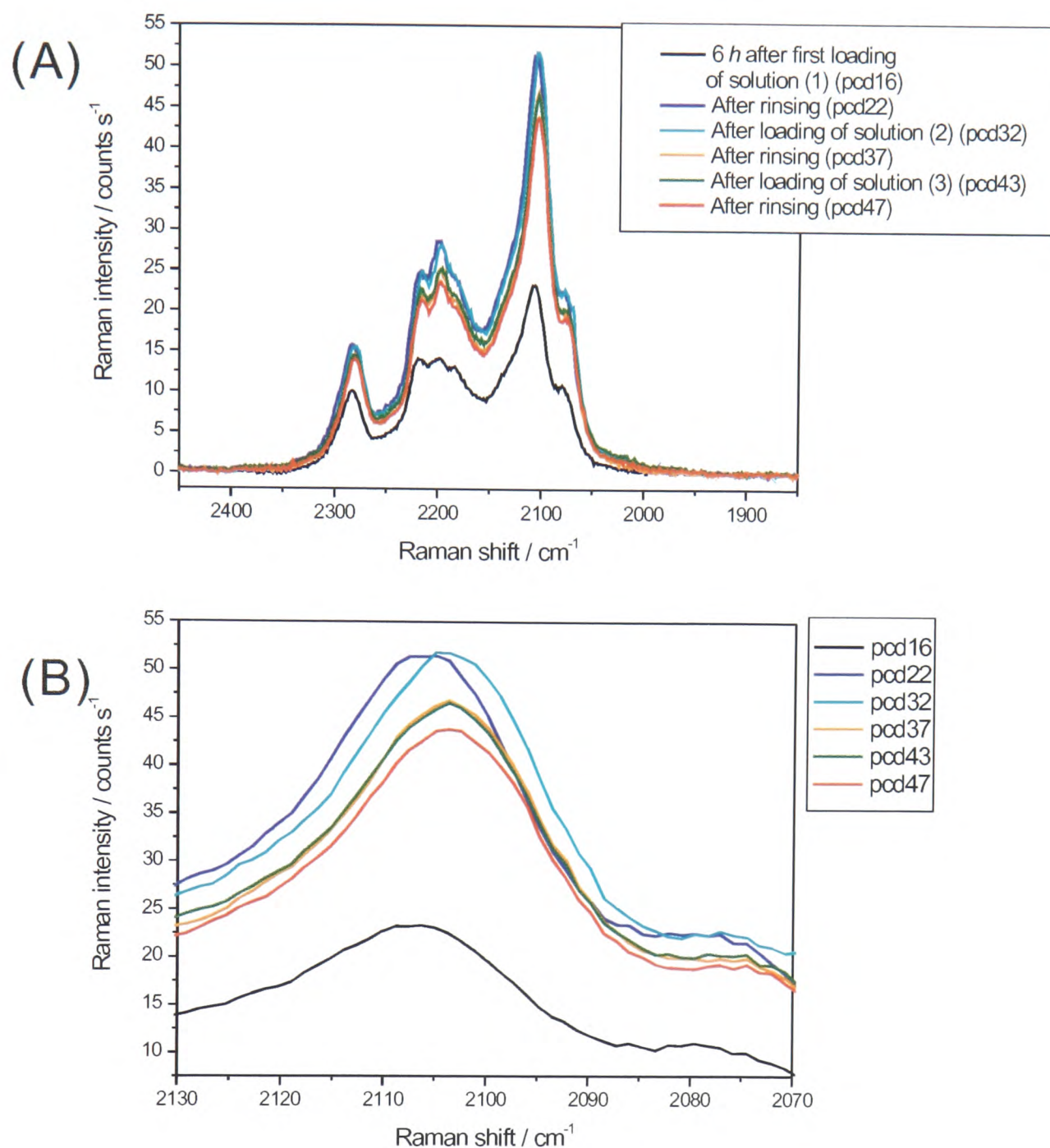


Figure 10.4 Time-dependent *p-pol* TIR-Raman spectra of the formation of a bilayer on silica from a micellar solution of d_{75} -DPPC and h -DDM. Other conditions as Fig. 10.1: 6 h after first loading of solution (1) of 0.1306 mg/mL (5.07 mg of d_{75} -DPPC and 27.6 mg of h -DDM) onto silica (pcd 16, in black), after rinsing (pcd 22, in blue), after loading solution (2) of 0.01306 mg/mL (pcd 32, in light blue), after rinsing (pcd 37, in orange), after loading solution (3) of 0.001306 mg/mL (pcd 43, in green), after rinsing (pcd 47, in red).

The first and striking observation is that the intensities of both the *s* and *p*-polarised spectra increase drastically in the C–D stretching region after rinsing away the first solution (scd 22, pcd 22). Since this increase occurs in both polarizations, it must be associated with an increase in the amount adsorbed. The increase in the CD₂ peaks is greatest in the *p*-polarised spectrum, from which we infer that the average tilt of the chains is decreasing. The most likely explanation is that dilution of the initial solution results in deposition of insoluble lipid onto the surface as mixed micelles breakdown. Concomitantly, loss of *DDM* from the bilayer by dissolution could allow adsorption of further lipid, though this needs to be confirmed by surfactant spectra in the C–H stretching region (see below). Changes in intensity on subsequent cycles of adsorption and rinsing are relatively small, but the ratio of *s* to *p*-polarised intensities increases suggesting a decreasing tilt of the chains. The second adsorption cycle, from a solution diluted ten times, does produce a pronounced red-shift in the frequency of the CD₂ symmetric stretch indicating that the packing density increased in the adsorbed layer.

The final lipid bilayer formed from mixed *h-DDM* / *d*₇₅-*DPPC* adsorption was compared with that formed by vesicle adsorption of pure *d*₇₅-*DPPC* vesicles (Figure 10.5). (The preparation was introduced in Chapter 5.14.) The vesicle solution was incubated onto silica substrate over chain melting temperature for 5 *h*, and then cooled to room temperature. Before the solution was washed out by *UHQ DW*, the first spectra (scd 1) were measured as shown in Figure 10.5. Rinsing out the vesicle solution (scd 2) resulted in a three-fold decrease in intensity, signifying the removal of loosely bound material. As time went on, the spectral intensities continued to decrease and finally approached a constant level (scd 8).

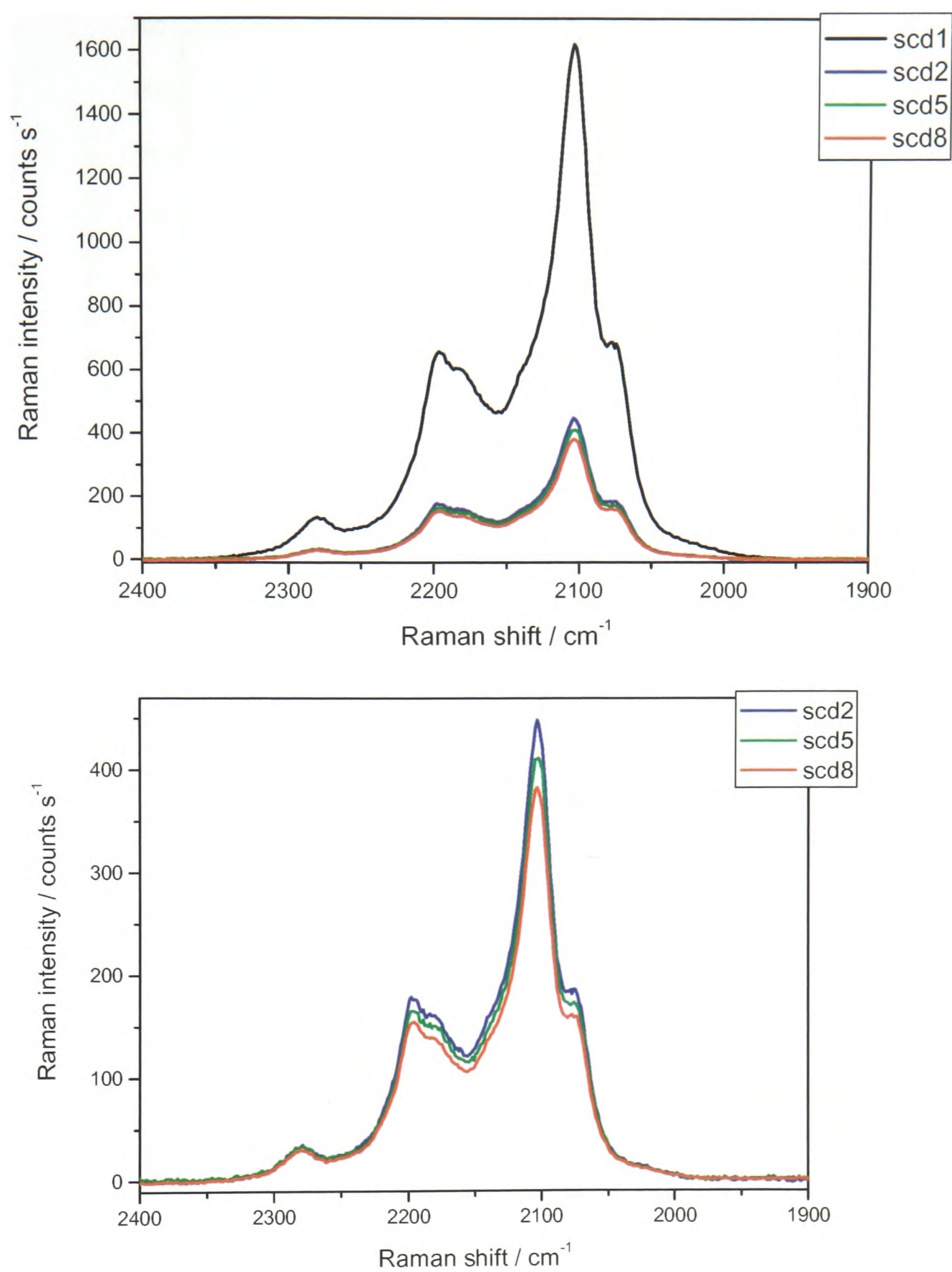


Figure 10.5 Time-dependent *s-pol* TIR-Raman spectra of adsorption of *d*₇₅-DPPC from its vesicle solution. Conditions as Fig. 10.1. Before rinsing with *UHQ* DW (scd 1, in black), after rinsing with *UHQ* DW (scd 2, in blue), 30 *min* after scd 1 (scd 5, in light green), 2 *h* after scd 1 (scd 8, in red).

