

STRUCTURE CHARACTERIZATION OF RECOMBINANT DEACYLATED HUMAN
PULMONARY SURFACTANT PROTEIN C AND ITS MUTANTS
USING INFRARED SPECTROSCOPY AND 2D CORRELATION ANALYSIS

by

YU ZHU

(Under the Direction of Richard A Dluhy)

ABSTRACT

Various FTIR spectroscopy sampling techniques are employed to characterize the structures of recombinant deacylated human pulmonary surfactant protein C (rdhSP-C) and its recombinant mutants in organic solvent, or in phospholipid environment. Two-dimensional (2D) correlation analysis techniques are applied to provide improved resolution enhancement for structure determination. Chapter 3 to Chapter 7 are based on individual manuscripts to be submitted.

Chapter 3 and Chapter 4 describe the investigations of a pH-dependent mechanism for misfolding of wild type rdhSP-C and its mutants using FTIR transmission spectroscopy and 2D correlation analysis. In Chapter 3, various 2D correlation analysis techniques are explored. Compared to generalized 2D correlation analysis, model-based 2D correlation analysis proves to be a straightforward and quantitative tool in protein structure determination. A pronounced α to β structure conversion is manifested for wild type rdhSP-C. In Chapter 4, as site-directed mutageneses moving from the N-terminus to the poly-valine segment, rdhS-C mutants unfold

into β -structures and unordered structures respectively. Chapter 5 describes the surface pressure induced structure changes of wild type rdhSP-C and one of the mutants (K11Q/R12Q) in phospholipid monolayers at the air/water interface. The combination of PM-IRRAS with model-based 2D correlation analysis proves to be very effective and suitable for investigation of model biomembrane system at the air/water interface. Chapter 6 describes the investigation of thermal induced structure changes of rdhSP-C Mut4 (K11Q/R12Q) in planar supported phospholipid bilayers. In addition, polarization ATR-FTIR is applied to elucidate the orientations of the phospholipid acyl chains and the β -sheet conformation of Mut4 respectively. Systematic studies on rdhSP-C and its mutants indicate that different structure motifs of rdhSP-C may be involved in the unfolding and misfolding process. In addition, environmental changes, i.e. from monolayer to bilayers, may also contribute to the formation of β -aggregations of rdhSP-C.

Chapter 7 describes the methodology using silver nanorods (AgNRs) based intrinsic surface enhanced Raman spectroscopy (SERS) and chemometrics in the differentiation of rotavirus strains.

INDEX WORDS: FTIR spectroscopy, polarization-modulation infrared reflection-absorption spectroscopy (PM-IRRAS), air/water interface, polarization ATR-FTIR, two dimensional (2D) correlation analysis, recombinant deacylated human pulmonary surfactant protein C (rdhSP-C), site-directed mutagenesis, protein secondary structure, phosphatidylcholine (PC), molecular orientation, Langmuir films, Langmuir-Blodgett transfer, SERS, silver nanorods (AgNRs), chemometrics.

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CHAPTER 1

INTRODUCTION

Pulmonary surfactant are substances existing in the lung alveolar aqueous lining films maintaining lung structure and facilitating the normal inspiration and expiration during breathing cycles, as well as preventing invading pathogens and particulates. Respiratory distress syndrome (RDS) is a fatal disease for premature baby born with underdeveloped lung, which is characterized by significant deficiency of pulmonary surfactant. The bulk of pulmonary surfactant consists of ~80% phosphatidylcholine, ~10% neutral lipids such as cholesterol, and ~10% pulmonary surfactant proteins. There are four pulmonary surfactant proteins. SP-A and SP-D are hydrophilic proteins responsible for host innate defense against the invading pathogens such as bacteria, virus, fungi, as well as particulates such as pollen. SP-B and SP-C are hydrophobic proteins that are mainly responsible for the adsorption, spreading and stability of the alveolar lining films during breathing cycles. Of the four pulmonary surfactant proteins, surfactant protein C (SP-C) is exclusively expressed in alveolar type II cell. The importance of SP-C has long been established with its functions in surfactant activity. SP-C together with surfactant protein B (SP-B) mediate the phase behaviors of phospholipids in alveolar lining films, which is of primary importance to maintain lung structures during normal breathing cycles. Recently, SP-C has been associated with interstitial lung disease, which is largely attributed to mutageneses in SP-C precursor polypeptide (proSP-C). In addition, misfolding of mature SP-C resembles the case of amyloid like fibril conformational diseases with characteristic

α to β conformation conversion. Biophysical studies on the structural-functional relationship of SP-C are therefore of primary importance to understand the mechanisms of misfolding of SP-C. The following chapters report several different biophysical studies related to the structure conversion of recombinant deacylated human SP-C (rdhSP-C) and its mutants expressed by a surfactant protein C baculovirus expression vector, which is developed by our collaborators lead by Dr. John E Baatz at the Medical University of South Carolina. **Fig1.1.** presents the primary structures of rdhSP-C wild type and its mutants.

Wild type rdhSP-C:

GSPGLNGIW-GIPCCPVHLKRLLIVVVVVLIVVVIVGALLMGL

rdhSPCmut3: C5S/C6S

GSPGLNGIW-GIPSSPVHLKRLLIVVVVVLIVVVIVGALLMGL

rdhSPCmut4: K11Q/R11Q

GSPGLNGIW-GIPCCPVHLQQLLIVVVVVLIVVVIVGALLMGL

rdhSPCmut6: L22P

GSPGLNGIW-GIPCCPVHLKRLLIVVVVVPVIVVVIVGALLMGL

rdhSPCmut7: V21P

GSPGLNGIW-GIPCCPVHLKRLLIVVVVPLIVVVIVGALLMGL

Fig1.1. Primary structures of rdhSP-C and its mutants. The additional ennea-peptide segment at the N-terminus is functioning to reduce the ability of the recombinants to irreversibly aggregate in the cells, and may also benefit to fluorescence study.

Various FTIR spectroscopy sampling techniques, including surface-sensitive techniques are applied to investigations on the structural-functional relationship of rdhSP-C and its mutants

with different mechanisms. Both theoretical and empirical aspects of these techniques are discussed in the literature review chapter.

FTIR provides a powerful tool in characterization of the structure of biomolecules. Vibrational modes of biomolecules differ depending upon the local environment, e.g. solvent, lipid matrix, etc. In the latter case, the interaction between the biomolecule and the matrix may also be investigated. Biophysical studies on folding and misfolding of proteins are of primary importance for elucidating protein functions in the biologic systems. Polypeptide and protein backbone have signature IR absorption in amide I region ($1700\text{-}1600\text{cm}^{-1}$) which is sensitive to secondary structure changes. Spectral-structure correlation in amide I region can therefore provides important information on folding and misfolding of proteins. However, absorption bands of all the conformations under amide I envelop are highly overlapped. Therefore, structure determination using amide I band must involve resolution enhancement techniques. Traditionally, Fourier self-deconvolution and curve fitting in combination with secondary derivative spectra are used. Each involves arbitrarily defined parameters such as band position and band width. Moreover, curve fitting is based on the assumption that the absorbance of all conformations under amide I envelop has the same extinction coefficient, which however is not true. Alternatively, 2D correlation analysis has been established to provide both resolution enhancement and information on sequential changes of intensity variations. In the following chapters, various 2D correlation analysis techniques are explored for protein structure determination, with more focused on model-based 2D correlation analyses, which are developed in Dluhy's group.

IR surface sampling techniques such as polarization modulation external reflection IR (PM-IRRAS) and ATR-FTIR spectroscopy, as well as surface techniques such as Langmuir-

Blodgett transfer were employed for investigation of wild type rdhSP-C and its mutant in lipid environment. Compared to other surface study techniques such as synchrotron X-ray diffraction, nonlinear secondary harmonic frequency generation (SHG) and sum frequency generation (SFG) techniques, PM-IRRAS and ATR-FTIR prove to be simple, economic yet powerful tools for biophysical studies on surface. PM-IRRAS in combination with Langmuir film study at the air/water interface is especially suitable for *in situ* studies of model pulmonary surfactant system which mimics the biological lung system. Polarization ATR-FTIR has been widely applied for investigation of biomembrane system. Important information such as molecular orientation may be quantitatively extracted from empirical measured dichroic ratios. It is of note that, despite of the large amount of investigations on the α to β structure conversion of SP-C, the orientation of β -sheet of SP-C in phospholipid bilayers has not been clearly characterized. In chapter 6, polarization ATR-FTIR spectroscopy is employed to investigate the orientation of β -sheet in lipid bilayers.

Finally, an intrinsic surface enhanced Raman spectroscopy (SERS) technique based on silver nanorods (AgNRs) platform is employed in identification and differentiation of rotavirus strains. Chemometrics is employed in data interpretation of SERS spectra. Compared to traditional techniques such as enzyme-linked immunosorbant assays (ELISA) and polymerase chain reaction (PCR), SERS has great potential to be an alternative approach for clinic diagnosis as a fast, economic and reliable method. The test report in the last chapter demonstrates these desirable properties of the intrinsic SERS based on AgNRs platform.

CHAPTER 2

LITERATURE REVIEW

2.1 Pulmonary Surfactant

Pulmonary surfactant are substances existing in the lung alveolar aqueous lining films, maintaining lung structure and facilitating the normal inspiration and expiration during breathing cycles, as well as preventing invading pathogens and particulates. Insufficiency in lung surfactant causes fatal respiratory deficiency syndrome (RDS) in premature infants or older children, or acute RDS (ARDS) in adults from lung injury. It is estimated that exogenous surfactant treatment can reduce the mortality rates associated with RDS by 40% to 60%. [1]

The bulk of pulmonary surfactant consists of ~80% phospholipids, ~10% neutral lipids such as cholesterol, and ~10% pulmonary surfactant proteins. Phosphatidylcholine (~80%) and phosphatidylglycerol (PG) (~10%) constitute the bulk of phospholipids, of which disaturated PC/PG are the major forms. Differentiated from other cell membranes, lung surfactant contains much higher portion of dipalmitoyl phosphatidylcholine (DPPC) (~ 40% of PC). Physical properties of phospholipids are determined by the type of the chemical moiety attached to the phosphate head group and the length of the hydrocarbon chains attached to the glycerol backbones. One of the important physical properties of phospholipids is the gel-to-liquid-crystal phase transition temperature (T_c). For example, T_c of (DPPC) is 41°C. The symmetric disaturated hydrocarbon chains (16:0) can pack tightly and form solid phase at high surface pressure up to ~70mN/m (i.e. low surface tension near 0mN/m). A DPPC-rich monolayer is proposed to be

essential to for alveolar lining film to attain near zero surface tension with minimal surface area compression to resist collapse at the end of expiration. However, DPPC alone cannot adsorb and re-spread quickly enough to incorporate into the aqueous monolayer upon inhalation. The presence of unsaturated phospholipids and acidic phospholipids, mainly PG, in pulmonary surfactants are implicated to enhance the fluidity and facilitate re-adsorption. [2] Other lipids including phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), as well as sphingolmyelin and fatty acids also exist in pulmonary surfactant in small amount. The structures of representative lipids are given in **Fig2.1**.

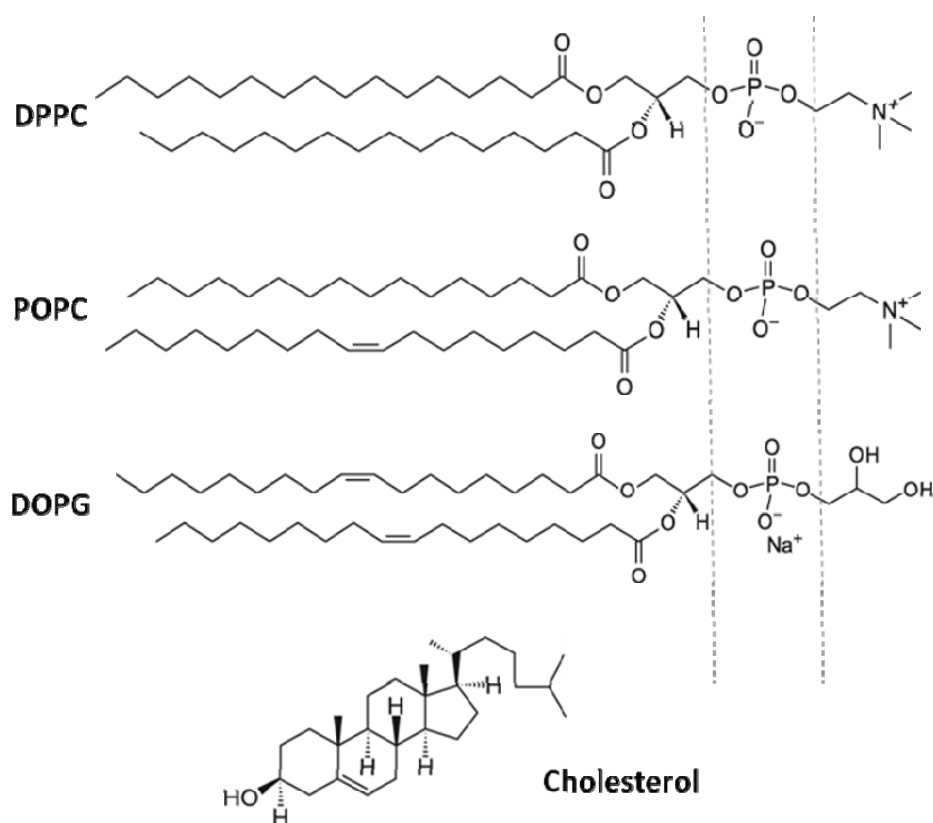


Fig2.1. Structures of the most common lipids.

Table2.1. Phase transition temperatures of selected phospholipids.

Glycerophospholipids	Fatty Chains (<i>sn</i> -1, <i>sn</i> -2)	Gel to liquid crystal phase transition $T_c(^{\circ}\text{C})$
DPPC	(C16:0, C16:0)	41
DPPG	(C16:0, C16:0)	41 (Na^+)
POPC	(C18:1, C18:0)	-2
DOPG	(C18:1, C18:1)	-18

Table2.1. listed the most common PC and PG. Full physiological function of lung surfactant depends on lipids-protein interfacial activities. There are four pulmonary surfactant proteins. SP- A and SP-D are hydrophilic proteins responsible for host innate defense against the invading pathogens such as bacteria, virus, fungi, as well as particulates such as pollen. SP-B and SP-C are hydrophobic proteins mainly responsible for the adsorption, spreading and stability of the alveolar lining films during the breathing cycles. Recent studies have found that SP-B plays crucial roles in trafficking of phospholipids and biosynthesis of SP-C. SP-B knock-out mice exhibited abnormal formation of lamellar body, an internal organelle for packing and restoring phospholipids with SP-B and SP-C, and dominant negative formation of SP-C. However, the discussion of SP-A, SP-D and SP-B is beyond of the scale of the presenting research work. Reviews on SP-A, SP-D and SP-B can be found in Ref. [3, 4]

Mature SP-C is a type II transmembrane lipoprotein exclusively expressed in pulmonary alveolar type II (ATII) cells. [5] Human SP-C proprotein (proSP-C) (21kDa) is synthesized at the endoplasmic reticulum with 191-197 amino acids, and then translocated to Golgi apparatus. After post-translocation and proteolytic cleavage, matured SP-C is secreted into the alveolar linings together with SP-B and phospholipids. [6] The molecular structure of mature SP-C is highly conserved through species. Four structure motifs of mature SP-C have been characterized

by NMR study in organic solvents: 1) two palmitoylated Cysteine residues (C5 and C6) surrounded by two Prolines at the N-terminus, 2) a positively charged Lys-Arg (K11 and R12) pair, 3) a very hydrophobic poly-valine segment, and 4) a heptapeptide C-terminus. [7] NMR study in addition indicated that mature SP-C in apolar solvent contains a rigid α -helix consisting of residues 9-34. The length of the α -helix is about 37Å, which is in agreement with the length to span in the lipid bilayers.