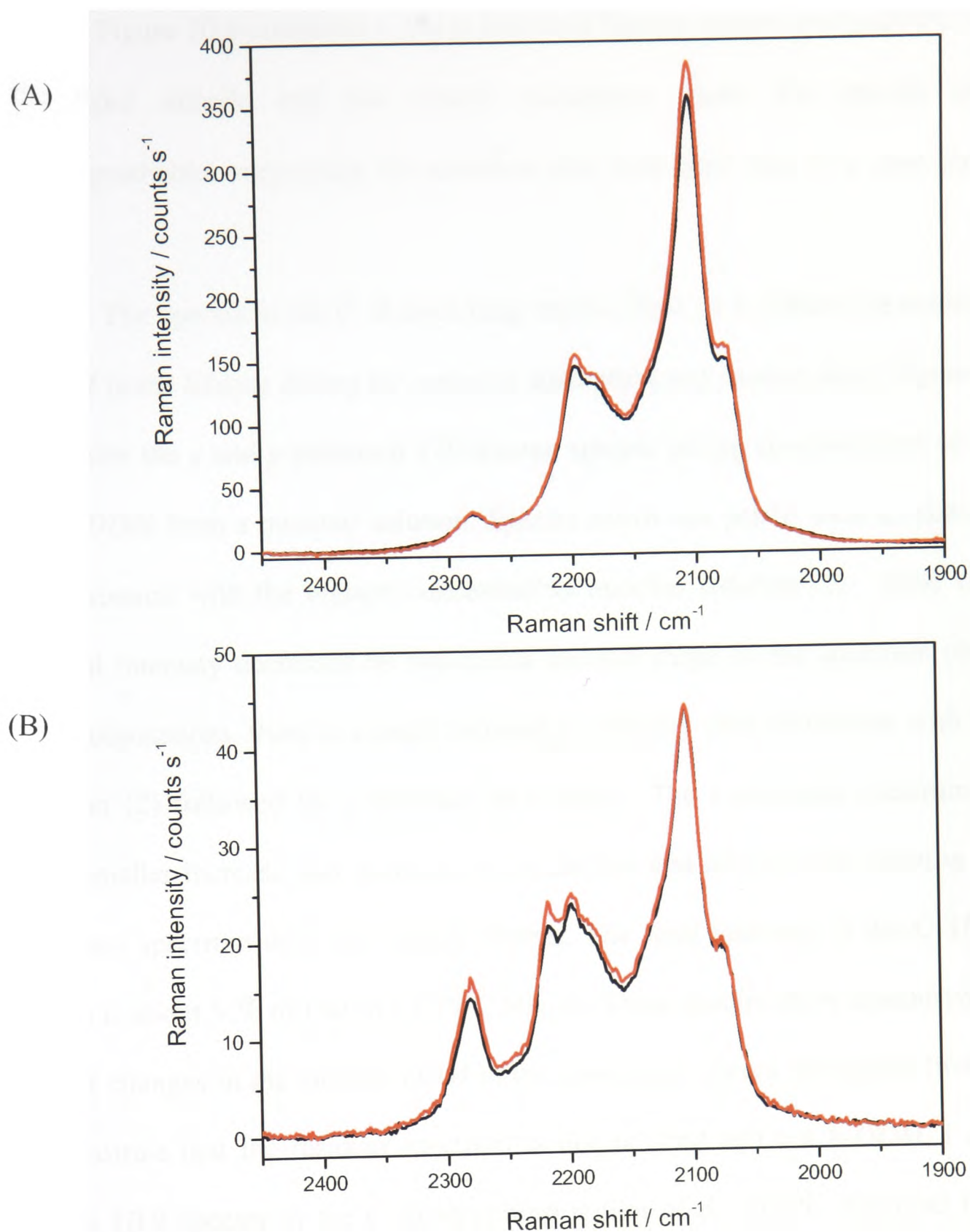


Figure 10.6 Comparison of the polarised Raman spectra of d_{75} -DPPC layer by co-adsorption from a micellar solution of h -DDM and by vesicle adsorption of pure d_{75} -DPPC. Conditions as in Fig. 10.1. (A) s -pol spectra of the final lipid layer formed by micellar solution (scd 47, in black) and by vesicle adsorption (scd 8, in red), (B) p -pol spectra of the final lipid layer formed by micellar solution (pcd 47, in black) and by vesicle adsorption (pcd 8, in red).

Figure 10.6 compares *s* and *p*-polarised Raman spectra of *d*₇₅-DPPC formed by the mixed micelle and the vesicle adsorption route: the spectra are almost indistinguishable, supporting the assertion that both give rise to a well-formed lipid bilayer.

The spectra in the C–H stretching region allow us to follow the evolution of the *h*-DDM in the bilayer during the series of adsorption and rinsing steps. Figures 10.7 and 10.8 show the *s* and *p*-polarised TIR-Raman spectra during co-adsorption of *d*₇₅-DPPC and *h*-DDM from a micellar solution. Spectra sch16 and pch16 were acquired after 6 *h* of incubation with the highest concentration micellar solution (1). After rinsing, the spectral intensity decreases by two-thirds and the shape of the spectrum changes. In both polarisations, there is a small increase in intensity after incubation with the diluted solution (2) followed by a decrease on rinsing. The *s*-polarised spectrum shows an even smaller increase and decrease on incubation and rinsing with solution (3); the *p*-polarised spectrum does not change further. The final intensity in the C–H stretching region is about 5 % of that in a DPPC bilayer. These spectra show conclusively that the largest changes in the amount DDM in the monolayer occurs during the first rinse. To demonstrate that the residual spectrum is due to lipid and not *h*-DDM, I compare in Figure 10.9 spectra in the C–H stretching region of *d*₇₅-DPPC adsorbed from mixed micelles with *h*-DDM and from vesicles. The spectra are identical to within a scale factor, with the fused vesicles being slightly more intense. There is thus no detectable amount of *h*-DDM remaining in the bilayer. (I would estimate that less than 1 % of the bilayer is by C-H bonds of *h*-DDM.)

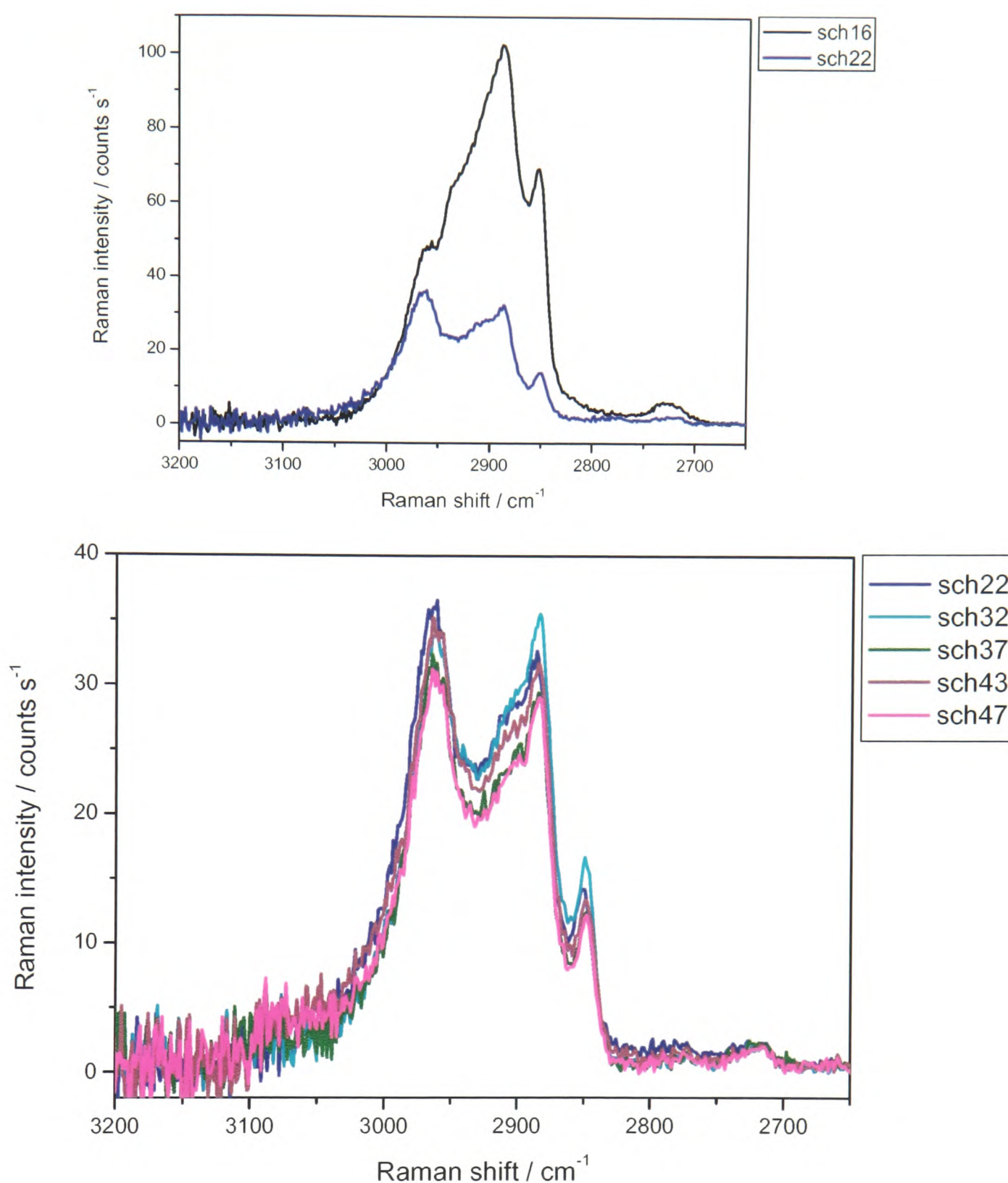


Figure 10.7 Time-dependent *s-pol* TIR-Raman spectra of bilayers adsorbed from mixed micelles of *h*-DDM and *d*₇₅-DPPC: C–H stretching region. Conditions as Fig. 10.1: 6 *h* after first loading of micellar solution (1) of 0.1306 *mg/mL* (5.07 *mg* of *d*₇₅-DPPC and 27.6 *mg* of *h*-DDM) (sch 16, in black), after rinsing (sch 22, in blue), after loading of solution (2) of 0.01306 *mg/mL* (sch 32, in light blue), after rinsing (sch 37, in green), after loading of solution of 0.001306 *mg/mL* (sch 43, in purple), after rinsing (sch 47, in pink).