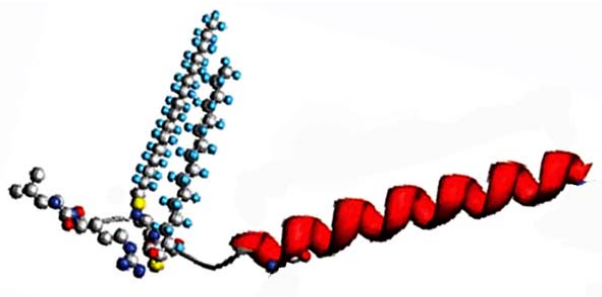


Fig2.2. Adapted molecular structure model of mature SP-C. (PDB ID: 1SPF)

Surface activity of mature SP-C together with SP-B in maintaining the alveolar structure during inspiration and expiration cycles has been extensively studied. SP-B and SP-C seem having overlapped functionality in mediation of phospholipids phase behavior during breathing cycles. [2, 8] Recently Serrano and Perez-Gil reviewed studies of pulmonary surfactant system at air/water interface including commonly employed physical techniques.[9] Structural-functional correlation studies of SP-C have focused on the function of the palmitoyl chains thiol-linked to the Cysteine pair. It was shown that palmitoylation is necessary for optimal surface active interaction of SP-C with phospholipids. [10] Most recently, Nakorn *et al* reported the role of palmitoylation with different length of α -helix. It is believed that palmitoylation not only mediated lipid-protein interaction within membrane, but also contributes to connection to neighboring bilayers to induce the formation of stacking bilayers. [11] Although the helical

polyvaline segment formed β -aggregations in apolar solvent, palmitoylation reduced the unfolding of SP-C into fibrillogenic intermediates. [12-14]

The role of SP-C in lung diseases has largely emerged thanks to the advancing knowledge on biosynthesis of SP-C. Misfolding of SP-C has been associated with interstitial lung diseases (ILD). The intrinsic propensity of the helical polyvaline segment to form β -structure was proposed to be a driving force in misfolding of SP-C. [15] In addition, studies have also focused on the genetic determinants for misfolding of SP-C. Mutageneses of SP-C have been found with proSP-C that subsequently results in abnormal synthesis, and misfolding of mature SP-C. [16] Of these studies, most were done with immunocytochemistry methods. [17-19] The role of the juxtamembrane Lys-Arg pair (corresponding to K11/R12 in mature SP-C) has been examined. The Lys-Arg pair were found having influence on palmitoylation by preventing trafficking of proSP-C out of ER. [18] In addition, the Lys-Arg pair plays role in determination of the orientation of proSP-C in ER membrane. It is of interesting to investigate how the Lys-Arg pair functions in mature SP-C in lipid bilayers.

2.2 Langmuir monolayer

Langmuir monolayers have been a fascinating area for physical chemistry and surface science with a long history. Insoluble amphiphilic molecules such as lipids can spontaneously form a monomolecular film on the surface of water. The thermodynamic characters of Langmuir monolayers have been extensively investigated. [20] One of the importance of Langmuir monolayers pertain to biomembrane studies lies in the fact that the effective internal lateral pressure of biological membranes and phospholipid bilayers cannot be directly measured. However, lateral compressibility, which is the derivative of the effective internal lateral pressure with respect to area, may be determined for lipid monolayer from the Π -A isotherms.

Furthermore, lateral compressibility is more relevant for partitioning of molecules into the membrane, or for conformational changes of embedded proteins. [21] The thermodynamic basis of bilayer-monolayer equivalence has been established and thoroughly discussed in Ref. [21] Many modern techniques has been employed for investigation of Langmuir films, such as fluorescence microscopy, vibrational spectroscopy, synchrotron X-ray diffraction and non-linear sum frequency generation (SFG). [22] All these techniques involve monolayer experiments on a Langmuir trough.

As stated above, the Π -A isotherm is directly measured for a monolayer on aqueous subphase. The surface tension (γ_0) of clean aqueous subphase such as water is reduced by the existence of the monolayer, which has surface tension (γ). The lateral change in the surface tension of a monolayer upon compression is monitored using a Wilhelmy plate in terms of surface pressure (π), which is defined as $\pi = \gamma_0 - \gamma$. At room temperature, the surface tension of water is about 72mN/m. Therefore, $\pi = 72 - \gamma$. The two-dimensional ordering of the monolayer at the aqueous subphase is directly reflected by the Π -A isotherm.

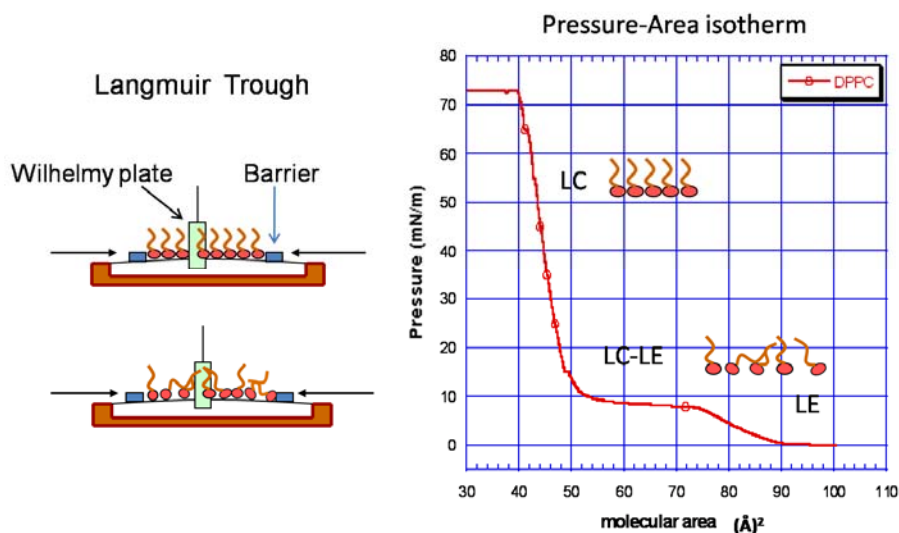


Fig2.3. Monolayer experiment with Langmuir trough.

Fig2.3. illustrated the instrumentation of Langmuir trough with a typical isotherm of DPPC monolayer on air/water surface. Vibrational spectroscopic measurements of the Langmuir monolayer therefore reveal *in situ* information such as phase transition and phase partition of phospholipids, as well as surface pressure induced protein conformational changes. Monolayer at the air/water interface can also be transferred to solid substrate by Langmuir-Blodgett transfer. Ordered multilayer thin films are usually obtained by sequential transfers of monolayers at a desired surface pressure. Depending on the property of the substrate, i.e. hydrophilic (such as glass, Ge, and quartz) or hydrophobic (such as silicon, Ag, Au, and silanized glass) three types of deposition are formed accordingly. [23, 24] **Fig2.4.** demonstrated the starting position of the substrates in deposition in corresponding to substrate properties, and the types formed from deposition. Transfer of phospholipids to hydrophilic substrate to form y-type multilayer is usually difficult, as the coupling of the second layer generally results in peel-off the first layer therefore prevents further deposition. A z-type may be achieved for phospholipids to form multiplayer on hydrophilic substrate. [25]

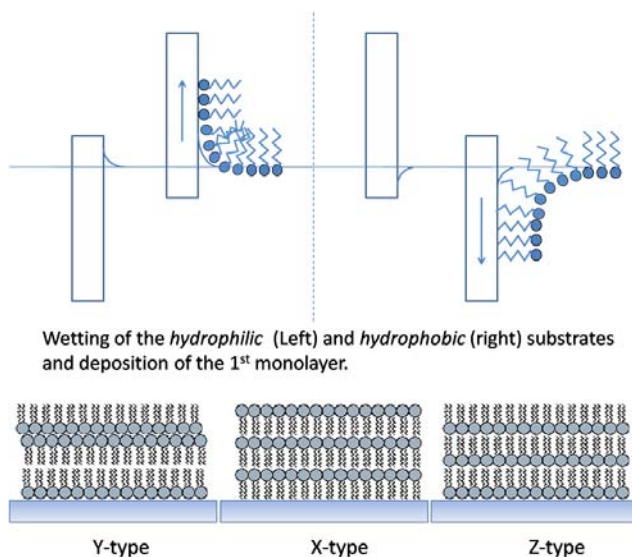


Fig2.4. Langmuir-Blodgett transfer of monolayers at the air/water interface.

2.3 FTIR Spectroscopy for Biomembrane Study

FTIR spectroscopy provides a powerful tool in characterization of the structure of biomolecules. Vibrational modes of the biomolecules may be investigated in different environments, e.g. solvent or lipid matrix. In the latter case, the interaction between the biomolecules and lipid molecules may also be investigated. Application of FTIR spectroscopy on biomembrane studies has advantages in terms of simple sample preparation and fast dynamic range. For example, X-ray diffraction studies require perfect crystallization of proteins, which may be difficult in case of membrane proteins. NMR spectroscopy has a much slower dynamics about 10^5Hz compared to IR ($\sim 10^{13}\text{Hz}$). In addition, resonance from lipid components can obscure signals from membrane proteins. [26] Whereas IR absorption of the secondary structure of proteins are generally separated from that of lipids. Various IR sampling techniques such as external reflection-absorption IR spectroscopy (IRRAS) at the air/water interface and attenuated internal reflectance IR spectroscopy (ATR-FTIR) in addition are employed for investigation of mimic biomembrane system *in situ* and on the surface, which may presents different natures from bulk samples.

IR radiation ranges from $1\mu\text{m}$ to 1mm in the electromagnetic spectrum. Mid-IR ranges from $4000\text{-}300\text{cm}^{-1}$ with fast dynamics of the order about 10^{-13}s . In classic mechanism, the principle governs IR spectroscopy can be modeled by a simple diatomic molecule of a harmonic oscillator. Vibrational frequency of the diatomic oscillator may be calculated by

$$\nu = \frac{1}{2\pi\sqrt{k/\mu}}, \text{ where } \mu = m_1m_2/m_1+m_2 \text{ is the reduced mass, } k \text{ is the elastic force constant. In}$$

quantum mechanism, the molecule is promoted from the ground state to the excited state only

when its dipole moment μ changes during interaction with the electric field, i.e. $\partial\mu/\partial Q \neq 0$, where Q is the normal coordinate system describing the motion of the atoms. [27]

Spectral-Structural Correlation

The vibrational frequencies of a molecule are determined by its three dimensional structures and its vibrational force field. Peptides and proteins backbones have characteristic vibrational modes called amide modes. In early studies of peptides and proteins, the characteristic frequencies of peptide amide group were empirically identified and correlated to the molecular structure using small molecules such as *N*-methylacetamide. [28, 29] Miyazawa *et al* first performed theoretical calculation of the normal modes of the vibration of *N*-methylacetamide, which advanced the understanding in spectra-structural correlation of peptides. [30] Later advances in computation by Krimm *et al* refined the vibrational force field model and extended the calculation to known polypeptide models such as β -poly(L-alanine) (antiparallel β -sheet) and α -poly(L-alanine) (α -helix). In comparison to the results from X-ray diffraction and empirical assigned vibrational frequencies, it was then established the basis for structural-spectral correlation for IR and Raman spectroscopy. [31] In summary, band assignment for both phospholipids and proteins are listed in **Table2.2** and **Table2.3**.

Analysis of β -sheet structure has been further promoted by the emerging of amyloid-fibril conformational diseases [32] and misfolding of proteins into amyloid like fibrils. [33] The structure of amyloid-fibril has been established by X-ray diffraction as a cross- β structure, i.e. the axis of the β -strands is perpendicular to the fibril axis. [33] IR frequency of amyloid fibril structure was clearly distinguished from native extended antiparallel β -sheet structure. [34] *Ab initio* simulation also predicated short “twisted” β -strands/hairpin structures to be distinguished from extended “planar” β -sheet. [35] On the other hand, it was suggested that natural β -sheet

proteins developed a negative strategy to prevent aggregation formation by adopting placed charges, short twisted strands and loops to avoid the formation of regular β -sheet edges. [36]

Table2.2 Characteristic vibrational modes of phospholipids in mid-IR region. [26, 37]

Functional group	Vibrational Mode	Approx. Freq. (cm^{-1})	Structure information
Hydrocarbon chain	CH ₃ asym.	2956	
	CH ₃ sym	2870	
	CH ₂ asym. str.	2920	Quantitative markers of chain conformations, i.e. <i>all-trans</i> or <i>gauche</i> .
	CH ₂ sym. str.	2850	
	CD ₂ asym. str.	2195-2200	
	CD ₂ sym. str.	2090-2100	
	CH ₂ scissoring	1462, 1473	Orthorhombic phase doublet
		1468	Hexagonal or triclinic phases
	CD ₂ scissoring	1086,1094	Orthorhombic phase doublet (perpendicular subcell packing)
		1089	Hexagonal or triclinic Phases
Polar region	C=O	1700-1750	Sensitive to hydrogen bonding, hydration
	PO ₂ ⁻ asym. str.	1220-1260	Sensitive to ion binding, hydration
	PO ₂ ⁻ sym str.	1090-1085	

Table2.3 Vibrational modes of protein secondary structures in mid-IR region. [31, 38]

Normal modes of protein backbone vibrations		Amide I vibrational modes	
mid-IR region (cm ⁻¹)	Potential energy distribution	Secondary structure	Frequency (cm ⁻¹)
Amide I (1700-1600)	C=O str. (83)+ CN str. (15)	Antiparallel β -sheet	1675-1695(w) 1640-1625(s)
Amide II (1520-1560)	NH ipb(49)+ CH str. (33)	β -turns	1660-1685
Amide III (1350-1200)	NH ipb(52)+ CC str. (18)	3_{10} -helix	1660-1670
Amide A (3400-3200)	N-H Str. (100)	α -helix	1648-1660
		Unordered (denatured)	1652-1660 (1640-1648)
		β -aggregated strands	1610-1628

2.3.1 (PM)IRRAS Spectroscopy at air/water interface

IRRAS has long been used to study thin films deposited on metallic substrates. The reflection spectrum strongly depends on the optical constants of the thin films and the substrate of the stratified system. A three-phase stratified system is illustrated in **Fig2.5**. Radiation of light on a flat metal surface results in reflection at the surface boundary and a phase shift from the incident light. The magnitude of the phase shift is polarization dependent. The perpendicular (s-) polarized incident light (E_y) undergoes a phase shift of π at the metallic surface. As a result, the sum electric field at surface is near zero for s-polarized light. The phase shift for the parallel (p-) incident light (E_x+E_z) depends on the incident angle. At grazing angle, the phase shift is about

$\pi/2$ with the sum electric field normal to the surface. IRRAS can therefore provide information on molecular orientations of surface molecules such as metallic supported polymers and biomembrane proteins. The intensity of the reflection-absorbance spectrum is determined by the ratio of the reflectance of the sample against the reflectance of the sample-free substrate: $A = -\log(\frac{R}{R_0})$.

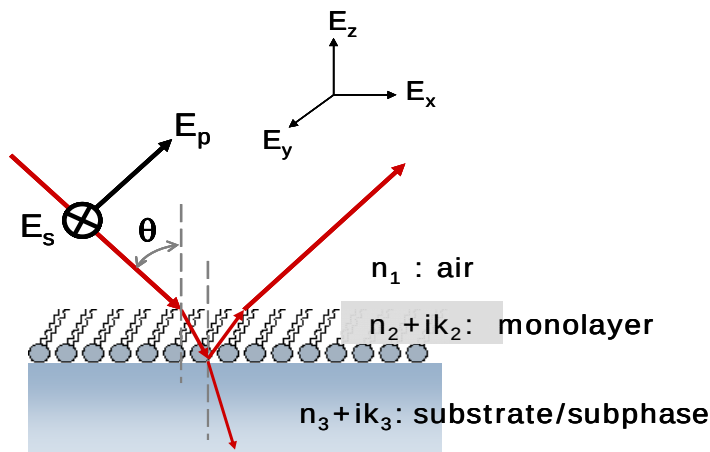


Fig2.5. An illustration of the electric fields at a three-phase stratified surface system. In case for external reflectance at the air/substrate interface, light travels from the rare medium (n_1) into the dense medium (n_3). $n_i + ik_i$ is the complex refractive index.