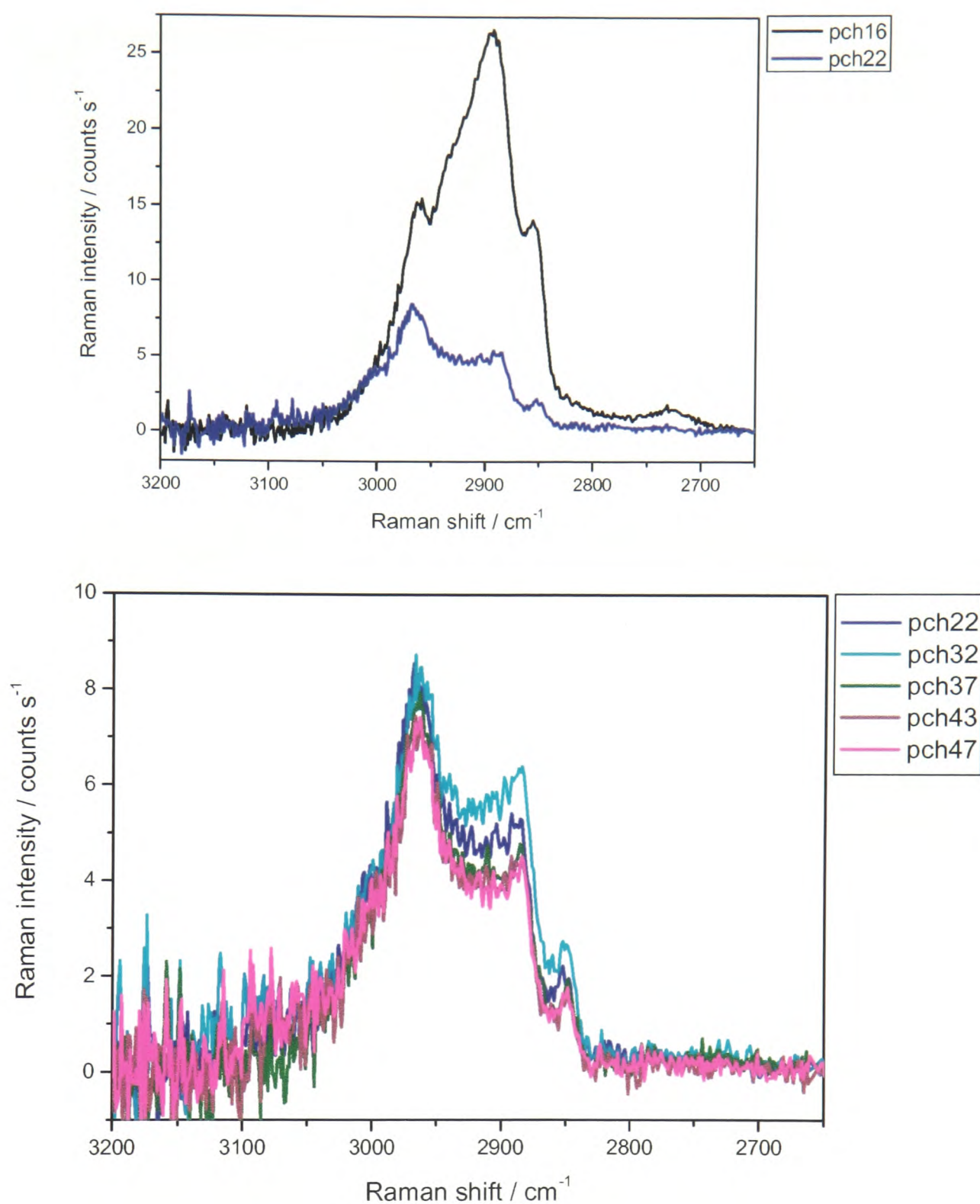


Figure 10.8 Time-dependent *p-pol* TIR-Raman spectra of bilayers adsorbed from mixed micelles of *h*-DDM and *d*₇₅-DPPC: C–H stretching region. Conditions as Fig. 10.1: 6 *h* after first loading of micellar solution (1) of 0.1306 *mg/mL* (5.07 *mg* of *d*₇₅-DPPC and 27.6 *mg* of *h*-DDM) (pch 16, in black), after rinsing (pch 22, in blue), after loading of solution (2) of 0.01306 *mg/mL* (pch 32, in light blue), after rinsing (pch 37, in green), after loading of solution of 0.001306 *mg/mL* (pch43, in purple), after rinsing (pch 47, in pink).

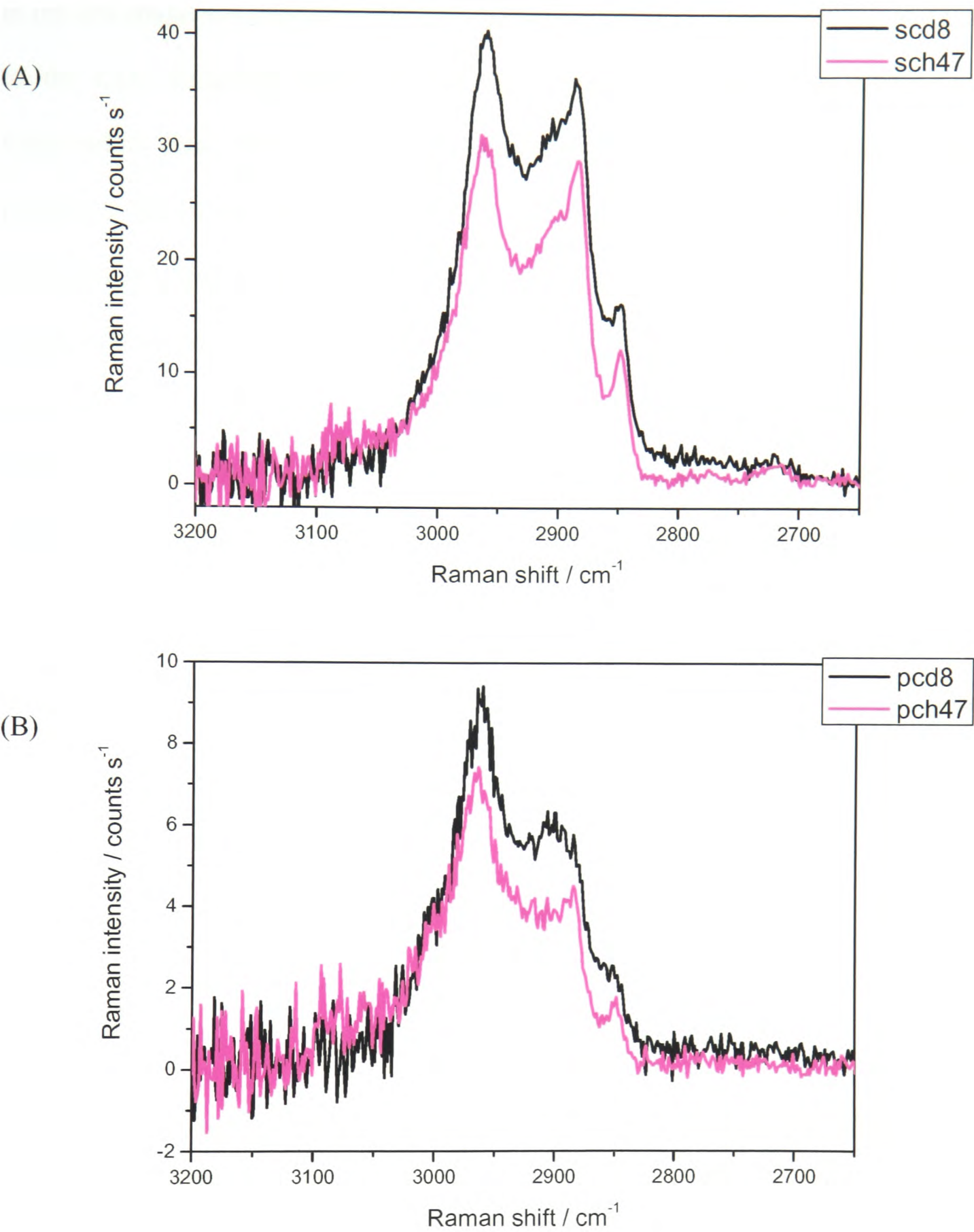


Figure 10.9 Comparison of the polarised Raman spectra of a d_{75} -DPPC *pslb* on silica formed by adsorption from *h*-DDM micellar solution (final stage) and by vesicle adsorption. Micellar solution (sch 47 and pch 47, in pink), vesicle adsorption (scd 8 and pcd 8 in black): (A) *s-pol* spectra, (B) *p-pol* spectra.

Finally, I present polarized spectra of a d_{75} -DPPC *pslb* made by vesicle fusion in the low frequency region ($< 1000\text{ cm}^{-1}$) (Figure 10.10). I draw attention particularly to the CD_2 scissoring mode at 984 cm^{-1} (which is polarised perpendicular the hydrocarbon chain axis) and the CD_2 twisting mode at 920 cm^{-1} (which is polarised parallel to the chain axis). These peaks appear at 1446 cm^{-1} and 1305 cm^{-1} in the hydrogenated lipids. The former is absent in the *p*-polarised spectrum but pronounced in the *s*-polarised. On the contrary the latter is stronger in the *p*-polarised than the *s*-polarised spectrum. These relative intensities demonstrate that the hydrocarbon acyl chains are very nearly vertical in the *pslb*. The experimental peak frequencies together with literature assignments and comparative frequencies are summarized in Table 10.2.

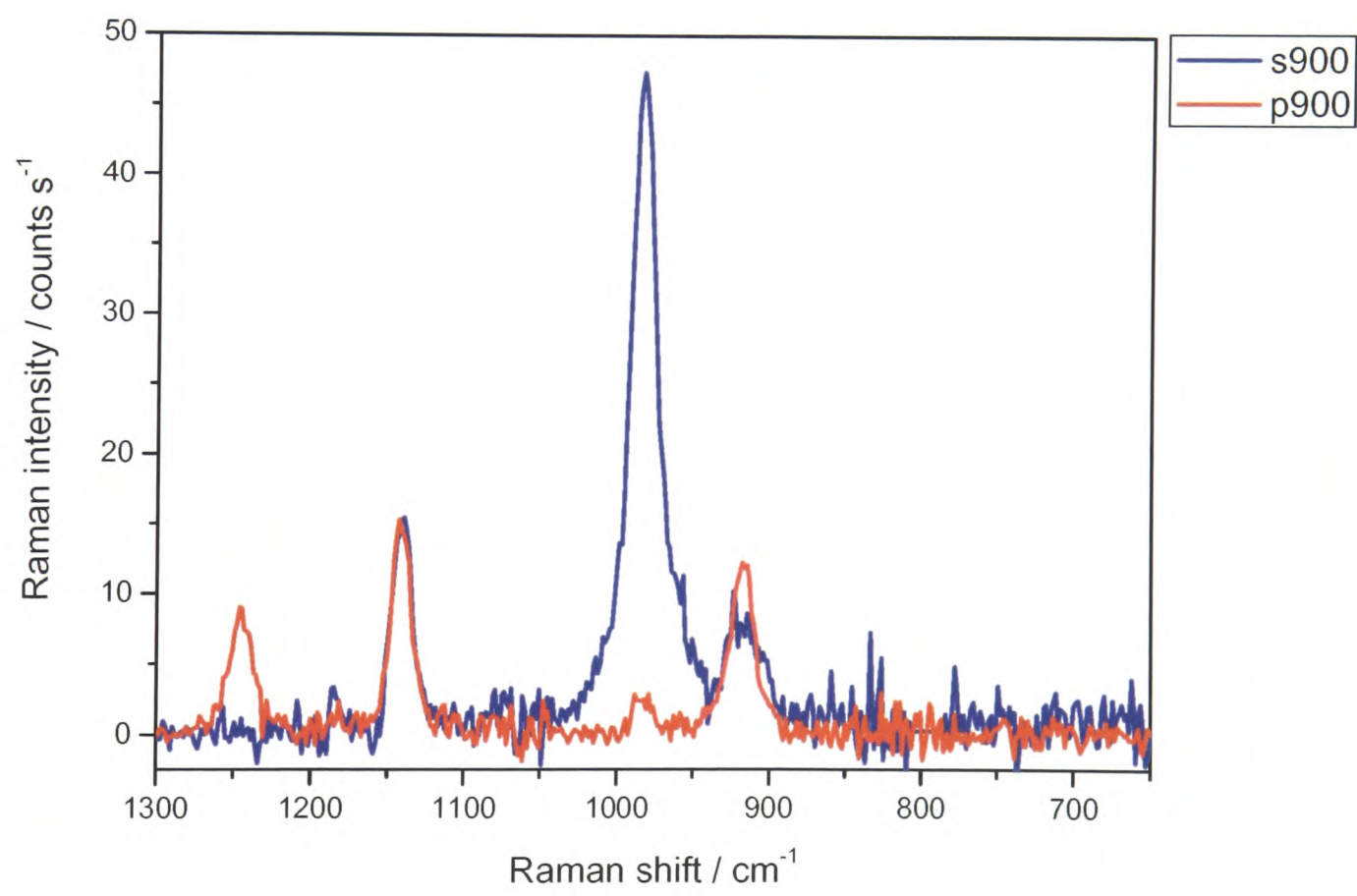


Figure 10.10 Polarised *TIR*-Raman spectra of d_{75} -DPPC *pslb* formed by vesicle adsorption: low frequency region: *s-pol* (in blue) and *p-pol* spectra (in red), Conditions as Fig. 10.1

Observed $\Delta \nu / \text{cm}^{-1}$		Reference $\Delta \nu$ s from literatures / cm^{-1}	Assignments
<i>s-pol</i>	<i>p-pol</i>		
-	1246.1 s	1249 ¹ C-C stretch 1249 ² CD ₂ wag	?
1139.6 s	1142.6 s	1145 ¹ C-C stretch 1144 ² CD skeletal mode and deformation	C-C skeletal stretching
983.5 s 961 m	988 m	984 ¹ 984 ² 967 ¹	CD ₂ scissoring
923.5 s 913 s	917.5 s	921 ³ 919 ¹ 918 ²	CD ₂ twisting

Table 10.2 Peak assignments of Raman spectra of *d*₇₅-DPPC adsorbed layer by micellar solution of DDM in the region of lower frequencies: strong (*s*), medium (*m*), weak (*w*), and shoulder (*sh*). ¹ crystalline solid (Bunow & Levin, 1977), ² aqueous dispersion (Garber, *et al*, 1978), ³ (Merajver, 1981), Round all peaks to nearest wavenumber.