In the mid 1980's, Dluhy and coworkers first demonstrated the feasibility of conducting IRRAS on air/water interface. [39, 40] Since then, successful work have been done in investigation of model biological system such as pulmonary surfactants. [37, 41, 42] For example, phase transition of phospholipids play important roles in maintaining lung structures during breathing cycles. Surface pressure induced phase transition of phospholipids has been directly demonstrated at the air/water interface by monitoring the frequency shift of the CH_2 band in the region ($3010\text{-}2750\text{cm}^{-1}$). As surface pressure increased, the symmetric (~ 2850) and the asymmetric (~ 2920) CH_2 stretch modes all down shifted to lower frequencies, indicating

ordered chain conformation.[43] IRRAS at air/water interface has also been used to demonstrate phase separation of binary phospholipids monolayer along, or in presence of proteins. Compared to IRRAS on air/metal surface, IRRAS on air/water interface possesses some distinguishing natures. First, both s- and p- polarization yield reflection at the air/water interface. Depending on the nature of polarization, negative bands may be observed as a result. The interpretation of IRRAS spectra at the air/water interface could be complicated. Second, water is a fairly weak substance for reflection. In addition, the abnormal dispersion of water bending mode results in a distortion of reflection spectrum at about $\sim 1645\text{cm}^{-1}$. Therefore, it is more difficult to acquire spectra with good signal to noise ratio for IRRAS at air/water interface. On the other hand, strong water vapor absorption in the region $1800\sim 1500\text{cm}^{-1}$ overlapped with amide I and amide II region, where information of protein secondary structure is mostly extracted. Two instrumental setups were developed to improve data acquisition. The Rutgers group uses a computer-controlled shuttle switching between a reference well and a sample well of the Langmuir trough. [44] In this way, signals from water vapor are compensated by the ratio of the sample spectrum (R) to the reference spectrum (R_0). This setup has been used in investigation on the pulmonary surfactant model systems. Monolayers containing PC-PG binary system or DPPC-deacylated-SP-C mixtures were investigated respectively. It was concluded that a “squeeze-out” of PG component at a high surface pressure ($50\text{-}60\text{mN/m}$) was achieved most for unsaturated PG and, in a concentration-dependent manner for POPG.[45] It was also shown that deacylated SP-C introduced fluidity for DPPC monolayer at surface pressure lower than 30mN/m , but had no effect on the structure or molecular orientations of DPPC nor that of SP-C itself at higher surface pressure.[46] In addition to characterization of thermodynamics of the modeled lung surfactant monolayers, The Rutgers group also investigated the secondary

structure and the orientation of SP-C in phospholipids monolayers, Investigation was done with various incident angles (30°-50°) of different polarization at the monolayer in order to determine the tilt angle of SP-C at the air/water interface. By comparing the measured spectra to the simulated ones, it was concluded that the SP-C helix tilt angle was changed from 24° relative to the surface normal in a lipid bilayers to ~70° in the DPPC/SP-C mixture monolayer. Meanwhile the chain tilt angle of DPPC decreased from ~26° in pure DPPC monolayer to ~10° in DPPC/SP-C monolayer.[47]

Alternatively, polarization-modulation IRRAS (PM-IRRAS) was developed by the French group. [48, 49] PM-IRRAS is a double modulation method. The output signal from the detector consists of two parts. One is the intensity modulation (I_+) induced by the moving mirror of the FTIR spectrometer. The other is the polarization modulation (I_-) induced by the photoelastic modulator. PM-IRRAS signal is given by the ratio of the two parts:

$$S = \frac{I_-}{I_+} = C \frac{J_2(\phi_0)(R_p - R_s)}{(R_p + R_s) + J_0(R_p - R_s)} \quad (2.1)$$

where J_2 and J_0 are the second- and zero-order Bessel functions of the maximum dephasing ϕ_0 introduced by the photoelastic modulator. Typical PM-IRRAS instrumentation utilizes two-channel electronics with a lock-in amplifier for signal process, as illustrated in **Fig2.6**.

The fast modulation of incident light between the p- and the s- polarization results in discrimination of the anisotropic absorptions of surface molecules from the isotropic absorption from the ambience, e.g. water vapor, therefore effectively enhances the spectral quality. As first demonstrated by Buffeteau *et al* for ultra-thin films deposited on metallic substrate, PM-IRRAS signal exhibit a linear dependent on the thickness and the extinction coefficient of the deposited film. For PM-IRRAS at the air/water interface, an optimized incident angle was determined at

76°. In addition to conformational investigations of Langmuir films, [50] PM-IRRAS has also been applied to studies on molecular orientation of biomolecules. [51]

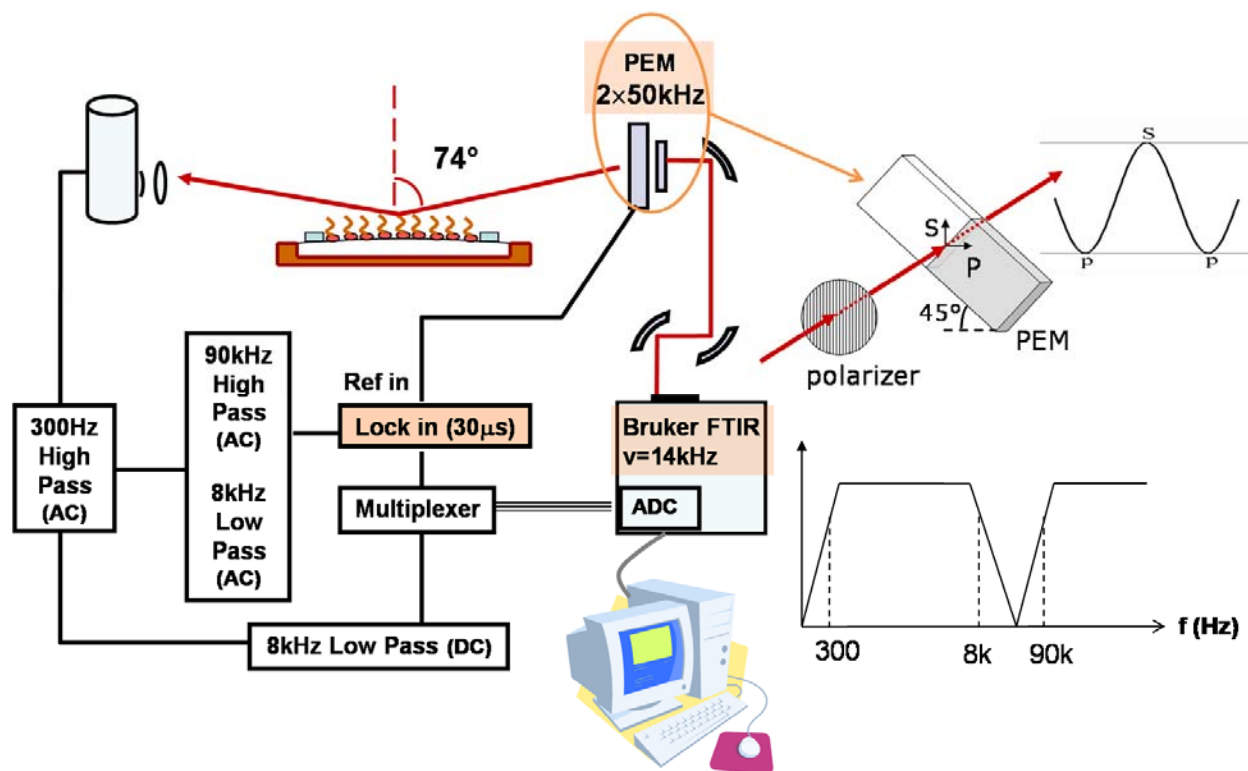


Fig2.6. Instrumentation of PM-IRRAS at the air/water interface.

Dluhy group have used IRRAS and PM-IRRAS in investigation of modeled pulmonary surfactant monolayer at the air/water interface. [42, 52]

2.3.2 ATR-FTIR Spectroscopy

ATR-FTIR has been one of the most powerful techniques used in biomembrane structure studies. Compared to studies conducted with bulk sample, ATR-FTIR spectrum of thin absorbed films have excellent signal to noise ratio. In addition, the problematic water absorption in IR region has been much reduced compared to transmission IR as the penetration depth is usually within several microns. Total Internal Reflection is a phenomenon occurring as the incident light propagating through an optical dense medium into an optical rare medium at an incident angle

larger than the critical angle θ_c , as determined by $\theta_c = \sin^{-1} n_{21}$, where $n_{21}=n_2/n_1$, n_1 and n_2 are the refractive indices for the internal reflection element (IRE) and the rare medium respectively. The incident and the reflected wave superimpose within the IRE and yield a standing wave normal to the total reflecting surface. The standing wave exponentially decays in the medium in contact with the IRE, i.e. attenuated. The average of the electric field resulted is a function of the penetration depth d_p .

$$E = E_0 \cdot \exp\left(-\frac{z}{d_p}\right) \quad (2.2)$$

$$d_p = \frac{\lambda / n_1}{2\pi \sqrt{\sin^2 \theta - n_{21}^2}} \quad (2.3)$$

Various geometry designs have been developed in ATR-FTIR experiments. [53] The most usual one is with a trapezoidal optical plate. Multiple reflections occur within the trapezoidal IRE. The number of reflections N is determined by the length l , the thickness t of the IRE, as well as the incident angle θ .

$$N = \frac{l}{t} \cdot \cot \theta \quad (2.4)$$

The absorption of a thin film in contact with IRE can be theoretically calculated by:

$$A = \frac{n_2}{n_1} \frac{\alpha t}{\log_{10} \cos \theta} \langle E^2 \rangle \quad (2.5)$$

where α is the absorption coefficient. One advantage with ATR-FTIR is that molecular orientation can be quantitatively determined with polarized incident light.

Sample preparation for ATR-FTIR studies

Various techniques are developed for preparation of model membrane systems. Multilayer may be formed on ATR crystal by direct evaporation of solvent followed by

hydration. Oriented multilayer may be deposited on IRE by Langmuir transfer technique. Hydrated sample of vesicles can form bilayers on IRE by self-assembling. Tamm et al proposed a modified way combining the Langmuir transfer with vesicle self-assembling. [54] In this “vesicle-fusion” method, a monolayer is first transferred to IRE by Langmuir transfer, the second layer is then coupled to the transferred monolayer by incubation of vesicle solutions on the monolayer. The kinetics of vesicle adsorption, fusion and desorption were investigated with fluorescence recovery after photobleaching (FRAP). The formation of a single bilayer on the quartz substrate was verified. [55]

Table2.4. Optical and Physical Properties of different IRE materials. [25]

Materials	Incident Angle (θ)	Refractive Index (n)	Hardness (Knoop)
Ge	30° 45° 60°	4.0	550
Si	30° 45° 60°	3.42	1150
ZnS	30° 45° 60°	2.42	355
KRS-5	30° 45° 60°	2.35	40

Determination of molecular orientation.

The applicability of determination of molecular orientation using polarized IR spectroscopy (transmission, external or internal reflectance IR spectroscopy) has rooted in the fact that IR absorbance is in proportion to the square of the product of the transition moment μ and the projection of the electric field E of the polarized IR radiation on μ , i.e. $A = |\mu E|^2 \cos^2 \phi$,

where ϕ is the angle between μ and $\mathbf{E}$. In an oriented system, the experimentally determined dichroic ratio, R represents an averaged ensemble of all orientation fluctuations of the transition moment.

$$R = \frac{A_{\parallel}}{A_{\perp}} = \frac{A_x + A_z}{A_y} \quad (2.6)$$

The spacial distribution of the transition moment is therefore defined by its distribution function in a fixed laboratory Cartesian coordinate. The orientation distribution of the transition moment around the defined molecular main axis may be expressed by conversion of the molecular crystallographic coordinates (a, b, c) into the laboratory Cartesian coordinates (x, y, z) using Euler angles. **Fig2.7.** illustrated uniaxial and biaxial distribution of the transition dipole moment in the fixed laboratory frame.

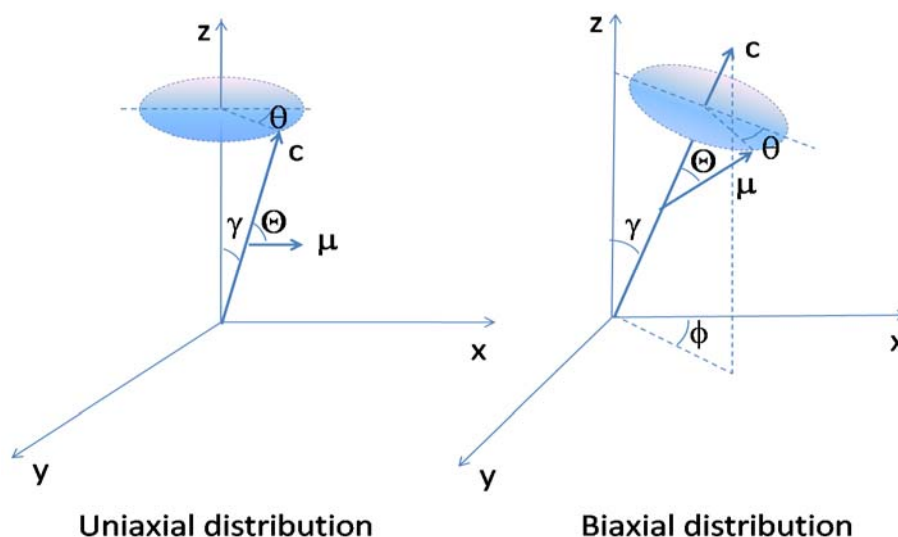


Fig2.7. Molecular orientation distributions of the transition dipole moments in a fixed laboratory frame.

Assuming the angle between a specific transition moment and the molecular main axis Θ is fixed, $\mu_i = \mu_i(\theta, \phi, \gamma, \Theta)$, the distribution function of μ_i is:

$$N_0 = \int_{\gamma} \int_{\phi} \int_{\theta} N(\theta, \phi, \gamma) d\theta d\phi d\gamma \quad (2.7)$$

The absorbance of the specific transition moment and its dichroic ratio are therefore given by:

$$A_i = \int_{\gamma} \int_{\phi} \int_{\theta} (\mu_i E_i)^2 N(\theta, \phi, \gamma) d\theta d\phi d\gamma \quad (2.8)$$

Therefore,

$$A_x = \langle (\mu \cdot E_x)^2 \rangle = \langle E_x^2 \rangle \langle \sin^2 \gamma \cos^2 \phi \rangle \quad (2.9)$$

$$A_y = \langle (\mu \cdot E_y)^2 \rangle = \langle E_y^2 \rangle \langle \sin^2 \gamma \sin^2 \phi \rangle \quad (2.10)$$

$$A_z = \langle (\mu \cdot E_z)^2 \rangle = \langle E_z^2 \rangle \langle \cos^2 \gamma \rangle \quad (2.11)$$

Calculation of molecular orientation can therefore be performed with the knowledge of empirically measured dichroic ratio, the amplitudes of mean square electronic fields and the optical constants of the stratified surface system.

Uniaxial Orientation Distribution Models. For molecules of cylindrical symmetry, transition moment is considered to be at a fixed angle Θ with respect to the molecular axis. Then the dichroic ratio is given by:

$$R = \frac{A_{\parallel}}{A_{\perp}} = \frac{A_x + A_z}{A_y} = \frac{\langle E_x^2 \rangle \langle \sin^2 \gamma \rangle + 2 \langle E_z^2 \rangle \langle \cos^2 \gamma \rangle}{\langle E_y^2 \rangle \langle \sin^2 \gamma \rangle} \quad (2.12)$$

The tilt angle γ of the molecular main axis can then be calculated directly. This method was applied by Ahn and Franses [56] to calcium stearate Langmuir-Blodgett monolayer deposited on ATR crystals. The ATR crystal was rotated by 90° to obtain different molecular orientation for dichroic ratio measurement.