10.3 Conclusion

Co-adsorption of *DPPC-DDM* micellar solutions onto which was introduced by Tiberg *et al* (Tiberg, *et al*, 2000) as an alternative way of build-up of supported lipid bilayer was measured *in situ* by polarised *TIR*-Raman spectroscopy. Our observation of the changes of Raman intensity in CH_2 vibration modes during the cycle of adsorption/rinsing is based on the crude assumption that the integrated area is proportional to the number of C-H bonds in the molecule times the surface excess. The prominent signal-to-noise ratio enabled to chase after the time-series changes of the polarised spectra of both *h-DPPC* and *h-DDM* in a cycle of rinsing and readsorption. Most adsorption of *h-DPPC* delivered by *h-DPPC/h-DDM* micellar solution was achieved within 5 *min* incubation and during subsequent cycle of rinsing by *UHQ DW* and readsorption by lower concentrated micellar solution, more adsorption as well as the removal of the loosely adsorbed surfactant attribute to the *h-DPPC* bilayer although the chain was more disordered than the *h-DPPC pslb* made by coverslip incubation (see Chapter 7 and experimental section of Chapter 5.7). CH_2 peaks were higher shifted. In order to discriminate adsorption of *h-DPPC* and removal of *h-DDM*, the *d₇₅-DPPC/h-DDM* micellar solution was used. After first solution of 0.1306 *mg/mL* was rinsed away, the *s* and *p*-polarised Raman intensities of the CD_2 stretching mode were drastically increased. The second adsorption cycle, from a solution diluted ten times, produce a pronounced red-shift in the frequency of the CD_2 symmetric stretching mode, which indicate that the packing density increased in the adsorption layer. To understand the final lipid layer we compared adsorption from *d₇₅-DPPC/h-DDM* micellar solution and from pure *d₇₅-DPPC* vesicle solution. These two spectra were almost same at the final stage of layer formation and provided a well-formed lipid bilayer. Furthermore we could

quantify how much surfactant remained in the final lipid bilayer after micellar adsorption kinetics completed by a comparison of the CH₂ stretching region of adsorbed layers from *d*₇₅-DPPC/*h*-DDM micellar solution and *d*₇₅-DPPC vesicular solution. The spectra were identical and indicating there was no detectable amount of *h*-DDM remaining in the bilayer. We conclude that our Raman technique is very useful of understanding the kinetic co-adsorption of DPPC and DDM from micellar solution to form a supported DPPC bilayer finally.

Chapter 11 Hydrolysis of a $DPPC$ *pslb* by PLA_2

This chapter demonstrates the application of *TIR*-Raman spectroscopy to studying the attack of a membrane-activated enzyme on a lipid membrane. The enzyme we chose was secretory porcine pancreatic phospholipase A_2 (PLA_2) (see Chapter 4.3), which has been studied in Oxford by the group of Dr. R. K. Thomas. The main purpose of the study was to observe changes in structure and loss of lysolipid from the bilayer during the course of reaction. In the process, we might also assess whether or not *TIR*-Raman scattering could reveal any useful information about the structure of a small protein interacting with a lipid bilayer. The putative *i*-face – the interfacial recognition region of PLA_2 – is always a challenging topic for membrane structural biologists. Structural information about the enzyme at the interface cannot be readily obtained by crystallographic methods due to difficulty of identification and positioning of the *i*-face in the presence of a lipid bilayer

Raman spectroscopy was used by Arêas access to investigate the structure of PLA_2 solution and in the crystalline phase (Arêas *et al*, 1989). These results look primitive and out-of-date compared to more recent data obtained by *ATR-FTIR* because the presence of lipid bilayer is required for formation of the enzyme in its active conformation. Notwithstanding, the Raman spectra of Arêas are the only reference data I know.

11.1 Hydrolysis of a $DPPC$ *pslb* by PLA_2 : lipid spectra

Figure 11.1 shows the *s*-polarised *TIR*-Raman spectra in the C-H stretching mode of a $DPPC$ *pslb* reacting with PLA_2 . In order to measure a time-profile of the Raman intensity in the C-H stretching bands during lipid hydrolysis, we used porcine pancreatic PLA_2 with sulfate ion (ammonium sulfate suspension), whereas the

measurement of amide I and III modes, which required relatively longer Raman acquisition (at least 20 *min* compared with 4 *min* in C-H stretching modes), was made using PLA_2 without sulfate ion (lyophilised powder form). With sulfate ions, the reaction was expected to proceed at a higher rate because anion can activate zwitterionic lipid headgroup for nucleophilic attack of PLA_2 interacting site. PLA_2 with an inactive lag phase was preferentially chosen because originally we were curious about the possibility of measuring the conformational change in PLA_2 in advance of interfacial activation. At the initial concentration of 0.1 *mg/mL*, catalytic hydrolysis leads to extensive decomposition of the *pslb* into fatty acid and lysophosphatidylcholine. Ideally, one would use a lipid specifically deuterated in one of the two acyl chains. Then Raman spectra would show clearly whether one or both chains was lost from the surface and the relative rates of loss. Such an isotopically substituted molecule was not available, but nevertheless we can draw several inferences from the data.

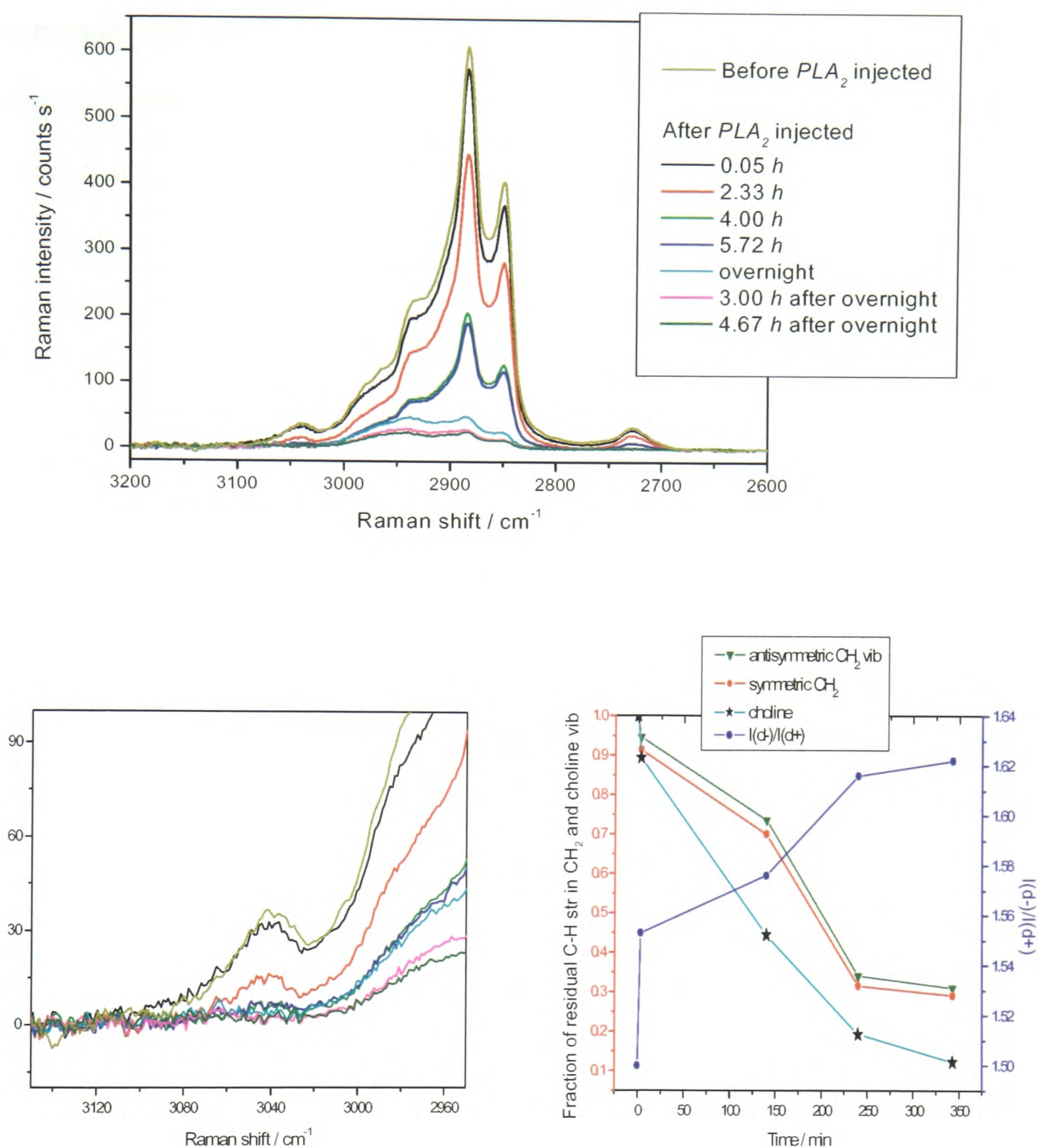


Figure 11.1 *s-pol* TIR-Raman spectra showing the reaction of a $DPPC$ *pslb* with PLA_2 in C-H stretching modes (top) and in C-H stretching in choline group (left bottom). The order parameters by the ratios of heights of $\nu_{as}(CH_2)$ to $\nu_s(CH_2)$, $I(d^-)/I(d^+)$ (blue) and fractions of residual C-H Raman intensities in $\nu_{as}(CH_2)$ (reverse triangle in green), $\nu_s(CH_2)$ (red circle), and choline group (black star) (percentages of Raman intensities of C-H stretching in choline group in time-series after PLA_2 injected relative to before PLA_2 injected) (right bottom). Before $DPPC$ *pslb* at 22 °C soaked in Tris 10 mM pH 7.4 before PLA_2 loading (dark yellow).

In interpreting the spectra in the C-H stretching region, we will first make the approximation that the enzyme is only a minor contributor to the spectra. This assumption will not be strictly true, but the weakness of the signal after incubation overnight (when, from the fingerprint region we know that there is the greatest amount of adsorbed enzyme) suggests that this approximation is reasonable. The region around the choline antisymmetric methyl stretch at 3040 cm^{-1} is shown in the lower left panel of Figure 11.1. The lower right-hand panel in Figure 11.1 shows intensity changes over the first 350 *min* for the methylene vibrations (both symmetric ($\nu_s(\text{CH}_2)$) at 2850 cm^{-1} and antisymmetric modes ($\nu_{as}(\text{CH}_2)$) at 2883 cm^{-1}) and $\nu_{as}(\text{CH}_3)$ of the choline group. The right-hand axis plots the ratio $I(d^-)/I(d^+)$, which as discussed earlier is a measure of conformational order in lipid chains.