Polarization ATR-FTIR has been routinely employed in determination of molecular orientations. [38, 57] The molecular orientation is generally expressed by an order parameter $\langle P_2(\cos \gamma) \rangle$, which depends on both the angular position and the shape of the orientation

distribution. [58] For molecules with uniaxial symmetry, the average distribution of the molecular axis with respect to the normal of the surface plane is expressed by the second order of Legendre polynomial:

$$S = \langle P_2 \cos(\gamma) \rangle = \frac{1}{2} (3 \langle \cos^2 \gamma \rangle - 1) \quad (2.13)$$

By convention, S has a value between -0.5 to 1.0. $S=1.0$ indicates perfect order perpendicular to the membrane surface, $S=-0.5$ indicates perfect order parallel to the membrane surface, and $S=0$ indicates an isotropic distribution or total disorder.

The relationship between anisotropic extinction coefficients $k_{x,y,z}$ and the order parameter (S) is given by Fraser's equation for uniaxial films: [59]

$$k_x = k_z = K[(S \sin^2 \Theta) / 2 + (1 - S) / 3] \quad (2.14.a)$$

$$k_y = K[S \cos^2 \Theta + (1 - S) / 3] \quad (2.14.b)$$

where, K is a constant, Θ is the angle between the transition moment and the molecular main axis. In combination of eq2.14.a and eq2.14.b, the value of the order parameter can be solved as a function of the dichroic ratio and the mean square electric field.

$$S = \frac{2(E_x^2 - RE_y^2 + E_z^2)}{(3 \cos^2 \Theta - 1)(E_x^2 - RE_y^2 - 2E_z^2)} \quad (2.15)$$

In case of DPPC, the transition moment of the all-trans symmetric CH_2 mode of the acyl chain is perpendicular to the chain axis, i.e. $\Theta=90^\circ$. The order parameter for the acyl chain of DPPC is:

$$S = \frac{(E_x^2 - RE_y^2 + E_z^2)}{(E_x^2 - RE_y^2 - 2E_z^2)} \quad (2.16)$$

In case of protein, the angle between the transition moment of α -helix at 1655cm^{-1} and the helix axis have been determined with different techniques, the value varies from 29° to 40° . In determination of the orientation of α -helix using the amide I band, Frey and Tamm takes the

fraction of α -helix of all conformations into account. The order parameter for α -helix is therefore calculated by

$$S = \frac{2(E_x^2 - RE_y^2 + E_z^2)}{f_H(3\cos^2\Theta)(E_x^2 - RE_y^2 - 2E_z^2)} \quad (2.17)$$

This formalism was applied in determination of the orientation of melittin in phospholipid bilayers. [57]

Biaxial Orientation Distribution Models. When molecular orientation takes an asymmetric biaxial distribution, such as β -sheet, calculation of the order parameters is no longer held as by eq2.13 and eq2.15. Instead, the orientation of β -sheet is calculated based on the molecular geometry in the fixed laboratory frame. Notice that the orientation of β -strands with respect to the membrane normal can still be evaluated by eq2.12.

The orientation of β -sheet is determined by the sheet orientation (α) with respect to the membrane normal and the β -strand orientation (β) within the sheet. Marsh introduced a model by measuring the dichroic ratios of the orthogonal transition moments of amide I and amide II respectively to determine the orientation of β -sheet. [60] The orientation (γ) of β -strands of the sheet relative to the membrane normal is related to the above two orientation parameters by: $\cos\gamma = \cos\alpha \cdot \cos\beta$. The amide I anti-parallel β -sheet at 1635cm^{-1} has transition moment $\nu_{\perp}(\pi,0)$ perpendicular to antiparallel β -sheet long axis. Calculation at 1635cm^{-1} resulted in a compound average orientation parameter which involves both γ and the azimuthal orientation of the sheet plane. Importantly, the tilt angle γ of β -strands of the sheet alone may be directly evaluated from amide II around 1530cm^{-1} , which has transition moment $\nu_{\parallel}(0,\pi)$ parallel to antiparallel β -sheet long axis. Therefore the combination of the dichroic ratios of the orthogonal amide I and amide II bands can provide discrete values of α and β . The weak component of anti-parallel β -sheet

around 1685cm^{-1} has the same transition moment orientation as that of the amide II band at 1530cm^{-1} . However, its application for orientation calculation could be limited if its intensity is too low.

Buffeteau *et al* determined orientation of the thin LB film of both α -helical polypeptide poly- γ -benzyl-L-glutamate (PBG) and antiparallel β -sheets of model synthetic peptide K(LK)₇ from the calculation of anisotropic optical constants of the oriented system in the rotation matrix. In this approach, it is not necessary to know the amplitudes of mean squared electric fields. [61, 62] The Rutgers group determined molecular orientation of Langmuir films by measuring IRRAS spectra at different incident angles and/or for different polarizations. [63, 64]

Factors influence the calculation of molecular orientation.

Mean squared electric fields. [65] Regardless of the model used to calculate molecular orientation, it is necessary to know the amplitude of mean squared electric fields of the oriented system. Three models have been used for calculations of mean squared electric fields of the stratified system. The same stratified three-phase system as described in **Fig2.5.** can be employed for ATR-FTIR. However, light travels in a reverse direction, i.e. from the dense medium to the rare medium.

Model 1. Harrick thick-film approximation (two-phase approximation). In this model, the film thickness is considered to be very small in comparison to the penetration depth of the evanescent wave ($d \ll \lambda$). The amplitudes of electric field of the evanescent wave penetrating into the thin film are therefore ignored and equivalent to that of the electric field at the interface in the support and bulk phase. At incident angle ϕ :

$$\langle E_x^2 \rangle = \frac{4 \cos^2 \phi_1 (\sin^2 \phi_1 - n_{31}^2)}{(1 - n_{31}^2) [(1 + n_{31}^2) \sin^2 \phi_1 - n_{31}^2]} \quad (2.18)$$

$$\langle E_y^2 \rangle = \frac{4 \cos^2 \phi_1}{(1 - n_{31}^2)} \quad (2.19)$$

$$\langle E_z^2 \rangle = \frac{4 \cos^2 \phi_1 \sin^2 \phi_1}{(1 - n_{31}^2)[(1 + n_{31}^2) \sin^2 \phi_1 - n_{31}^2]} \quad (2.20)$$

Model 2. Harrick thin-film approximation (three-phase approximation). Compared to the two-phase approximation, the thin-film approximation is significantly different from the two-phase approximation in the z direction of the electric field. The amplitudes of electric field of the evanescent wave is considered remaining constant as $d \ll \lambda$. As compared to eq2.20, the mean square electric field in the z direction is expressed by

$$\langle E_z^2 \rangle = \frac{4 n_{32}^4 \cos^2 \phi_1 \sin^2 \phi_1}{(1 - n_{31}^2)[(1 + n_{31}^2) \sin^2 \phi_1 - n_{31}^2]} \quad (2.21)$$

Model 3. Thickness-and-absorption-dependent three-phase approximation. Axelsen and Citra [66] calculated the electric field in a more strict way by taking the thickness and absorption of the film into account. Reflection and absorption are considered for the semifinal phases and intermediate phases respectively. This model was applied to the calculation of α -helix tilt angles of polypeptides. In comparison to the two-phase and three-phase approximations, it was concluded that the two-phase approximation was more suitable for supported monolayers and bilayers with thickness $d \ll \lambda$, while both thin film approximation and thickness-and-absorption-dependent three-phase approximation may resulted erroneous and unphysical conclusion.

Picard et al [67] investigated the above three approximations for different bilayers of phospholipids, i.e. dry films and hydrated films of bilayers formed by self-assembling of phospholipids from SUVs. The calculations of the chain tilt angle using three models yielded apparent differences for dry films, but gave reasonable accuracy for hydrated films. Further, the

accuracy of chain tilt angle was more susceptible to the variation of dichroic ratio for hydrated films than for dry films. It was concluded that the accuracy of refractive indices of the oriented system and that of the dichroic ratio may affect the determination of molecular axial tilt angle. For films thicker than 200Å and up to 0.5µm, the stricter thickness-and-absorption-dependent approximation should be adopted. For non-uniaxial oriented system such as β -sheet and heterogeneous system of protein-lipid mixtures, Harrick thin film approximation may not be applicable.

Optical constants. Accurate calculation of molecular orientation also depends on the accurate knowledge of the optical constants. It has been estimated that the change of refractive index by 0.1 may lead to a change in the measurement of the tilt chain angle up to 5° or even more. [56] Experimental measurement of optical constants can be done with various geometry setup, such as external reflection or ATR-FTIR, or use a combination of polarized transmission IR with external reflection IR. Kramer-Kronig Transformation has been widely used to calculate optical constants from spectrum. Isotropic optical constants can be obtained by measurement of one constant, e.g. the extinction coefficient k , and calculate the other constant, i.e. refractive index n herein, and vice versa. [68, 69] Anisotropic optical constants can be further calculated using Fraser's equation as shown previously. Buffeteau et al [70] reported a general method to determine optical constants from IR reflectance and transmittance measurements. Using this scheme, they investigated oriented LB films using PMIRRAS, [51] as well as thin lipid films using ATR-FTIR. [67] Alternatively, anisotropic optical constants were directly calculated using the rotation matrix formalism, which was applied for uniaxial system such as α -helix and biaxial systems such as β -sheet. [61] Mendelsohn et al calculated the oriented system using anisotropic

optical constants obtained from Fraser's equation [63] or similar as using rotation matrix [71] respectively.

2.4 Two Dimensional (2D) Correlation analysis

2D correlation analysis for IR spectroscopy was introduced by Noda in the early 1990's. The concept was borrowed from 2D-NMR. When an external perturbation, such as concentration, thermal, pH or surface pressure, is introduced to an experimental system, the intensity variations may be recognized for a set of variables. [72]

1. Generalized 2D correlation.

For a set of perturbation-introduced spectra, the intensity variation $y(\nu, t)$ during a fixed time frame ($T_{min} \sim T_{max}$) is a function of the wavenumber variable ν and the external variable t (such as concentration, pH, temperature etc.). The dynamic spectrum $\tilde{y}(\nu, t)$ in response to the external perturbation is introduced by comparing $y(\nu, t)$ to a reference spectrum $\bar{y}(\nu, t)$ (eq2.22). The reference spectrum may be chosen as the original state of the system, or a conveniently defined reference point either at the beginning or at the end of the measurement. As a common practice, the reference spectrum may be the average spectrum calculated by eq2.23.

$$\tilde{y}(\nu, t) = \begin{cases} y(\nu, t) - \bar{y}(\nu) & \text{for } T_{min} \leq t \leq T_{max} \\ 0 & \text{otherwise} \end{cases} \quad (2.22)$$

$$\bar{y}(\nu) = \frac{1}{T_{max} - T_{min}} \int_{T_{min}}^{T_{max}} y(\nu, t) dt \quad (2.23)$$

2D correlation analysis is in nature based on statistical cross analysis for pattern recognition. Cross correlation of the intensity variations of two variables ν_1 and ν_2 can be expressed by a correlation function:

$$X(\nu_1, \nu_2) = \langle \tilde{y}(\nu_1, t) \cdot \tilde{y}(\nu_2, t) \rangle = \Phi(\nu_1, \nu_2) + i\Psi(\nu_1, \nu_2) \quad (2.24)$$

$$\Phi(\nu_1, \nu_2) + i\Psi(\nu_1, \nu_2) = \frac{1}{\pi(T_{\max} - T_{\min})} \times \int_0^\infty \tilde{Y}_1(\omega) \cdot \tilde{Y}_2^*(\omega) d\omega \quad (2.25)$$

To simplify mathematical calculation, $X(\nu_1, \nu_2)$ is treated as a complex number function comprising the two orthogonal components: the real and the imaginary components (eq2.25). The real and the imaginary components can be then calculated respectively by Fourier transformation.

$$\tilde{Y}_1(\omega) = \int_{-\infty}^{\infty} \tilde{y}(\nu_1, t) e^{-i\omega t} dt \quad (2.26.a)$$

$$\tilde{Y}_2^*(\omega) = \int_{-\infty}^{\infty} \tilde{y}(\nu_2, t) e^{+i\omega t} dt \quad (2.26.b)$$

Eq2.27 and Eq2.28 directly result in the synchronous correlation map for $\Phi(\nu_1, \nu_2)$ and the asynchronous correlation map for $\Psi(\nu_1, \nu_2)$ respectively.

$$\Phi(\nu_1, \nu_2) = \frac{1}{m-1} \sum_{j=1}^m \tilde{y}_j(\nu_1) \cdot \tilde{y}_j(\nu_2) \quad (2.27)$$

$$\Psi(\nu_1, \nu_2) = \frac{1}{m-1} \sum_{j=1}^m \tilde{y}_j(\nu_1) \cdot \sum_{k=1}^m N_{j,k} \cdot \tilde{y}_j(\nu_2) \quad (2.28)$$

$$N_{jk} = \begin{cases} 0 & \text{if } j = k \\ \frac{1}{\pi(k-j)} & \text{otherwise} \end{cases} \quad (2.29)$$

where N_{jk} is Hilbert-Noda transformation for simplifying numerical calculation of the asynchronous correlation.

Generalized 2D correlation synchronous map is featured with diagonal auto-peaks and off-diagonal cross peaks. Diagonal auto-peaks are corresponding to the ensemble of peaks resolved in experimental 1D spectra. Off-diagonal cross peaks reveals linear (in phase) intensity change relationship between a pair of peaks. Peaks that simultaneously increasing or decreasing are presented as positive or negative cross peaks respectively. Generalized 2D correlation

asynchronous map is featured only with off-diagonal cross peaks. Only when the intensity changes of a pair of peaks is non-linear (out of phase) will the cross peak present in the contour map. Interpretation of asynchronous map based on Noda's rules provides qualitative information on sequential changes in peak intensities. The sign of the cross peak indicates either advanced or lagged intensity change of one peak compare to the other. However, interpretation of the sequence is not very straightforward. In addition, the whole sequence of intensity changes of all peaks is derived by comparing one pair of peaks to another pair of peaks, therefore usually cannot be fully resolved. Nonetheless, generalized 2D correlation has been proved to be a powerful tool in both structural and kinetic analyses. Examples include the application of generalized 2D correlation in studies of basic molecules such *N*-Methylacetamide [73] and water [74], and macro molecules such as polymers [75] or liquid crystals [76] with either pressure or polarization perturbation.

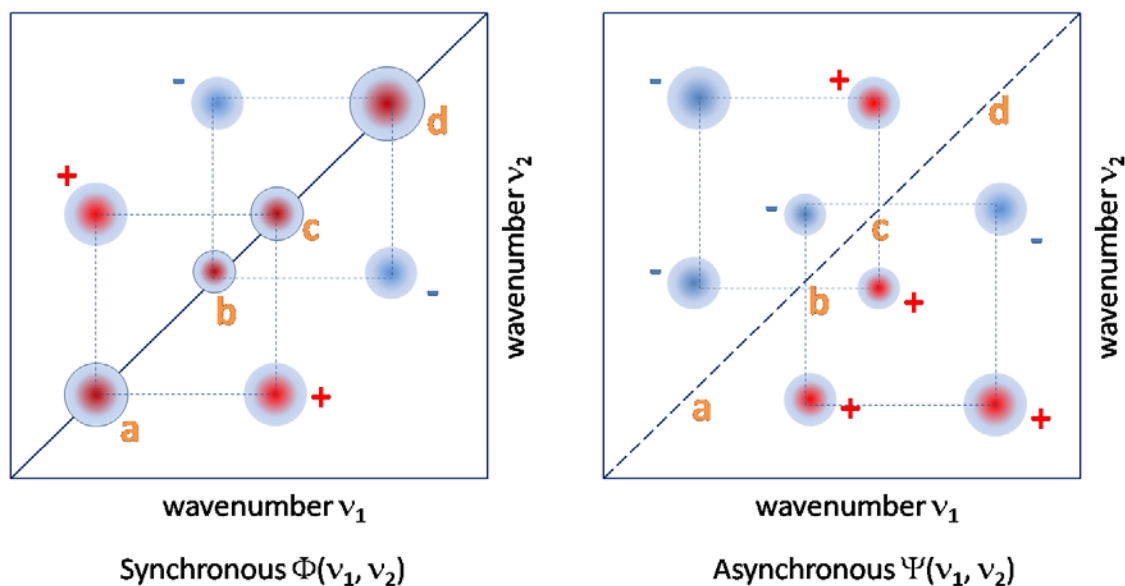


Fig2.8. Generalized 2D correlation contour maps.