Figure 11.2 shows both *s* and *p*-polarised spectra in the fingerprint region at various reaction times of a *DPPC* bilayer with PLA_2 . Figure 11.3 shows the intensity of characteristic peaks in Fig. 11.2 as a function of time.

We can draw the following conclusions.

- (i) The reaction does not show a pronounced lag phase, or at least, any lag phase is much less than 2 *h* long.
- (ii) The reaction is more or less complete after 4 *h*. The red and green spectra in Fig. 11.1 and the pale and dark blue spectra in Fig. 11.2 (*s-pol*) are almost the same. The intensity carbonyl peaks has fallen to less than 20 % of the original intensity by this time.
- (iii) The intensity of the choline $\nu_{as}(\text{CH}_3)$ drops in tandem with the carbonyl group and has almost disappeared after 4 *h*, showing that the relatively soluble lysolipid is lost from the bilayer.
- (iv) After 4 *h*, the spectrum in the C-H region still looks like a lipid spectrum with >30 % of the original intensity. The ratio $I(d^-)/I(d^+)$ increases slightly as the

reaction proceeds. These observations suggest that greater fraction of the palmitic acid product of the reaction remains on the surface in the form of well-ordered domains of fatty acid.

- (v) In the *s*-polarised spectra, the CH_2 scissoring mode decreases in intensity more slowly than the twisting mode, while in the *p*-polarised spectra the scissoring mode decreases more rapidly. As discussed in Chapter 8, these changes show that the hydrocarbon chains are less tilted with time, consistent with the inference regarding islands of fatty acid on the surface.

After overnight reaction, the lineshapes and peak positions change, the CH_2 modes weaken and there is a substantial increase in the intensity of peptide bands in the Amide I region. These observations suggest that the fatty acid has slowly dissolved/been removed from the surface and that the residual peaks are largely due to adsorbed peptide.

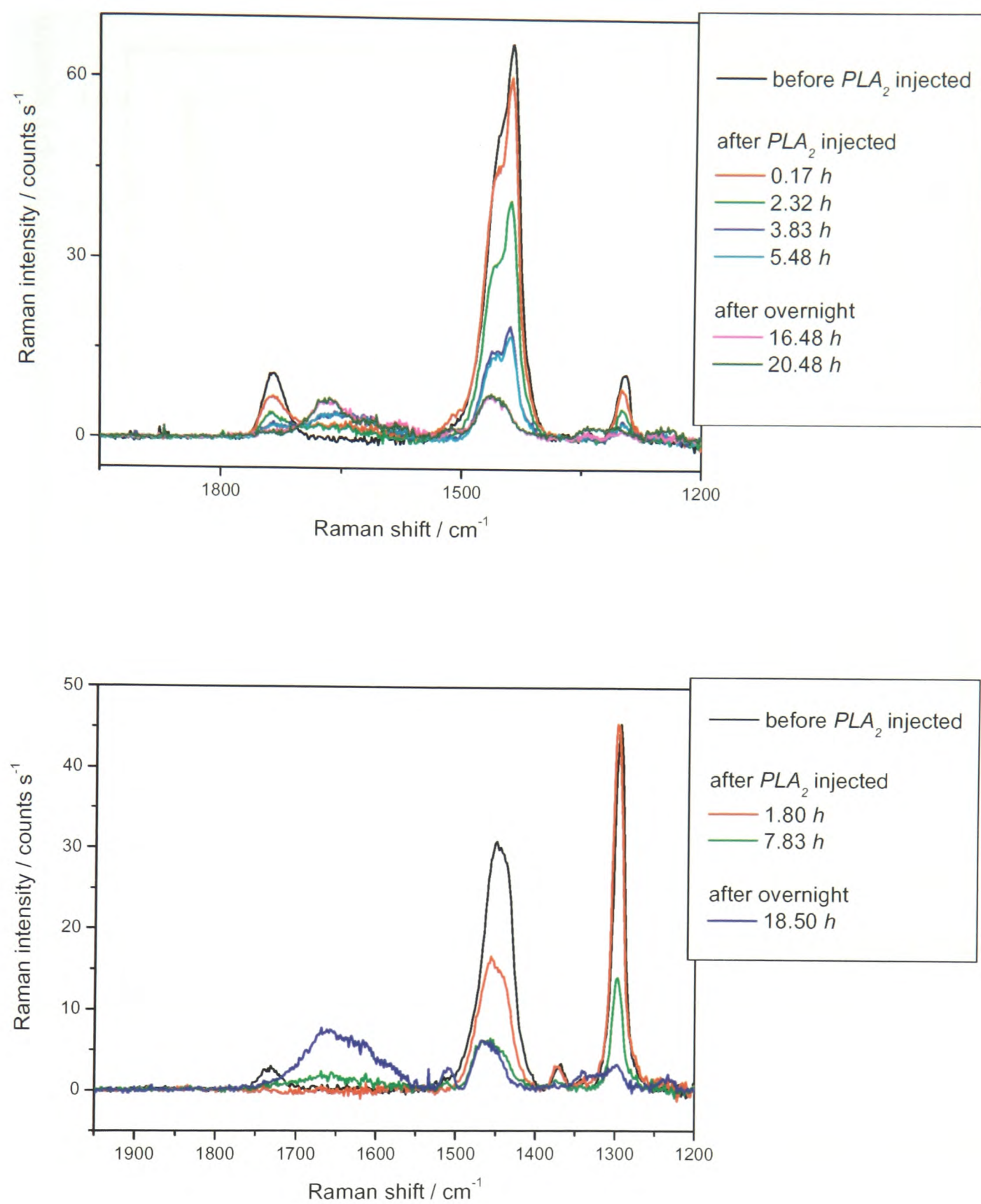


Figure 11.2 *s* (upper) and *p*-polarised (lower) *TIR*-Raman spectra showing the reaction of a *DPPC pslb* with PLA_2 in the fingerprint region. Reaction times are noted in the legend for each spectrum. Reference spectrum of *DPPC pslb* at 22.6 °C (in black).

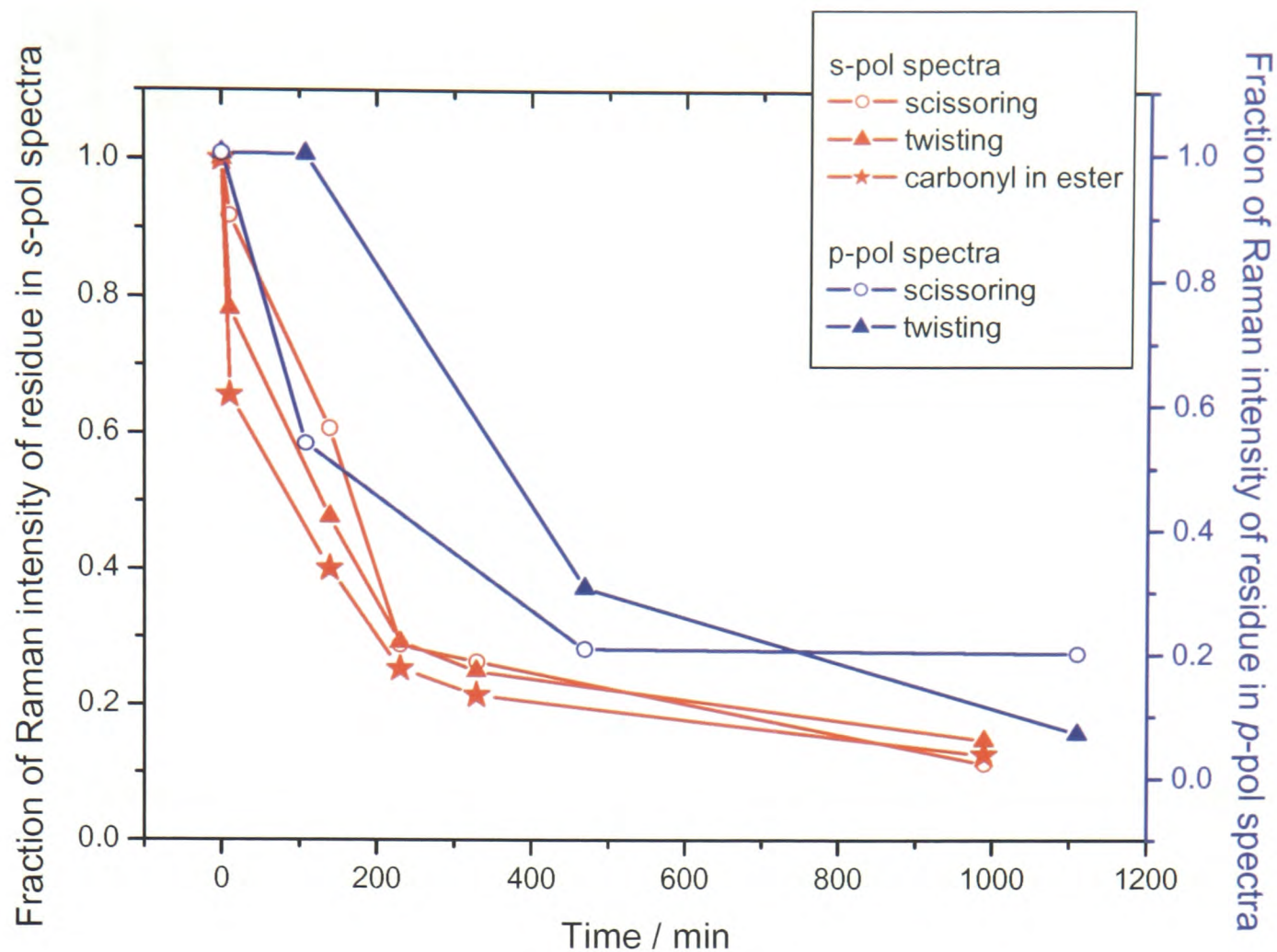


Figure 11.3 Relative intensity of CH_2 scissoring and twisting modes and carbonyl in ester group (only for *s-pol* spectra) in the *s* (red) and *p-pol* spectra (blue). Intensities were normalised by the Raman intensity before injection of PLA_2 .

The effect of sulphate on the rate of reaction is shown in Figure 11.4, which plots the choline $\nu_{as}(CH_3)$ intensity in *s*-polarised spectra as a function of time with and without sulfate ion. There is not a large difference in the reaction rates, although the rate in the presence of sulfate is somewhat more rapid.