Application of generalized 2D correlation on protein structure determination provided a new tool for resolution enhancement for the highly overlapped conformations under amide I

band. Compared to traditional Fourier self-convolution and curve fitting, there is no arbitrary parameters such as band position and band width to be assumed. It is also free from the assumption that the absorption coefficients are the same for all the conformations. Some of the early applications of generalized 2D correlation analysis on proteins studies include monitoring protein thermal-denaturation in aqueous solutions, [77] and investigation of myoglobin secondary structure using H-D exchange of the amino protons as external perturbation. [78] Hetero-correlation between mid-IR and NIR region was employed in thermal unfolding of ribonuclease A. [79] Dluhy's group combined 2D correlation analysis with IRRAS investigation of model biological monolayer at the air/water interfaces. A review has come out recently. [80]

Despite of the apparent advantages with generalized 2D correlation analysis, there may be artifacts generalized in asynchronous map due to the susceptibility to the choice of reference spectrum, spectral noises and overlapping effects, e.g.[81]. In conclusion, data preprocess such as normalization is essential for qualified 2D correlation[82]. Interpretation of 2D maps is usually complicated by intensity variation, band shift and band broadenings in experimental spectra. These factors has been investigated with both simulated spectra and real systems, e.g. [83, 84]. Error sources have also been explored. [85]

In physical studies, it is desirable to acquire knowledge of physical property-spectra relationship under external perturbations, e.g. phase transition of phospholipids. However, due to the statistical root of generalized 2D correlation analysis, intensity variations are presented as averaged ensemble over the duration of external perturbation. Spectral marks at the crucial point of physical properties are therefore lost. Thomas *et al* [86] developed the so called *windowed auto-correlation analysis* to address this issue. In windowed auto-correlation method, the whole data set is divided into small subsets containing spectra corresponding to a certain range (i.e. the

window) of the external perturbation. The scale of the subset, i.e. the width of the window is defined arbitrarily. Started from the initial spectrum (the beginning point of perturbation), the window is translated along the external axis by including one successive spectrum while taking out the beginning spectrum in the window. Auto-correlation (synchronous) is then done on each subset formed. Results from individual subsets are put together and plotted against the external perturbation variables. This method directly reveals physical property-spectrum correlation in the 2D map, which otherwise is deduced from a set of stacked spectra. Morita et al extended windowed-auto correlation analysis into generalized perturbation-correlation moving-window 2D correlation analysis. [87]

2. Model-based 2D Asynchronous Correlation Analysis.

As mentioned above, generalized 2D correlation analysis is very susceptible to spectral noises. In addition, the sequential changes of intensity variations can only be qualitatively deduced. Model-based 2D correlation analysis [88, 89] have been developed by Dluhy's group to quantitatively and directly determine the relative sequential changes of intensity variations. Considering that experimental dynamic spectrum may theoretically taking any analytical waveform, correlation can therefore be performed between the experimental spectra and mathematical functions of certain waveform with a chosen parameter which quantitatively determines out-of-phase intensity variations. Two analytical waveforms, i.e. sinusoidal and exponential, have been used to simulate the experimental spectra. The corresponding methods are $\beta\nu$ correlation $k\nu$ correlation respectively. In $\beta\nu$ correlation, a parameter of phase angle is defined as: $\beta = \tan^{-1}(\frac{\Psi}{\Phi})$, where Φ represents synchronous correlation intensity and Ψ represents asynchronous correlation intensity. The physical meaning of β is to describe the out-of-phase relationship of the intensity variations. Calculation of $\beta\nu$ correlation is given by eq2.30.

$$\Psi(\nu_1, \nu_2) = \frac{1}{m-1} \sum_{j=1}^m \tilde{y}_j(\nu, n_j) \cdot \sum_{k=1}^m N_{j,k} \cdot \sin(k\phi + \beta) \quad (2.30)$$

Upon implementation of $\beta\nu$ asynchronous correlation to dynamic spectra, a new parameter β_{eff} is naturally introduced to quantitative the *relative* out-of-phase relationship of intensity variations. As a result, $\beta\nu$ correlation possesses advantages that noise-induced artifacts are much more reduced upon correlation to noise-free analytical functions. Second and most importantly, sequential intensity variations are quantitatively measured. Third, the correlation is done simultaneously for all interested peaks with respect to the reference point, therefore the sequence for intensity variations of all peaks are fully resolved and may be compared as of chosen or in a whole.

A second model was explored with exponential function. A rate parameter k is defined to quantitative rate changes of intensity variations. Similar to $\beta\nu$ correlation, a new parameter k_{eff} is naturally introduced when performing correlation to dynamic spectra. The analytical form of $k\nu$ correlation is expressed by eq2.31. Compared to $\beta\nu$ correlation, $k\nu$ correlation possesses all the advantages as of $\beta\nu$ correlation. More importantly, $k\nu$ correlation further distinguishes the direction of relative rate changes of intensity variations, i.e. increase vs. decrease. In addition, two-step changes in intensity variation may also be resolved with $k\nu$ correlation.

$$\Psi(\nu_1, \nu_2) = \frac{1}{m-1} \sum_{j=1}^m \tilde{y}_j(\nu, n_j) \cdot \sum_{k=1}^m N_{j,k} \cdot \exp(-kt + R) \quad (2.31)$$

A complete review of the application of $\beta\nu$ and $k\nu$ correlations of biological monolayers is available in Ref. [80].

In this dissertation, $\beta\nu$ and $k\nu$ correlation was applied to structural characterization of rhdSP-C and its mutants in organic solvent or in lieu of phospholipids.

There have been many other applications and advances in 2D correlation analysis. A recent review has been given by Noda in 2006. [90] One example is a true combination of 2D correlation with chemometrics [91, 92] With this method, generalized 2D correlation is carried out based on a reconstructed data matrix of the significant scores and loading vectors obtained from the principal component analysis (PCA). As a result, spectral noises are much suppressed and therefore yield improved data quality for asynchronous 2D correlation analysis.

A book written by Noda and Ozaki was published in 2004, which systematically discussed 2D correlation analysis over various spectroscopic techniques and serves a good reference for 2D correlation analysis. [93]

2.5 Surface Enhanced Raman Spectroscopy (SERS)

1. Theory of Raman and SERS in general

Light scattering consists of two types. Rayleigh scattering originates from elastic collision of the incident radiation with sample molecules, i.e., the scattered light is at the same frequency as the incident light ν_0 . Rayleigh scattering accounts for 10^{-4} - 10^{-3} of the intensity of the incident excitation. Of interest for Raman spectroscopy is Raman scattering. The intensity of Raman scattering is about 3-5 orders weaker than Rayleigh scattering. In another words, only 1 in 10^{6-8} photons undergo Raman scattering. [94] Raman scattering originates from non-elastic collision of the incident light with sample molecules. As a result, the frequencies of Raman scattering shift from the excitation light, as expressed by $\nu = \nu_0 \pm \nu_m$, where ν_m is the Raman shift corresponding to the transition vibrational or rotational mode measured in Raman spectrum. $\nu = \nu_0 - \nu_m$ is referred as Stokes lines, and $\nu = \nu_0 + \nu_m$ is anti-Stokes lines. The vibrational energy level of IR and Raman is compared in **Fig 2.9**.

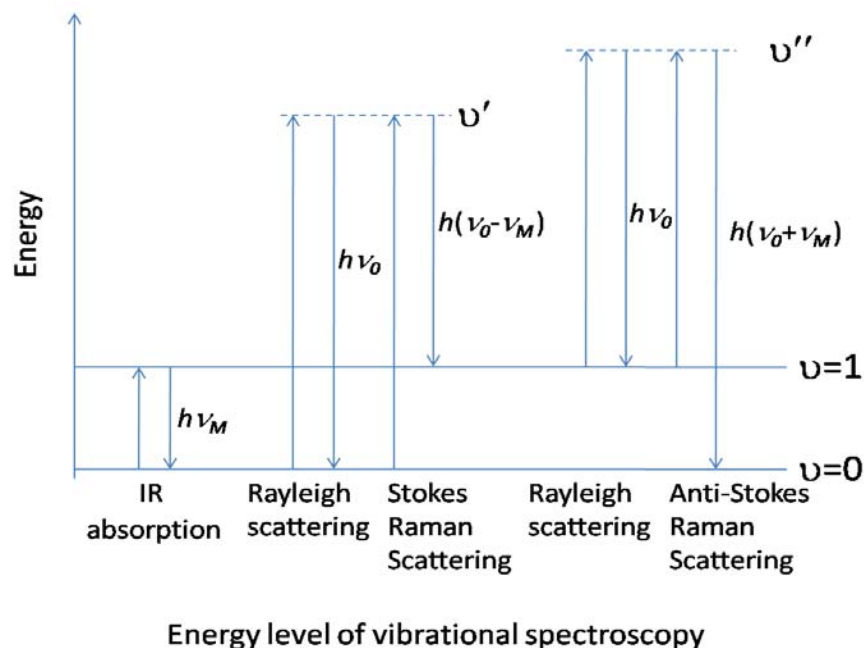


Fig2.9. Comparison of the energy level of IR and Raman spectroscopy.

In classic theory for light scattering, both Rayleigh and Raman scattering are generated by oscillating electric dipoles induced by incident excitation radiation. The relation of the induced dipole moment vector μ and the incident electric field vector E is expressed in form of a power series:

$$\mu = \alpha E + \frac{1}{2} \beta EE + \frac{1}{2} \gamma EEE + \dots \quad (2.32)$$

where α is the polarizability, β the hyperpolarizability and γ the second hyperpolarizability of the molecule. Comparatively, IR spectroscopy measures the change of transition dipole moment $\partial\mu/\partial Q$, while Raman spectroscopy measures the change of induced polarizability $\partial\alpha/\partial Q$, where Q is the normal coordinate associated with the vibrational mode at wavenumber ν . $\partial\alpha/\partial Q$ may be regarded simply as the change of electric clouds of the molecular bond. Therefore, IR and Raman are complementary in considering molecular symmetry. [95] Although Raman scattering is a fairly weak phenomenon, Raman spectroscopy has become

feasible for routine application mainly due to the advances in laser technology for excitation source and detectors such as charge coupled device (CCD).

Surface enhanced (Resonance) Raman spectroscopy (SERS/SERRS) was first demonstrated by Fleischmann *et al* in 1974 in experiments in studying adsorption of pyridine at a roughened silver electrode. The same phenomenon was observed later by others. Strong Raman scattering was observed up to a factor of 10^6 . At about the same time, Van Duyne and Creighton with their coworkers respectively concluded that such huge enhancement cannot be attributed to increased surface area. Since then, various metals have been demonstrated with SERS effect, with silver giving the largest enhancement. [96] SERS/SERRS have gained great popularity especially in biomedical and bio-diagnosis areas. Up to date, huge Raman enhancement up to 10^{14} for single molecule detection has been reported. [97] The discussion in the following is strict to SERS pertaining to the research work presented.

Despite of intensive studies on SERS, the theoretical model for explaining SERS effect is very much complicate and yet still controversial. A detailed review of SERS theories can be found in Ref.[96]. In general, Raman intensity is in proportion to the square of the induced dipole, which is a function of the molecular polarizability and the electric field (eq2.32). SERS enhancement may therefore originate from the electromagnetic field or polarizability induced by chemical interactions. Accordingly, two mechanisms for SERS enhancement have been proposed, i.e. electromagnetic enhancement and chemical enhancement.

In electromagnetic mechanism, conduction electrons bound to metal surface exist as surface plasmons, which are excited by incident electromagnetic wave. Plasma resonances provide a major contribution to electromagnetic enhancement. At smooth flat metal surface, surface plasmons can only propagate in the direction parallel to metal surface. At roughened

metal surface, surface plasmons are no longer confined and can be excited in both parallel and perpendicular directions. Metallic nanostructures possess similar electromagnetic resonance as surface plasmon. Therefore, local fields with large polarization are induced upon irradiation with incident light on the surface. As a result, the analyte molecules experience a largely enhanced electric field compared to the applied incident electric field. The intensity of SERS may be expressed as:

$$I_{SERS} \propto \left| \frac{E_{local}(\omega_0)}{E_0(\omega_0)} \right|^2 \left| \frac{E_{local}(\omega_s)}{E_0(\omega_s)} \right|^2 \approx \left| \frac{E_{local}(\omega_0)^4}{E_0(\omega_0)} \right| \quad (2.33)$$

where ω_0 and ω_s are the incident and scattering frequency respectively.

In the electromagnetic model, Raman-active analytes are not necessarily in contact with the metallic surface, but located within the enhanced electric field, which decays as $1/r^3$ away from the surface. r is the distance between the dipole and the surface. It has been noticed that surface morphology such as the size, shape and spacing of nanostructures contribute to SERS enhancement effects. Electromagnetic models have been approximated for various nanostructures, such as isolated metallic nanospheres, prolate spheroids and ellipsoids. [98-100] As an example, for nanostructures of dimensions much smaller than the excitation wavelength, the effective electric field is approximated by electrostatic “Rayleigh approximation”. The effective electric field is a function of the depolarization factor, the dielectric constants of the bulk metal and the surrounding medium respectively, as well as the incident electric field. For large metallic nanostructures with dimensions beyond “Rayleigh limit”, however, more complicated treatment using electrodynamic formalism is required. It is predicated that with prolate ellipsoid nanostructures, the plasmon resonance shifts to red, while the enhancement is largest in the region of the highest curvature, i.e. near the tip of prolate and near the waist of oblate isolated

ellipsoids. The enhancement spectrum will have two peaks, corresponding to the enhancement of the incident field and the scattering field respectively. In case of coupled nanostructures, the high fields were found at the interstitial locations. It was concluded that a SERS spectrum would be dominated by emissions from molecules occupying these sites. [96, 98, 100]

In chemical charge transfer mechanism, molecules chemisorb on roughened metal surface. Charge transfer between the absorbate molecules and the conduction bands of the metal surface result in apparent increase of molecular polarizability. Effective enhancement depends on absorbate-metal bonds therefore is short ranged (0.1-0.5nm). [100] The absorbates are required to be directly in contact with metallic nanostructures. Chemical effect helps explained some observed discrepancy in comparison of SERS spectra to ordinary Raman spectra, such as peak intensity and band shift of molecules like CO, N₂ and C₆H₆. As chemical mechanism is applicable to specific absorbate-metal systems but not general ones, it has not acquired much popularity. In the debate between electromagnetic mechanism and chemical mechanism, it may also be the case as pointed by Moskovits [96] that each makes contribution, that “*SERS derives its enhancement... almost entirely from molecular coupling to electromagnetic resonances of small, coupled metal particles. ...the electromagnetic effect operates on the system that actually exists at the surface and that may be modified ...through bond formation with surface adsorption sites.*”

2. Application of SERS in biological analysis

Since the observation SERS, the interest in SERS has been largely extended from surface science to the application of SERS as a bioanalytical tool. To implement SERS as a bioanalytical tool, there are two issues to be addressed effectively. First, effective signal enhancement relies on the manufacture of reproducible homogeneous metallic nanostructures as a platform for

effective absorption of analytes, which is however a challenging task. Compared to solution based hydrosols, immobilized colloids on solid substrates provide much better reproducibility and easier control. Various methods have been introduced to fabricate reproducible and homogeneous metallic nano-structures. Detailed reviews are given by Vo-Dinh [100, 101] On the other hand, metallic nano-structures by themselves provide no selectivity for absorbates nor signal differentiation (in terms of the same vibrational mode) between different species. Alternative approaches are therefore required to control adsorption of specific absorbates. There are two types SERS developed for selective SERS detection. One is *extrinsic* SERS which generally involves functionalization of metallic nano-structures to capture analytes, or derivatization of molecules to be effectively attached to SERS substrates. [102] One very much active area is in fabrication of SERS-based immunoassay platform. [103] In this case, metallic nano-particles are immobilized and coated with Raman reporter molecules. Specific antibody is then chosen and attached to reporter-coated metallic nano-particles. With the specificity of the labeling antibody, the functionalized metallic nanoparticles are then used for SERS detection of target biomolecules. Recently, single molecule detection using extrinsic SERS has been achieved. [104]

Another type is *intrinsic* SERS which employ direct SERS signal detection in combination with chemometrics for bioanalysis. [105, 106] Recently, intrinsic SERS detection interfaced with a scanning electron microscopy was reported. [107] Silver nanorods based SERS (Ag-NRs SERS) as employed in this research work is a typical example of intrinsic SERS.

Silver nanorod array based SERS (Ag-NRs SERS). Among various metals having been tested as SERS materials, Ag gives strongest enhancement by visible light excitation, followed by Cu and Au. Silver also gains popularity due to economic concern. A technique using oblique

angle vapor-deposition (OAD) was adapted to manufacture Ag nanorods arrays as high sensitive SERS platform (Ag-NRs SERS) with an enhancement factor about 10^8 . [108] The large enhancement has been attributed to the high aspect ratio of the cylindrical nanostructure [109] and anisotropic nature of the films. [110, 111] Examination of the UV-vis spectra of Ag-NRs arrays revealed a relationship of the length of the Ag rods and the enhancement. It was found out that for very small rods (film thickness $< 200\text{\AA}$), Ag nanorods presented isotropic property. For long rods (film thickness $> 200\text{\AA}$), there are at least two plasmon resonance peaks for Ag-NR arrays, which was attributed to large aspect ratio and overlap of nanorods, as well as imperfect shape and alignment of the rods. [112] For the same Ag-NR array system, polarized SERS revealed that the anisotropic SERS scattering was primarily attributed to the lateral arrangement of the three dimensional tilted nanorods lattice. It was proposed that highly localized plasmon modes are created by strong electromagnetic coupling between adjacent Ag nanorods. [113] This Ag-NRs based intrinsic SERS has been applied to detect respiratory virus. [106, 114] Distinct spectral differences were observed between RSV viral strains. Chemometric analysis further distinguished closely related RSV strains.

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CHAPTER 3

STRUCTURE CHARACTERIZATION OF RECOMBINANT HUMAN DEACYLATED SURFACTANT PROTEIN C USING FTIR SPECTROSCOPY AND 2D CORRELATION ANALYSIS¹

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Abstract

Recombinant deacylated human surfactant protein C (rhdSP-C) was expressed by a baculovirus expression vector and purified. The secondary structure of rhdSP-C was determined using transmission FTIR spectroscopy and 2D correlation analysis. A pH-dependent α to β unfolding was observed similar to native mature SP-C. The presenting study explored various 2D correlation analysis methods in protein structure determination. As a specific case, heterospectral correlation between two spectral regions, i.e. amide I region and the NHs region distinguished the origins of two spectral features at 1658cm^{-1} and 1645cm^{-1} , which were unambiguously assigned to α -helix and unordered conformations respectively without the employment of H-D exchanges. The pH-conformation relationship was also revealed using windowed autocorrelation analysis. It was demonstrated in the presenting study that FTIR spectroscopy in combination with 2D correlation analysis was powerful in resolving the highly overlapped conformations under amide I envelope.

Keywords: recombinant deacylated human surfactant protein C (rhdSP-C), pH titration, structure conversion, FTIR transmission spectroscopy, generalized 2D correlation analysis, model-based 2D correlation analysis, windowed autocorrelation analysis, heterospectral correlation analysis.

Introduction

Human pulmonary surfactants consist of 85% phospholipids, 4-8% neutral lipids and about 10% surfactant proteins. Phosphatidylcholine (PC) and anionic phosphatidylglycerol (PG) constitute the bulk of phospholipids, with saturated PCs such as 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) being essential to resist lung collapse at the end of exhalation attributed to the relatively high gel-to-liquid-crystal phase transition temperature ($T_c \sim 41^\circ\text{C}$) of the acyl chains. PGs are essential for re-adsorption and fast re-spreading of the surfactant proteins cooperated into the aqueous alveolar linings upon inhalation. [1, 2] There are four pulmonary surfactant apoproteins. Surfactant protein A and D (SP-A and SP-D) are hydrophilic and mainly responsible for the innate host defense against the invading pathogens such as viruses and bacteria, as well as particulates such as pollen. In contrast, surfactant proteins B and C (SP-B and SP-C) are small hydrophobic proteins associated with phospholipids to maintain the normal mechanical breathing cycle, facilitate the dynamic adsorption, spreading and the stability of the lung lining films. [3]

Mature SP-C is a unique small (4.2kDa) hydrophobic lipoprotein exclusively expressed in pulmonary alveolar type II (ATII) cells. The precursor protein of SP-C (proSP-C, 21kDa) is synthesized at the endoplasmic reticulum with 191-197 amino acids, and then translocated to Golgi apparatus. After post-translocation and proteolytic cleavage, matured SP-C is secreted into the alveolar linings together with SP-B and phospholipids. [4] The molecular structure of mature SP-C determined by NMR in organic solvents consists of 1) two palmitoylated Cysteine residues (C5 and C6) surrounded by two Prolines at the N-terminus, 2) a positively charged Lys-Arg (K11 and R12) pair, 3) a very hydrophobic poly-valine segment and 4) a heptapeptide C-terminus. [5] It has been determined that SP-C is a type II transmembrane protein. [6] NMR

study on SP-C in organic solvent indicates a α -helical conformation consisting of residues 9-34. The length of the α -helix is about 37Å which is in agreement with the length to span in the lipid bilayer. [7]

Recently, the understanding of SP-C biosynthesis has indicated an emerging role of SP-C mutations in conformational lung disease. Genetic mutations in the SP-C gene (*sftpc*) occurring in the domains of proSP-C have been associated to lung diseases such as idiopathic pulmonary fibrosis (IPF), pulmonary alveolar proteinosis (PAP), and less commonly, familial and sporadic forms of fatal interstitial lung disease (ILD). [8] In the case of PAP, mature SP-C is misfolded, transforming from a helical structure to a β -sheet structure that forms amyloid-like fibrils, which is possibly linked to partially or complete deacylated SP-C. It is also indicated that certain structure motifs such as the positively charged Lys-Arg pair may influence the normal biosynthesis pathway and aftermath secretion events. [9] The question of what specific sequence domain elements determine the structural and functional characteristics of SP-C still has yet to be thoroughly explored and is complicated further by its ability to transform between two unique secondary structures.

The current study investigated a pH-induced structure change of the wild type of recombinant deacylated human surfactant protein C (rdhSP-C WT) using FTIR transmission spectroscopy. IR spectroscopy is a powerful tool in protein structure determination. Spectral-structural correlation between the vibrational modes in the amide I region (1700-1600cm⁻¹) and the protein backbone secondary structure has been very well established in agreement with the theoretical predictions for both soluble proteins and membrane proteins [10, 11] FT-IR spectroscopy is particularly valuable for studies of membrane proteins and their interactions with

lipid matrix in terms of non-invasive, fast dynamics (10^{-13} sec. compared to NMR 10^{-5} sec.), and it does not require perfect crystallization as in X-ray diffraction.

Generalized 2D correlation analysis was developed by Noda in 1993 as a resolution enhancement technique. [12] Arbitrarily chosen of parameters is avoid in generalized 2D correlation analysis in contrast to conventional resolution enhancement techniques such as self Fourier deconvolution and curve fitting. The dynamic spectral features in response to external perturbation such as temperature, pH and pressure are evaluated by synchronous and asynchronous contour maps. The synchronous correlation analysis maps the simultaneous or linear occurrence of two intensity variations at ν_1 and ν_2 . The asynchronous correlation analysis maps the sequential or non-linear occurrence of intensity variations at ν_1 and ν_2 . The non-linear occurrence of two intensity variations can be *qualitatively* extracted based on the Noda's rules. Generalized 2D correlation analysis has been used in dynamic studies on protein secondary structures with hydrogen-deuterium exchange, and structural modifications of proteins in response to thermal and pressure perturbation. [13-15] It has also been applied to investigations of Langmuir films and model pulmonary surfactant systems at the air/water interface. [13-15] [16] However, generalized 2D correlation is very sensitive to spectral noise, baseline fluctuations, frequency shifts and bandwidth changes in the experimental data set. Asynchronous correlation is especially prone to artifact peaks introduced by the abovementioned factors. Pretreatment of the spectra such as normalization is an essential. [17]

Heterospectral correlation can also be applied to two different spectral signals, which is helpful in band assignment for less understood spectral features. Recently, correlation between distinct frequencies has been proposed to predict protein secondary structures. [18] Herein, we reported the correlation between the NH stretching region (NHs) and the amide I region, which

proved to be valuable to unambiguously distinguish the helical conformation from the unstructured conformation under the highly overlapped amide I envelope in this specific study. Usually, IR absorptions of α -helix and unordered structures are distinguished from each other by peptide backbone H-D exchange experiment.

Model-based 2D asynchronous correlation methods were developed by Dluhy et al. [19] [20] Generally, the waveform of the dynamic spectra can be modeled with certain analytical functions. In the model-based 2D asynchronous correlation analysis, a set of experimental spectra were correlated to appropriate model functions, being either sinusoidal ($\beta\nu$) or exponential ($k\nu$) in this case. Compared to the generalized 2D correlation, noise-induced artificial peaks are much reduced. More importantly, model-based 2D asynchronous analysis provides straightforward *quantitative* analysis of the relative rate changes in intensities of the interested spectral features. Model-based 2D asynchronous analysis has also been successfully utilized in studies model lung surfactant system at the air/water phase transition of phospholipids monolayer on the air/water interface with surface pressure perturbation. [21] [17]

In order to reveal pH-conformation relationship, *windowed autocorrelation analysis* was also employed in the presenting study. Due to the statistical nature of 2D correlation analysis, spectral features of physical importance, such as phase transitions, are averaged. *Windowed autocorrelation analysis* was developed by Richardson et al [22] to directly reveal spectral features in terms of the temperature-phase transition relationship of a liquid crystal. In windowed autocorrelation analysis, synchronous correlation was performed on a subset of the spectral matrix. A translating window containing a subset of spectra corresponding to a sub-range of the external perturbation is moved along the external perturbation axis. Auto-correlation is performed on each subset of spectra. The correlation results are then collectively plotted against

the external perturbation variations. The resultant external perturbation-spectral frequency contour map reveals the physical property-structural relationship.

Materials and Methods

Production and Purification of Recombinants of Human SP-C

A human SP-C cDNA (pre-pro form containing entire coding region, kindly provided by Dr. Steven Glasser, University of Cincinnati) was subcloned into the pGEM-11Zf(+) vector (Promega, Madison, WI), two modifications of the SP-C sequence were then created sequentially using reverse complementary primers and the PCR-based method of the QuikChange XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), and a construct with both modifications was then transferred into the 6xHis-encoding baculovirus transfer vector pAcHLT-A (BD Biosciences Pharmingen, San Jose, CA) for subsequent insect cell expression. Forward (F) and reverse (R) primers for the first site-directed mutagenesis

(F: CCGGGGCCGACATATGGTTTGGCATTCCCTG;

R: CAGGGAATGCCAAACCATATGTCTGGCCCCGG) were designed to introduce an NdeI restriction enzyme site immediately upstream from the sequence encoding the shorter, mature form of the SP-C protein (i.e., nucleotides 244-348 of the Genbank reference sequence *NM_003018*). Primers for the second site-directed mutagenesis

(Forward: GCTCATGGGTCTCTGATAGCACATGAGCCAG;

Reverse: CTGGCTCATGTGCTATCAGAGACCCATGAGC) were designed to introduce stop codons immediately following the mature SP-C sequence (i.e., after nucleotide 348 of GenBank *NM_003018*). The NdeI/PstI-flanked insert containing this modified SP-C construct was then excised from pGEM-11Z and ligated into the corresponding restriction sites in pAcHLT-A.

Recombinant baculovirus was produced by co-transfection of linearized BaculoGold DNA (BD Biosciences Pharmingen) and the pAChLT-A transfer vector containing the modified mature SP-C construct into Sf9 insect cells using calcium phosphate precipitation. After obtaining large stocks of high titer virus ($\geq 10^8$ pfu/ml) by several rounds of amplification (using low multiplicity of infection of 0.1 MOI), the 6xHis-tagged, mature SP-C fusion protein was expressed by infection (5 MOI) of Sf9 (2×10^6 cells/ml) grown in SF-900 II serum-free medium (Invitrogen/Gibco, Carlsbad, CA) in spinner flasks. Cells were harvested 3 days after infection. Pellets of infected Sf9 cells were washed with phosphate buffered saline and resuspended in approximately 1 ml (10^8 cells) of freshly-prepared lysis buffer without NaCl (2 M urea, 1% Triton X-100, 0.5% NP-40, 10 mM imidazole, 2 mM MgCl_2 , 20 mM Tris pH 8.0; supplemented with a protease inhibitor cocktail of 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 5 $\mu\text{g/ml}$ aprotinin, 35 μM bestatin hydrochloride, 25 μM N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64), 20 μM leupeptin hemisulfate, and 10 μM pepstatin A). Suspensions were then incubated with 250 U/ml benzonase (Sigma Aldrich, St. Louis, MO) for 60 min at room temperature. Lysates were then supplemented with 300 mM NaCl, cleared by centrifugation at $13000 \times g$ for 5 min, and supernatants were transferred to microfuge tubes containing pre-equilibrated Ni-NTA agarose (100 μl per ml lysate) (Qiagen, Valencia, CA) and incubated with rocking at 4°C for 4 hrs to overnight. Agarose beads were then washed 4 times with lysis buffer (containing 300 mM NaCl and no protease inhibitors) before detachment of SP-C fusion protein by resuspension of beads in 0.3 ml elution buffer (2 M urea, 1% Triton X-100, 0.5% NP-40, 250 mM imidazole, 20 mM Tris pH 8.0, 150 mM NaCl) and incubating for 2 hrs at room temp with rocking. For removal of 6xHis tag, eluates were diluted 4-fold with diluent (20 mM Tris pH 8, 150 mM NaCl, 3 mM

CaCl₂) and incubated with biotinylated thrombin (1 U/ml; Novagen/EMD, San Diego, CA) for 18 hrs at ambient temperature. Thrombin and cleaved 6xHis tag peptides were then removed by incubation with rocking for 2 h at room temp in a pre-equilibrated mixture of 100 µl Ni-NTA agarose and 50 µl streptavidin agarose (Novagen/EMD) per ml of digest solution. The supernatant containing purified SP-C was clarified using 0.45 µm cellulose acetate Spin-x centrifuge tube filters (Corning Inc., Corning, NY).