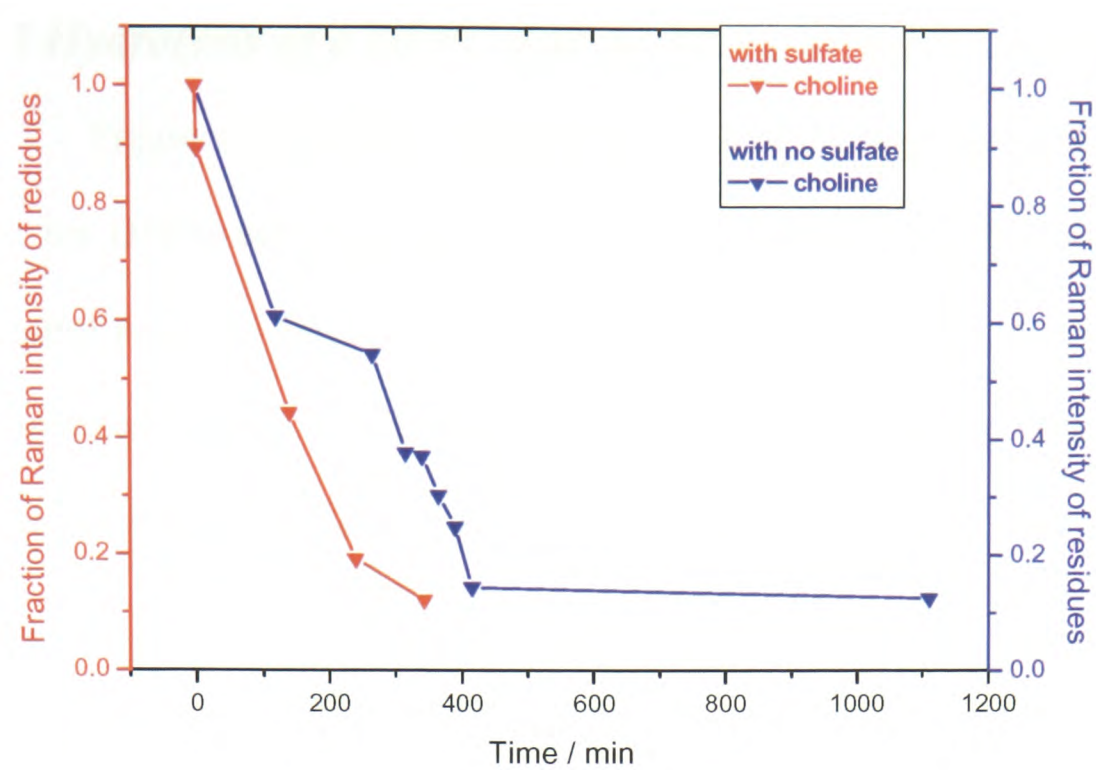


Figure 11.4 Time-dependent decrease of fraction of *s-pol* C-H Raman intensity in choline to the intact Raman intensity of no PLA_2 reaction for PLA_2 with (in blue) and without sulfate ion (in red)

11.2 Hydrolysis of a $DPPC$ $pslb$ by PLA_2 : protein spectra

Figure 11.5 shows s -polarised spectra of the fingerprint region as a function of time for $DPPC$ reacting with PLA_2 with no sulfate. The behaviour of the lipid and carbonyl peaks is similar to that described in Section 11.1, but there are much more pronounced Amide I and Amide III bands, suggesting the adsorption/deposition of larger amounts of enzyme in the second experiment.

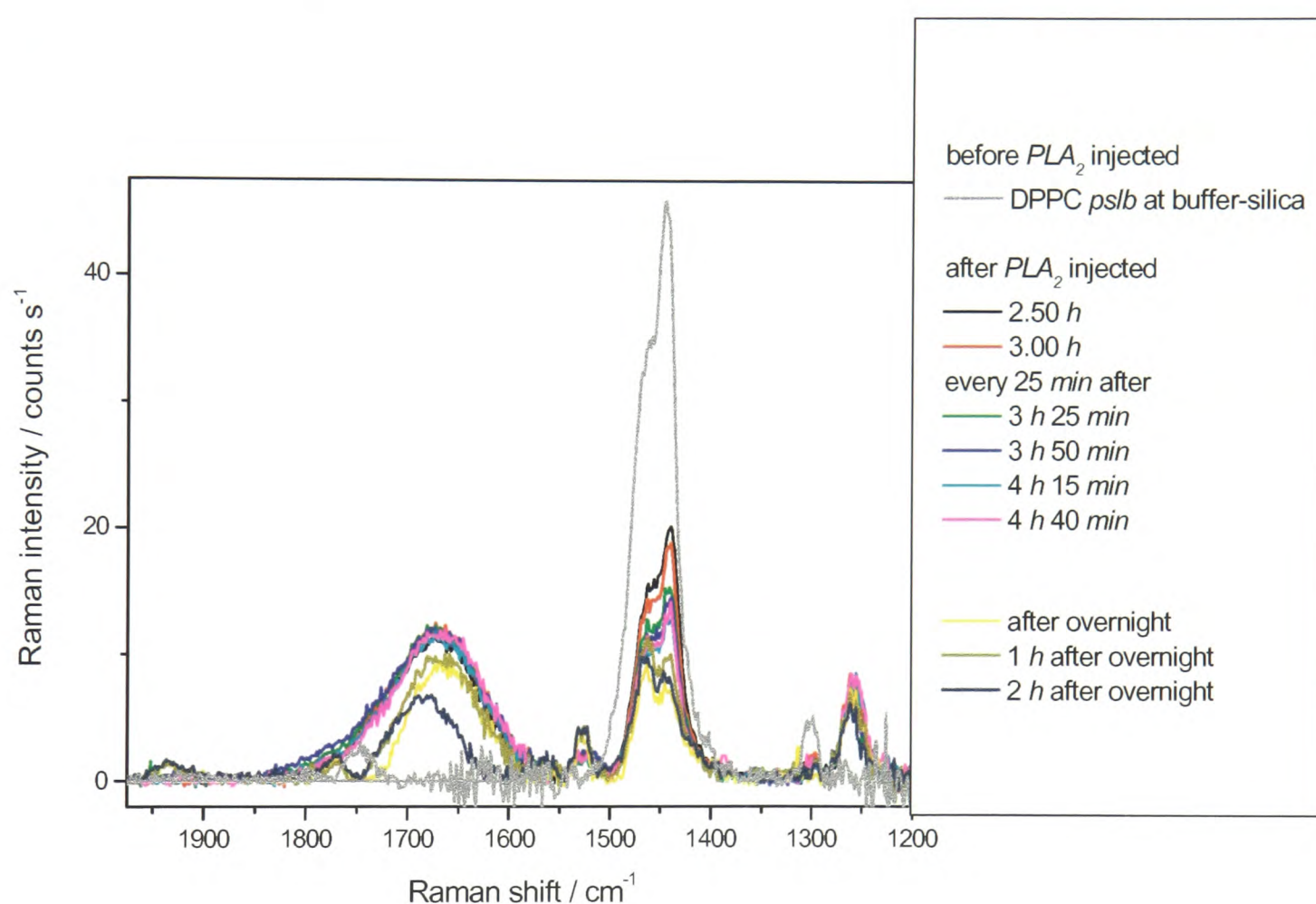


Figure 11.5 s -pol TIR-Raman spectra showing the reaction of a $DPPC$ $pslb$ with PLA_2 : fingerprint region. Reaction time: 2.5 h (black), 3 h (red), and then after every 25 min , 3 h 25 min (light green), 3 h 50 min (blue), 4 h 15 min (light blue), 4 h 40 min (pink), and after overnight (yellow), 1 h after overnight (dark blue), 2 h after overnight. Reference spectrum of $DPPC$ $pslb$ at 22.6 °C (gray). Conditions as Fig. 10.1.

Pronounced Amide I and Amide III bands are present in all the spectra during the time when most of the lipid is being hydrolysed. After reaction overnight, the shapes of these Amide bands changed and new peaks appear around 1770 and 1930 cm^{-1} . We have previously suggested that most of the reaction products are removed during overnight immersion in buffer and that the residual Raman signal is due largely to adsorbed enzyme. We hypothesize that the changes in the protein spectra were due to denaturation of the enzyme on patches of glass left bare by dissolution of the products of lipidolysis. To test this hypothesis, we acquired Raman spectra from the PLA_2 adsorbed onto bare hydrophilic silica without a phospholipid bilayer. Spectra of denatured PLA_2 are compared with the spectra from the lipidolysis experiment in Figures 11.6 and 11.7 studies. A three-fold higher protein concentration was used in the bare silica experiment, since we did not know how well PLA_2 would adsorb on hydrophilic silica.

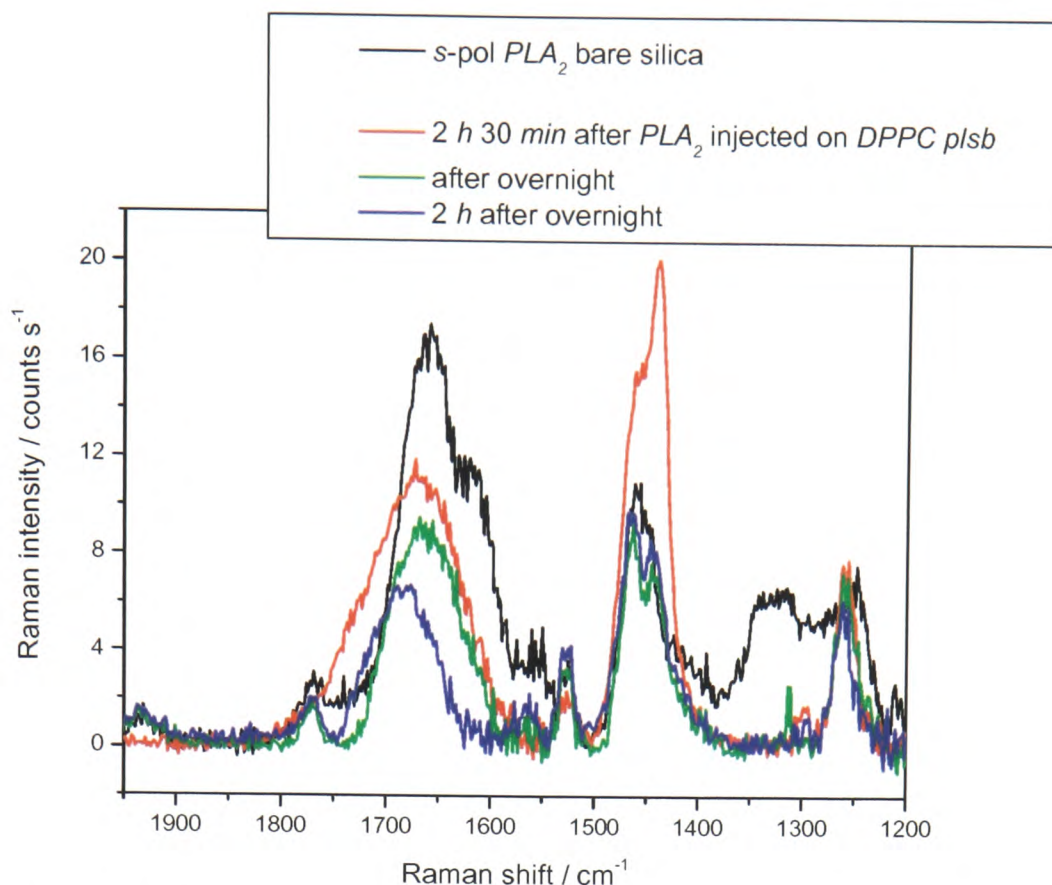


Figure 11.6 Comparison of *s-pol* TIR Raman spectra of PLA_2 reacting with $DPPC$ *pslb* and absorbed onto bare silica: 2 h 30 min after PLA_2 loading (in red), after overnight (in light green), 2 h after overnight (in blue), *s-pol* PLA_2 on bare silica (2 h after PLA_2 loaded onto clean hydrophilic bare silica window, in black). Conditions as Fig. 11.1.