Final purification of mature rhSP-C was performed on a Perseptive BioCAD Sprint perfusion liquid chromatography system using a Vydac Protein C8 column and binary gradient elution using 2% ethanol + 0.1mM TFA in H₂O (solution A) and 100% methanol + 0.1 mM TFA (solution B). Peak detection was achieved using dual wavelength detection at 215 and 280 nm. Molecular mass and Triton X-100-free preparations were verified by MALDI TOF mass spectrometry using sinapinic acid as the carrier matrix. A single peak of MH⁺ of 3694.8 was consistent with deacylated mature rhSP-C, referred to hereafter as rhdSP-C. Verification of rhdSP-C purity was also performed using SDS PAGE using 4-12% bis-tris SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) under reducing conditions (50 mM tris(2-carboxyethyl)phosphine hydrochloride) in MES running buffer and either subjected to silver staining (Owl Separation Systems, Portsmouth, NH). To verify removal of the 6xHis tag Western blots were performed with electro-transfer to nitrocellulose with tris-glycine buffer (+20% CH₃OH) for immunodetection via penta-his mAb (Qiagen Corp.), followed by HRP-conjugated secondary antibodies and SuperSignal West Dura chemiluminescence kit (both Pierce Biotechnology, Rockford, IL). Visualization of silver stained gels or immunoblots was performed on a FluorChem 8900 Imager (Alpha Innotech, San Leandro, CA). Concentration of rhdSP-C protein was determined by micro bicinchoninic acid (BCA) assay (Sigma/Aldrich).

pH Titration and Transmission IR Experiments

The wild type rhdSP-C generated by above-mentioned methods was dialyzed against 80% ethanol to get rid of the spectral interference from TFA. An aliquot of dialyzed samples (200 μ L) was then subject to pH titration. The initial pH of the sample was about 4. First, the spectrum of the initial pH value was collected. The sample was then titrated using 0.01M HCl or 0.01M NaOH within the range of 3~9. After each titration, an aliquot of sample (8 μ L) was cast on the CaF₂ crystal window using a microliter syringe, and dried up to film by purging with dry air in a chamber. The transmission spectra were collected with a Bruker EQUINOX55 spectrometer in the mid-IR range (3500-800cm⁻¹) with a DLaTGS detector. Spectral resolution at 4cm⁻¹ with 2-level zero filling and a Blackman-Harris 4 Terms apodization function were adopted. Totally 256 scans were performed.

Data analysis was performed with Grams/32 AI software. Linear baseline correction was performed on truncated amide I region (1570-1750cm⁻¹), and then normalized to the CH region. Curve fitting was also performed using Lorentzian lineshape in the amide I region.

2D correlation analysis

Numerical computation of 2D correlation analyses were performed following the algorithms in eq1-5. The dynamic spectra set $\tilde{y}(\nu, \mathbf{p})$ were acquired by subtracting the reference spectrum, typically the average spectrum $\bar{y}$. Calculation of the asynchronous correlation was simplified using the Hilbert-Noda transformation (eq3) instead of the cumbersome fast Fourier transform.

$$\Phi(\nu_1, \nu_2) = \frac{1}{n-1} \sum_{j=1}^n \tilde{y}(\nu_1, \mathbf{p}_j) \cdot \tilde{y}(\nu_2, \mathbf{p}_j) \quad (1)$$

$$\Psi(\nu_1, \nu_2) = \frac{1}{n-1} \sum_{j=1}^n \tilde{y}(\nu_1, \mathbf{p}_j) \sum_{k=1}^n M_{jk} \cdot \tilde{y}(\nu_2, \mathbf{p}_j) \quad (2)$$

$$M_{jk} = \begin{cases} 0 & \text{if } j=k \\ 1/\pi(k-j) & \text{otherwise} \end{cases} \quad (3)$$

In the $\beta\nu$ asynchronous correlation (eq4), the experimental spectra were correlated to the sinusoidal function $\sin(k\phi+\beta)$, where β is the phase angle defined by $\beta = \tan^{-1}(\frac{\Psi}{\Phi})$ ($0^\circ \leq \beta < 360^\circ$). $k=10^\circ$, so that the correlation was performed with $\sim 1/4$ of a cycle of the sinusoid function. The temporal order of two signal features at ν_1 and ν_2 can be quantitatively measured by the effective angle $\beta_e = \beta + 90^\circ$ in the $\beta\nu$ correlation maps. [23]

$$\Psi(\nu, \beta) = \frac{1}{N-1} \sum_{j=0}^{N-1} \tilde{y}(\nu, n_j) \sum_{k=0}^{N-1} M_{jk} \sin(k\phi + \beta) \quad (4)$$

In the $k\nu$ asynchronous correlation (eq4), the experimental spectra were correlated to the exponential function $\exp(-kt+R)$, where k is the rate constant describing the relative rate changes in intensity variations. The effective rate constant k_{eff} is defined as the point the maximum correlation intensity at particular frequencies in the $k\nu$ correlation map. [20]

$$\Psi(\nu, k) = \frac{1}{N-1} \sum_{j=0}^{N-1} \tilde{y}(\nu, n_j) \sum_{k=0}^{N-1} M_{jk} \exp(-kt + R) \quad (5)$$

Windowed autocorrelation was calculated following the formalism by Richardson et al. [21] Each window contains 4 spectra translating along the pH axis.

All data set were baseline-corrected and normalized before 2D correlation analysis in the amide I region of $1570\text{-}1750\text{cm}^{-1}$ and the NHs region ($3100\text{-}3500\text{cm}^{-1}$). All the algorithms were carried out with MATLAB programs (v6.1, The MathWorks, Natick, MA) written in our lab.

Results and Discussion

Fig3.1.a. illustrated the representative transmission spectra of rhdSP-C upon pH titration in the region of $3500\text{-}800\text{cm}^{-1}$. As mentioned above, samples were dialyzed against 80% ethanol

to remove the spectral interference from TFA at 1673cm^{-1} . Before dialysis, the amide I band presented as a single broadened envelope (data not shown), which is usually observed for proteins. It was of note that the bands were much resolved for the dialyzed samples after dialysis. Two regions were examined to determine the spectral-structure correlation of rhdSP-C: the amide I region ($1600\text{-}1700\text{cm}^{-1}$) containing the backbone vibration mode of proteins, and the highly localized NHs bands ($3200\text{-}3500\text{cm}^{-1}$). Band assignment was given in **Table3.1.** for the secondary structure of rhdSP-C WT. At low pH, peaks are resolved in the amide I region at 1659 and 1633, and in the NHs region at 3355. As pH increased, a shoulder appeared at approximately 1679 in the amide I region. At high pH of titration, the amide I region was predominant with a band at 1645, while a peak at 3390 showed up in the NHs region. In the amide I region, the α -helix (1659) and the β -sheet (1633) were the major conformation throughout pH titration. β -turn conformation were increased as pH increased.

The IR spectral-structure correlation has been established for both the amide I region and the NHs regions. The amide I envelop consists of overlapped absorptions of different protein conformations. The α -helix absorption for soluble proteins are usually in the range of 1648 to 1660 cm^{-1} . Membrane proteins may acquire α -helix structure at higher wavenumber in lipid environment as a result of stretching of the hydrogen bond. The assignment of the 1659cm^{-1} to α -helix was in agreement with the prediction for polyp(L-alanine) and α -helix with 14-16 residues. [11] It was also possible that a mixture of α -helix with 3_{10} helix ($1658\text{-}1666\text{cm}^{-1}$) coexisted. In the presenting study, the band position of α -helix was a little bit higher compared to that of bovine dSP-C in apolar solvent (C:M in 1:1) ($\sim 1656\text{cm}^{-1}$) as previously reported. [24] We hypothesize that 80% ethanol presented as a hydrated environment for rhdSP-C, which could contribute to the up-shift of α -helix absorption.

β -structures of proteins usually presents as overlapped features in the 1630-1640 and 1660-1690 region. Conventionally, extended antiparallel β -sheet are featured with a splitting of a high intensity-low wavenumber component at $\sim 1633\text{cm}^{-1}$ and in some cases a low intensity-high wavenumber component at $\sim 1683\text{cm}^{-1}$. Theoretical modeling of extended antiparallel β -sheet showed that the splitting is due to transition dipole coupling (TDC). In absence of TDC, the calculated absorption could be down to around 1670cm^{-1} . [11] The discrimination of β -sheet and β -turns was proposed to be based on the ratio of the high to low frequencies. Usually, $I_{1690-1660}:I_{1645-1635}=2:1-3:2$ is indicative of β -turns, while $I_{1690-1660}:I_{1640:1620}=1:10-1:8$ is indicative of β -sheet. [10] In the present study, the frequency at 1679cm^{-1} was therefore assigned to β -turn structure and 1633cm^{-1} to antiparallel β -sheet respectively. Native proteins usually contain unstructured conformation. The predominant band at 1645cm^{-1} of rhdSP-C WT at the highest pH (8.24) of titration was however indicative of denatured protein structure.

The structure conversion from α to β conformation of the protein has been considered to be indicative of the formation of β -aggregates resembling amyloid like fibrils with cross- β structure. [25] Characterization of the α to β conformational conversion using FTIR spectroscopy usually identifies strong absorption of extending β -sheet around 1635cm^{-1} and a concomitant band around $1610-1630\text{cm}^{-1}$ for β -aggregation. In the presenting transmission spectra, however, no peak was appreciable in the region around 1620cm^{-1} in the transmission spectra, though a fairly weak peak around $1610-1619\text{cm}^{-1}$ could be assigned in curve fitting procedure (data not shown). In this case, spectral-structure correlation purely based on the IR spectra and conventional resolution enhancement showed little formation of β -aggregations. As

will be discussed in the following, 2D correlation analysis provided better resolution for the β -structure.

In the presenting study, pronounced NHs bands were also resolved with dialyzed sample. The NH stretching region is a highly localized region. The NHs frequency is usually not sensitive to the chain conformation but depends strongly on the strength of the $\text{N-H}\cdots\text{O}=\text{C}$ hydrogen bond. The normally observed NHs bands are centered at 3310cm^{-1} (amide A) and 3270cm^{-1} (amide B). $3300\sim 3500\text{cm}^{-1}$ has a limited sensitivity in determine the secondary structure. IR band at $3380\text{--}3340\text{cm}^{-1}$ has been assigned to β structures for modeled polypeptides [26] In case of rhdSP-C, two bands centered at 3355cm^{-1} and 3390cm^{-1} were resolved in transmission spectra in response to pH titration. The band at 3355cm^{-1} was assigned to hydrogen-bonded α/β conformations. The band at 3390cm^{-1} has no reported assignment. The concomitant presence of 3390cm^{-1} with 1645cm^{-1} in amide I region at pH8.24 indicated that IR absorption at 3390cm^{-1} could be attributed to random coils, as clearly demonstrated with 2D correlation analysis discussed in the following.

Generalized 2D correlation analysis

In generalized 2D correlation contour maps, peaks are resolved at 1679, 1644 and 1633cm^{-1} (fairly weak) in synchronous map (**Fig3.2.a.**), in agreement with the transmission spectra. The cross peaks were resolved for peak pairs of $1645=1658\text{cm}^{-1}$ and $1632=1658\text{cm}^{-1}$. “=” denoted that the intensity changes of these peaks were changed linearly. In the asynchronous map (**Fig3.2.b.**), negative cross peaks were resolved for two pairs: $1658<1683$ and $1633<1683$. “<” denoted that both 1658cm^{-1} and 1633cm^{-1} were changed after 1683cm^{-1} according to Noda’s rule. [25] The positive cross peak pair of 1645cm^{-1} and 1683cm^{-1} indicated that the change in intensity of 1645cm^{-1} was ahead of that of 1683cm^{-1} . It was of import to notice the “new” band

resolved at 1683cm^{-1} was fairly strong. As discussed above, this band was not apparent in the original spectra under the overlapped amide I envelop. 2D correlation analysis therefore demonstrated to be powerful in resolution enhancement. Since 2D correlation is based on statistic method, the acquired resolution presented an average result.

The heterospectral correlation between the amide I region and the NHs region was illustrated in **Fig3.3**. In the synchronous map, IR absorption at 3355cm^{-1} was positively correlated to two bands at 1659cm^{-1} and 1632cm^{-1} respectively, but negatively correlated to 1645cm^{-1} . The correlations of 3390cm^{-1} to these three amide I bands were in reversed signs. In the asynchronous map, the band at 1679cm^{-1} was positively correlated to 3355cm^{-1} and negatively correlated to 3390cm^{-1} . The reversed signs for 1658cm^{-1} and 1645cm^{-1} clearly indicated that they were of different spectral origins.

Fig3.4. showed $\beta\nu$ and $k\nu$ correlation maps for rdhSP-C of the amide I region. Quantitative evaluation of the relative changes in intensity variations were given in **Table3.2**. In **Fig3.4.a.**, the predominant bands in amide I region were resolved at 1679cm^{-1} with a shoulder at 1659cm^{-1} . Two less intense bands were resolved at 1645cm^{-1} and 1632cm^{-1} respectively. These bands were corresponding to the conformations assigned in transmission spectra. The β_e values were indicative of the relative intensity change rates for each conformation in the order: $1645/\beta_{e214.9}(\text{random coils}) > 1632/\beta_{e31.2}(\beta\text{-sheet}) > 1659/\beta_{e22.7}(\alpha\text{-helix}) > 1679/\beta_{e11.6}(\beta\text{-turns})$. The largest β_e value for 1645cm^{-1} was attributed to the fast denaturing of the protein upon titration above pH7.

In the $k\nu$ map (**Fig3.4.b.**), A broadened band of weak intensity was resolved centered at $\sim 1684\text{cm}^{-1}$ with the $k_{\text{eff}}=1.7$. Other bands were resolved at the same wavenumbers as in the $\beta\nu$ map. Of importance was that the peak centered at $\sim 1684\text{cm}^{-1}$ was in agreement with the result

from generalized 2D asynchronous correlation analysis. A similar band position was reported with bovine deacylated SP-C, [27] which was attributed to short twist β -strands based on *ab initio* calculations on β -sheet structure. It was also proposed that IR absorption of the structure element for β -aggregation was distinctive from that of the extended antiparallel β -sheet, which was characterized by the split of the high-intensity-low-frequency and the low-intensity-high-frequency bands. [24] A very weak and broad band in the region of 1620-1600 cm^{-1} was appreciable in both $\beta\nu$ and kv maps. It was indicative that β -aggregations were only formed at fairly low level for rdhSP-C wild type.

In both $\beta\nu$ and kv maps of the NHs region (**Fig3.5.**), the rate changes of the band at 3390 cm^{-1} was faster than that at 3355 cm^{-1} . In comparing to $\beta\nu$ and kv maps of the amide I region, it may be seen that IR absorption at 3390 cm^{-1} was associated to the band at 1645 cm^{-1} , while the band at 3355 cm^{-1} was associated to 1659 cm^{-1} and 1632 cm^{-1} . Therefore, the band at 3390 cm^{-1} may be assigned to the same structure as that at 1645 cm^{-1} , while the band at 3355 cm^{-1} was corresponding to two different conformations changing concurrently.

Windowed autocorrelation analysis on pH-dependent conformational changes of rdhSP-C was presented in **Fig3.6.** Two pH regions were revealed in association with structural changes. Below pH 4.5, rdhSP-C was predominant with β structure. Above pH 5, rdhSP-C presented coexistence of different conformations, with α -helix and β -sheet predominant between pH 5.5-6, while β -turns predominant around pH 6.5. This result was in agreement with the experimental observation of a “jump” around pH 5 during titration. It could be speculated that the protein structure was tolerant to environmental perturbations within certain threshold.

Although no pronounced peak was resolved for β -aggregation in the transmission spectra of rdhSP-C wild type upon pH titration, both generalized 2D correlation analysis and model-

based 2D correlation analysis revealed an increase in β -strands as indicated by 1684cm^{-1} . In conclusion, a α to β structure conversion was manifested for rdhSP-C wild type with 2D correlation analysis. It was also implied in model-based 2D correlation analyses that very limited β -aggregation could exist for rdhSP-C.

Conclusion

Biophysical characterization of SP-C used to be limited due to the difficulties to produce and purify this very hydrophobic protein in quantity. In the presenting study, rdhSP-C was produced and purified in quantity using baculovirus expression system. The combination of various 2D correlation analysis techniques was proved to be powerful in the determination of the pH-dependent structural changes of rdhSP-C. The pH-dependent α to β conformational conversion of rdhSP-C was manifested with 2D correlation analysis. The relative rate changes in intensities of different conformations were quantitatively determined using model-based $\beta\nu$ and kv correlation analyses. In the presenting study, heterospectral correlation between amide I and NHs regions indicated the different origins for bands at 1659cm^{-1} and 1645cm^{-1} . These two bands may be attributed to both α -helix and unordered structures, which were usually differentiated by H-D exchange experiment. Windowed autocorrelation analysis indicated a tolerant threshold for rdhSP-C to maintain certain conformations. The presenting work provided a solid case for structural conversion of rdhSP-C using combination of FTIR spectroscopic analysis with 2D correlation analysis. In parallel, works were also done on the site-directed rdhSP-C mutants (reported elsewhere). In both cases, the combination of FTIR spectroscopic analysis with 2D correlation analysis provides a powerful tool for the determination of structural-functional relationship.