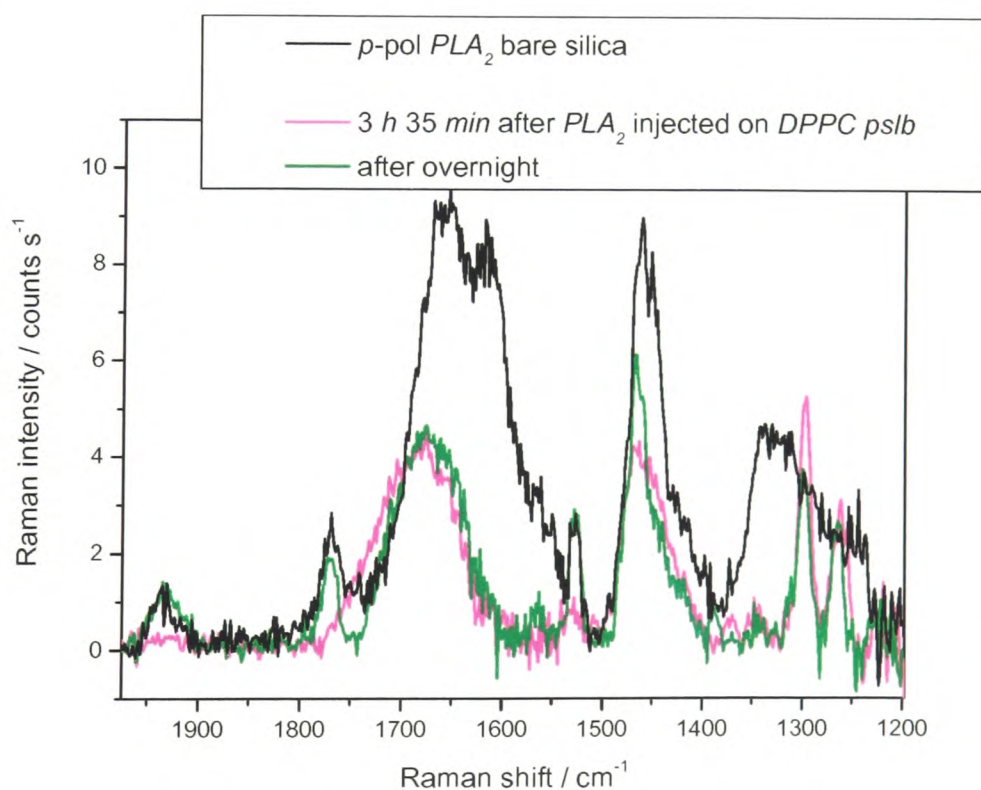


Figure 11.7 Comparison of *p-pol* TIR Raman spectra of PLA_2 reacting with $DPPC$ *pslb* and absorbed onto bare silica: 3 h 35 min after PLA_2 loading (in pink), after overnight (in light green), *p-pol* PLA_2 bare silica (2 h 30 min after PLA_2 loaded onto clean hydrophilic bare silica window, in black). Conditions as Fig. 11.1

The spectra of PLA_2 on glass are more complex than those on the $DPPC$ bilayer, but we can see clearly that the two peaks at 1769 and 1934 cm^{-1} in both *s* and *p-pol* are present in PLA_2 on glass, but absent at early times for PLA_2 reacting with a $DPPC$ bilayer. The tyrosine peak 1527 cm^{-1} in both *s* and *p*-polarized, which strengthens at longer times in the reaction time-series, is also prominent and at the same frequency in the denatured PLA_2 on glass. It appears probable therefore that the changes that we observe overnight are due to dissolution of lipid reaction products leaving bare glass upon which the protein adsorbs.

I have tentatively analysed the Amide I and Amide III regions by the method of smoothing and second derivatives described in Chapter 9. A quartic filter with a width of 33 points was employed. The results are shown in Figure 11.8 for the whole fingerprint region and in Figure 11.9 for the Amide I and Amide III regions.

In the PLA_2 alone on bare silica, the amide I modes are dissected into a strongest peak of 1654 cm^{-1} for α -helical structure correlated with the peak at 1317 cm^{-1} in amide III modes and strong shoulder around 1678 cm^{-1} for most of turns (random coil structure) supported by the peak at 1246 cm^{-1} in amide III modes. The peak at 1611 cm^{-1} might indicate the existence of β -sheet structure, but there is no corresponding peak at amide III and the 1600 cm^{-1} region also includes aromatic amino acids such as tryptophan (W^3), phenylalanine (F^5 , F^{63} , F^{94} , F^{106}), and tyrosine (Y^{35} , Y^{38} , Y^{52} , Y^{69} , Y^{73} , Y^{75} , Y^{111} , Y^{123}). (Arêas, *et al*, 1989; Carey, 1982; Miyazawa, 1960; Sane, *et al*, 1999; Tu, 1982). The peak at 1526 cm^{-1} indicates the presence of tyrosine (Iconomidou, *et al*, 2000) and it exceeds the neighboring peak at 1553 cm^{-1} which is representative of tryptophan $\omega 3$ mode because there are 8 tyrosine residues and only a single tryptophan residue in this PLA_2 . The CH_2 bending modes are observed at the broad and strong peak around 1450 cm^{-1} . The peaks at 1341, 1770, 1935 cm^{-1} could not be assigned.

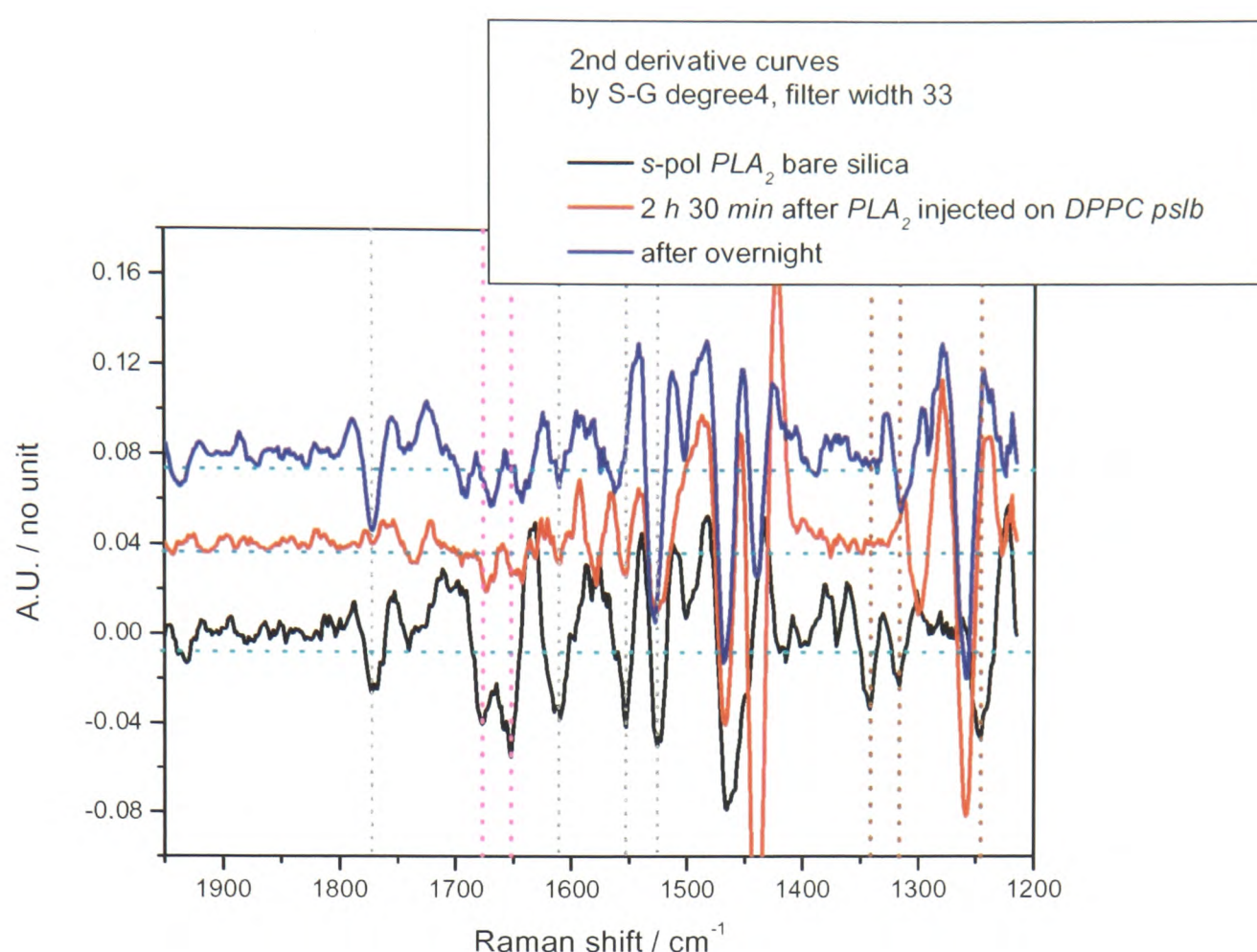


Figure 11.8 Comparison of second derivative curves of *s-pol* TIR-Raman spectra of PLA_2 -hydrolysed $DPPC$ *pslb* and absorbed onto bare silica window with no $DPPC$ supported bilayer (inactive form) in *Tris* 10 mM pH 7.4: 2 h 30 min after PLA_2 loading (in red), overnight after (in blue), *s-pol* PLA_2 bare silica (2 h after PLA_2 loaded onto clean hydrophilic bare silica window (in black)). The 2nd derivative spectra were treated by Savitzky-Golay algorithm of smoothing quadratic filter with the filter width of 33. The minimum position of the 2nd derivative curve in the spectral region of between 1800 and 1900 cm^{-1} limits reading of minima in each 2nd derivative curve (by a dotted line in light blue). The peak positions in consideration are indicated by dotted lines for amide I (by a dotted line in pink) and amide III modes (by a dotted line in brown).

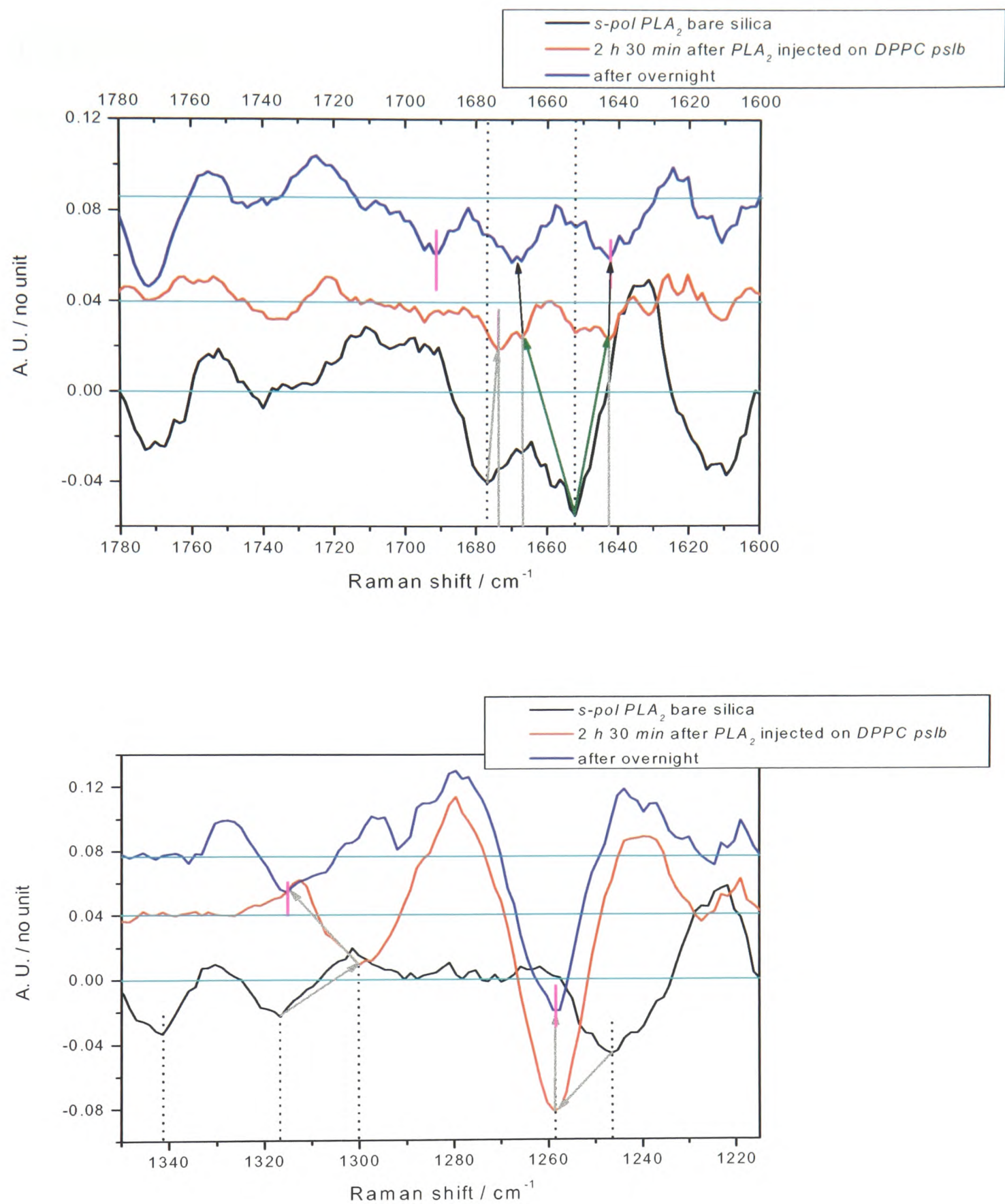


Figure 11.9 In amide I (upper) and III regions (lower), the changes of possible peak positions which are indicative of secondary structures in the second derivative curves of *s-pol* TIR-Raman spectra of PLA_2 -hydrolysed $DPPC$ $pslb$: 2 h 30 min after injection (in red), after overnight (in blue), *s-pol* PLA_2 bare silica (in black) are the same as noted in Figure 11.6. The second derivative curves are determined as described in Figure 11.8.

In the presence of lipid bilayer, the characteristic peaks of PLA_2 in the amide I and III regions occur at different position from denatured enzyme on silica. Notably, the 1611 cm^{-1} and 1341 cm^{-1} peaks in the denatured enzyme are absent in the peptide adsorbed to the lipid bilayer. In the lipid-activated enzyme, the α -helical peak presented at $\sim 1653\text{ cm}^{-1}$ is less intense and there is greater intensity at higher frequencies in the Amide I region.

The amide III region the lipid-activated enzyme has a strong peak at 1260 cm^{-1} characteristic of random coil structure. A second band at 1300 cm^{-1} overlaps the CH_2 twisting mode but increase in intensity at long time so likely to be arisen in part from the protein. This region cautiously may be belonging to α -helical structure, but as we observe the split of 1653 cm^{-1} into the two peaks of 1668 and 1648 cm^{-1} , the occurrence at 1300 cm^{-1} is correlated with the presence of α -helix, but not similar to the typical α -helical structure which is typically characterised at 1653 cm^{-1} . Rather than 1668 cm^{-1} higher shifted peak at 1668 cm^{-1} supports the more dynamic α -helix conformation in part of the protein structure (it has been proposed by Tatulian's work by *ATR-FTIR* study (Tatulian, *et al*, 1997) with the correlated peak at 1300 cm^{-1} in the lipid-activated state.

11.3 Conclusion

The data presented here are only by way of a preliminary study and would have to be repeated to confirm the conclusions. Several experimental problems were noted, which would need to be solved to obtain conclusive data. First, the lag phase observed in previous experiments was not evident in our measurements. Second, the intensities of the protein bands in the Raman spectra were variable and scattering was observed from aggregates on the surface of the silica window. These observations suggest the presence of contaminants, possibly ions leaching from the coverslip or other glassware.

Nevertheless, I was able to show that the time-course of the reaction could be followed by Raman scattering. From the intensity of bands due to different vibrational modes, I could conclude that the lysolipid was lost from the surface and that well-ordered patches of fatty acid remained – without the need for isotopically substituted lipids.

While I was able to observe significant difference between the secondary structure of PLA_2 interacting with a lipid bilayer and with bare glass, additional experiments would be required to address the question of conformational changes in the PLA_2 caused by membrane activation. These experiments would need to eliminate the precipitation of aggregated enzyme on the substrate.

Chapter 12 Conclusion

In the thesis we principally achieved the development of an unenhanced Raman spectrometer with sufficient sensitivity for the detection for supported single lipid bilayers and demonstrated its optimised performance for understanding the structure-function relationship of membrane peptide/protein interacted with planar supported lipid bilayers in water/solid interface.

A solid-supported lipid bilayer is a model biomembrane and a platform for studying membrane peptides/proteins. The spontaneous liposomal adsorption of various phosphatidylcholines (*PC*) (*DMPC*, *DPPC* for the saturated *PC* of different chain length (*DMPC* for dimyristoyl of 14, *DPPC* for dipalmitoyl of 16 hydrocarbon) and *POPC* for one palmitoyl with the partial unsaturated *PC* in *sn*-2 position of oleoyl of 18 hydrocarbon) with/without membrane-spanning peptide (like gramicidin D) formed the lipid layer on the silica substrate under an aqueous environment and the study of fusion kinetics by atomic force microscopy *in situ* revealed by direct imaging from the early to the final stages of adsorption that formation of planar supported lipid layer (*pslb*) was complete.

To investigate single lipid bilayers at water/silica interface, I applied total internal reflection (*TIR*) geometry in order to maximise electrical field at interface with polarisation of incidence and detection controllable. The bilayer formation was confirmed by comparison of Raman intensity of *C₁₆TAB* in the C-H stretching modes. The prominent Raman sensitivity of 350 counts per second (*cps*) in C-H stretching and 60 *cps* in C-H bending region was attained to a various *PC* bilayer by the penetration depth of 290 nm with incident angle of 68 ° just above the critical angle (~ 65.8 °) at water/silica interface. The sample heating was checked using different laser intensities

at the temperature where Raman intensities are sensitive with small change of temperature (like 22.5 °C for *DMPC pslb*) and there was no heating damage by the fact that Raman spectra could be overlapped by scaling factors multiplied.

The phase transitions of *pslbs* of *DMPC* and *DPPC* in 2-D space at water-silica interface was also studied and the melting region of L_α (liquid crystalline) to L_β (gel) lamellar phases in both lipids was broader and lower shifted of 2-5 °C than in vesicle, which contrasted with the sharp and narrow temperature widths by differential scanning calorimetry (*DSC*) explained by van't Hoff formalism. In *DPPC pslb*, intermediate phase was observed and it might be considered to P_β (ripple phase).

Gramicidin D (*gD*), a membrane-spanning peptide, was incorporated into small unilemmellar vesicles in solution using *TFE* as cosolvent for preferentially preparing the channel form and their accommodation of *DMPC* bilayer in single-stranded channel form was examined by the *CD* and fluorescence spectroscopy. For the polarised *TIR*-Raman measurements, the *DMPC pslb* embedding *gD* was formed at water/silica interface and results of sufficient resolution of 2-3 *cps* in amide I and III modes to assign the *gD* structure in lipid bilayer qualitatively. My polarised *TIR*-Raman spectra were correlated with the 3-D coordinates by solid-state *NMR* spectra with and they corresponded to showing the single-stranded C-terminal-to-C-terminal $\beta^{6.3}$ π -helical conformation as a channel structure in *DMPC pslb* at water/silica interface.

Also the *DMPC pslb* over its main phase transition temperature was interacted with membrane-penetrating indolicidin of a microbial peptide and they were probed *in situ* by polarised *TIR*-Raman spectroscopy. With the perturbation of indolicidin into the *pslb* detected by *CCD* imaging of surface while indolicidin interacted, the time-course spectra acquired every 10 *min* for amide I and III modes presented the predominant part of β -turn conformation with random coil structure through the entire period of the interaction at membrane-active state.

DPPC adsorption from a micellar solution with nonionic sugar-based surfactant of *n*-dodecyl- β -D-maltoside (*DDM*) was measured *in situ* by polarised *TIR*-Raman spectroscopy. This alternative way to produce *pslb* was dissected using time-series spectra. Adsorption kinetics by a mixed micellar solution delivering lipid to water/silica interface was studied in the time-series acquisition of Raman spectra using *d*₇₅-*DPPC* and hydrogenated *DDM* (*h-DDM*) to discriminate how much lipid and surfactant is involved during the adsorption process.

Finally membrane-hydrolytic enzyme reaction of porcine pancreatic phospholipase A₂ (*PLA*₂) with the preformed *DPPC pslb* at water-silica interface was studied by time-dependent polarised *TIR*-Raman measurement at room temperature. The subsequently decreased Raman intensity of the C-H stretching vibrations made us sure that most lysolipid left in the time-course disruption of *pslb*. Also an increase of chain ordering inferred the accumulation of fatty acid while the phospholipids were hydrolysed and it attributed to the allosteric kinetics as well as initial interfacial activation without any lag phase. The conformation of *PLA*₂ was detected in amide I and III modes in active state with *DPPC pslb* and it was very different from inactive form on bare silica with more dynamic α -helical motif evolved and dominant random coil structure.

I make a conclusion that our *TIR*-Raman technique with polarisation was successfully developed to probe the single lipid bilayers at water-silica interface and it could be applied to investigate the secondary structure of membrane-peptides/proteins in static and dynamic states. For achievement, model biomembranes of *DMPC* and *DPPC pslb* were built on by interaction with membrane-spanning peptide of *gD*, membrane-associating microbial peptide of indolicidin, and membrane-hydrolytic enzyme of *PLA*₂.

We launched an initial step of this potentially powerful technique, and

performed successfully an unprecedented work in Raman spectroscopic research. In addition it was very useful of probing a model membrane of supported single lipid bilayer, but it has some limits to be overcome in future:

1. It has still problematic water-subtraction in the fingerprint region as long as a membrane-associating (which penetrates and perturbs the lipid bilayer) peptide is involved. The subtraction was relatively easier than *ATR-FTIR* which is a complementary technique (actually it is a kind of motivation to use Raman scattering), and it especially works well on the subtraction in the C-H stretching region. But most of informative data relative to the secondary structure of peptide/protein belong to the fingerprint region where O-H peak pronounced.

2. Our unenhanced Raman scattering could maximise excitation *E*-field using *TIR* geometry with enormous intensity like 1.2 W for 600 s acquisition. The peptide/protein is very much vulnerable to strong laser intensity. So we might be thinking of introducing and modifying our current technique with *UV* laser and resonance effect (like *UV*-resonance Raman).

3. When a peptide/protein perturbs a supported lipid bilayer, aggregates or holes caused by peptide/protein action can evolve, and they behave like scattering sites which may distort the Raman bands. In our experiments of indolicidin and *PLA*₂, I had to be very careful to get the reproducible Raman spectra because of some kind of scattering.

Nevertheless now we demonstrated a potency of this technique at the starting point of research involved in membrane world, and we are well-placed to extend the study of membrane biophysics in the future.

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