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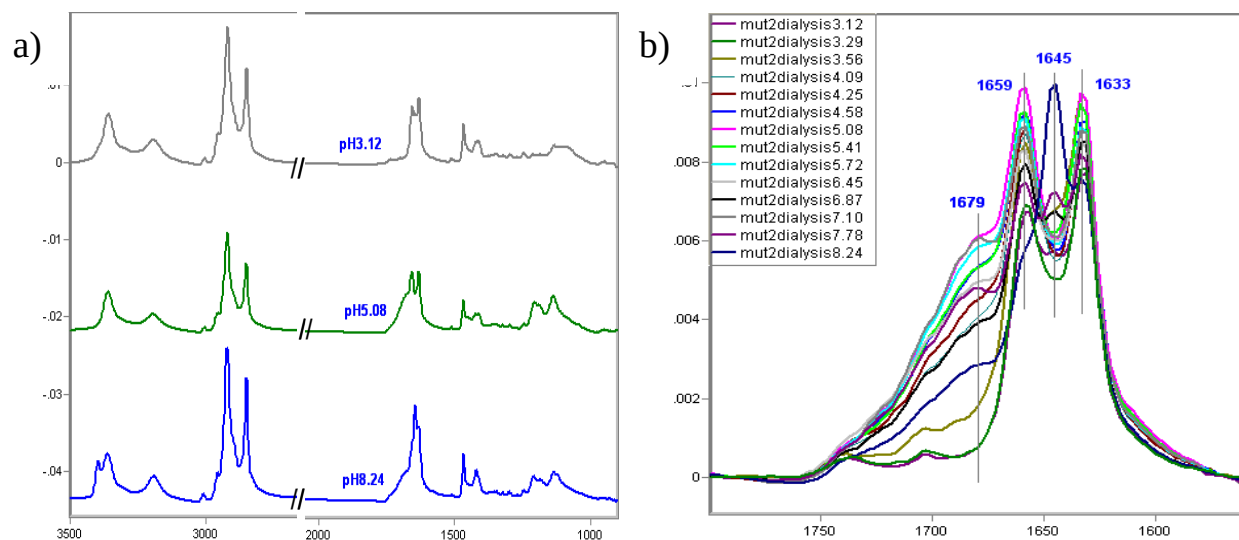


Fig3.1. Transmission spectra of wild type rdhSP-C. Peaks were resolved in NHs region and amide I region for the secondary structure. a) representative spectra of rdhSP-C in the region of 3500-800 cm^{-1} , b) overlaid spectra of wild type rdhSP-C in amide I region upon pH titration.

Table3.1. Peak assignment for the secondary structure of rdhSP-C wild type.

Wavenumber (cm ⁻¹)		Conformation Assignment
Amide I region	1679	β -turns
	1659	α -helix
	1645	Random coils
	1633	β -sheet
NHs region	3355	α/β conformation
	3390	Random coils

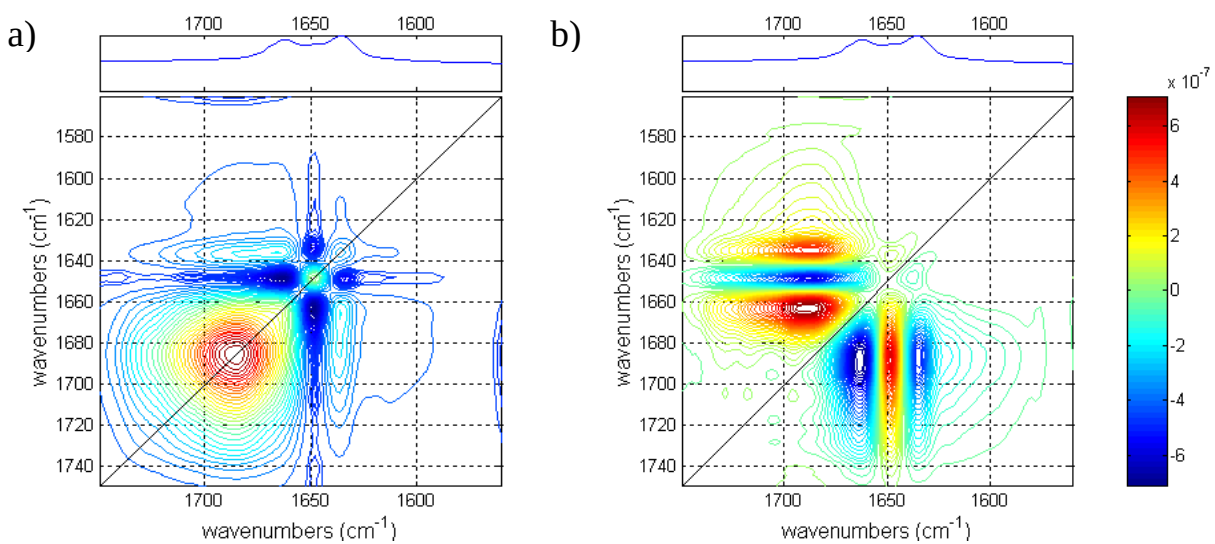
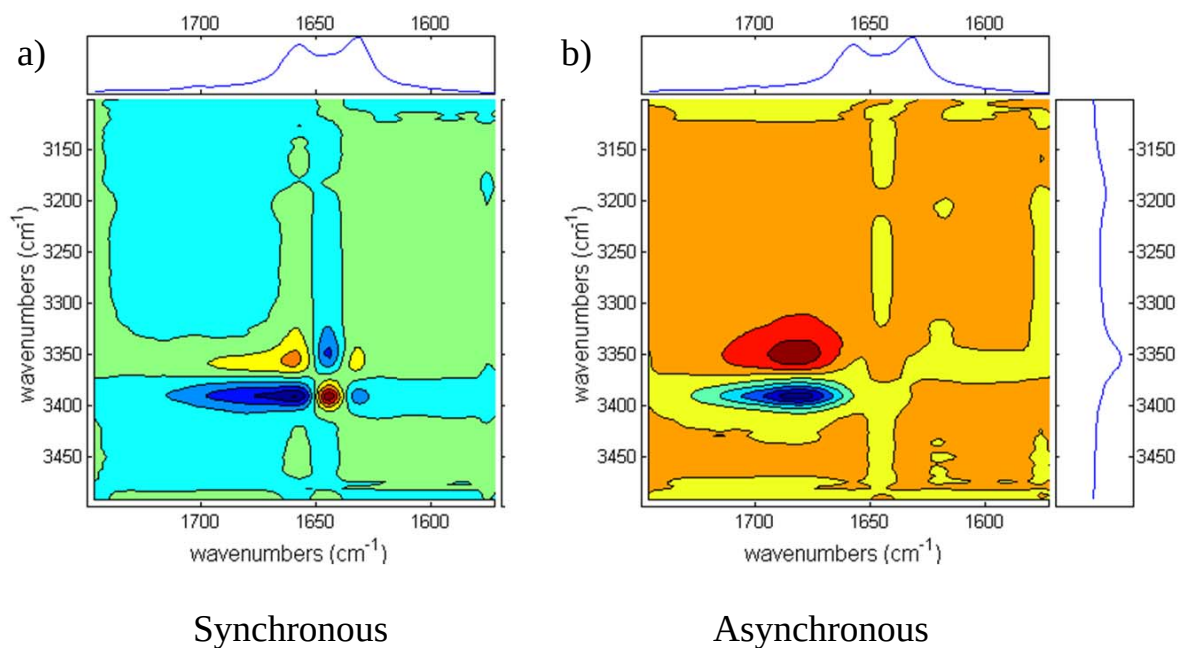


Fig3.2. Generalized 2D correlation analysis of rdhSP-C wild type secondary structure.

a) synchronous map, b) asynchronous map.



	Frequency (cm ⁻¹)	3355	3390
Synchronous	1659	+	-
	1645	-	+
	1633	+	-
Asynchronous	1679	+	-

Fig3.3. Heterospectral correlation between the amide I region (1750~1570cm⁻¹) and the NHs region (3500~3100cm⁻¹).

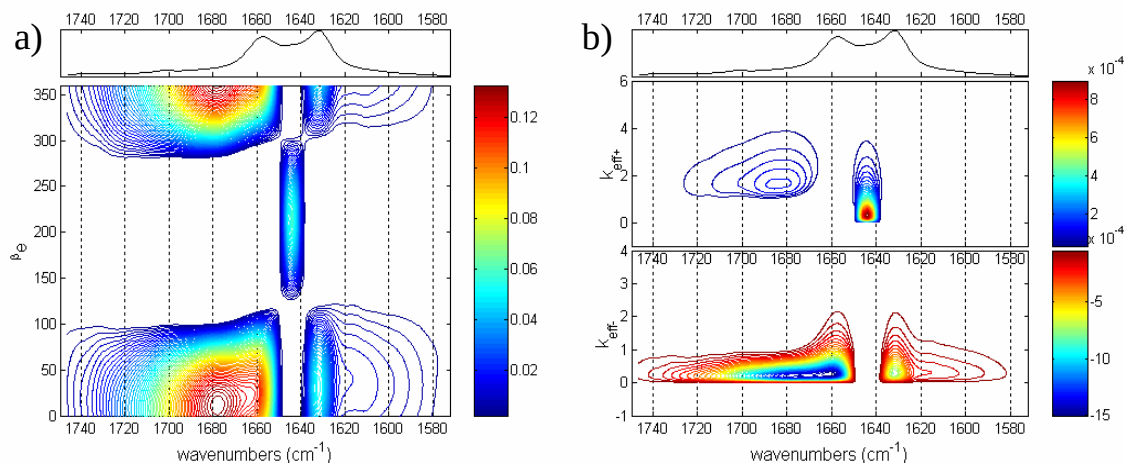


Fig3.4. a) $\beta\nu$ correlation analysis, b) $k\nu$ correlation analysis of rdhSP-C wild type in amide I region.

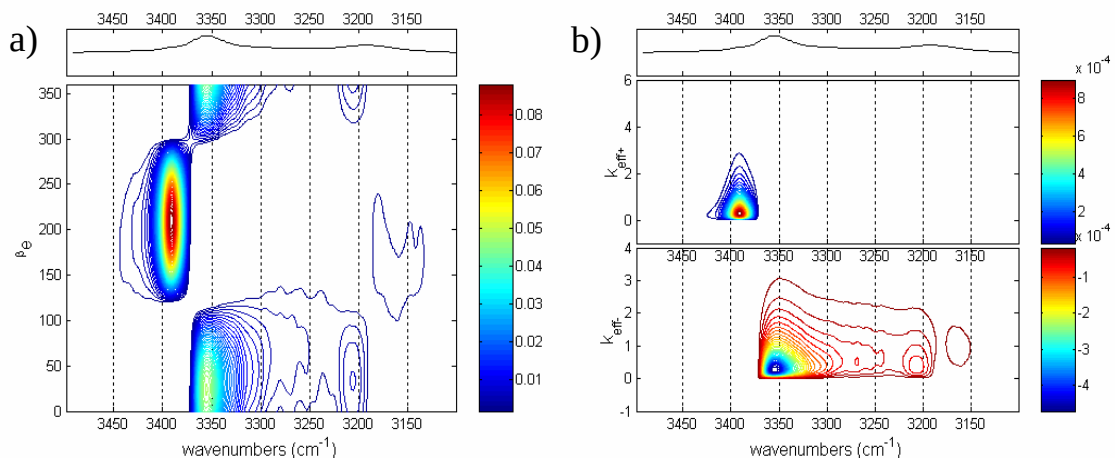


Fig3.5. a) $\beta\nu$ correlation analysis, b) $k\nu$ correlation analysis of rdhSP-C wild type in NHs region.

Table3.2. Quantitative evaluation of the relative changes in intensity variations of rdhSP-C wild type with β_{eff} and k_{eff} values in the NHs region and the amide I region respectively.

Vibrational modes	Frequency (cm⁻¹)	β_e	k_{eff+}	k_{eff-}
NHs region	3390	211.2	0.3	-
	3355	23.9	-	0.3
Amide I region	1684	-	1.7	-
	1679	11.6	-	0.2
	1659	22.7	-	0.3
	1645	214.9	0.3	-
	1632	31.2	-	0.3

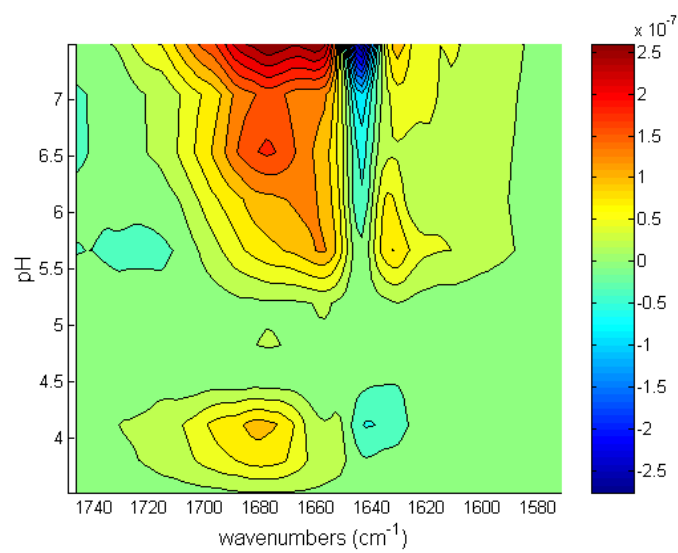


Fig3.6. Windowed auto-correlation of rdhSP-C wild type.

CHAPTER 4

PH-DEPENDENT UNFOLDING OF MUTANTS OF RECOMBINANT HUMAN DEACYLATED SURFACTANT PROTIEN C¹

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Abstract

Lung diseases associated with misfolding of human pulmonary surfactant protein C (SP-C) resembles the case of amyloid fibril diseases with a significant α to β structure conversion. To investigate the functional importance of certain structural motifs of SP-C in the aggregation process, site-directed mutageneses of the recombinant deacylated human SP-C were carried out at the N-terminal palmitoylation sites C5S/C6S, the positively charged Lys-Arg pair, as well as in the poly-valine segment (V21P and L22P). Structural analysis using FTIR transmission spectroscopy coupled with 2D correlation analysis revealed distinct conformational changes of these mutants in response to pH titration. The results showed that the responses of the mutants to pH perturbation were in very different ways for mutagenesis at the N-terminus from that within the poly-valine segments. As the mutagenesis moving from the N-terminus to the poly-valine segment, the secondary structure of the mutants unfolded into β -structure and unordered structure respectively. It was indicated that the formation of β -aggregation did not require the formation of disulfide bond crosslinks in deacylated N-terminus.

Keywords: recombinant deacylated human surfactant protein C (rdhSP-C), site-directed mutagenesis, N-terminus, poly-valine segment C5S/C6S, K11Q/R12Q, L22P, V21P, FTIR transmission spectroscopy, generalized 2D correlation analysis, model-based 2D correlation analysis, windowed autocorrelation analysis.

Introduction

Human pulmonary surfactants consist of 85% phospholipids, 4-8% neutral lipids and about 10% surfactant proteins. Phosphatidylcholines (PC~46%) and anionic phosphatidylglycerol (PG ~10%) constitute the bulk of phospholipids. Disaturated PCs such as 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) are essential to resist lung collapse at the end of exhalation attributed to its high gel-to-liquid-crystal phase transition temperature ($T_c \sim 41^\circ\text{C}$) of the acyl chains. Anionic PGs and unsaturated phospholipids are essential to retain fluidity of the aqueous alveolar linings for re-adsorption and fast re-spreading of the surfactant materials upon inhalation. [1] There are four pulmonary surfactant apoproteins. Surfactant protein A and D (SP-A and SP-D) are hydrophilic and mainly responsible for the innate host defense against the invading pathogens such as viruses and bacteria, as well as particulates such as pollen. Surfactant protein B and C (SP-B and SP-C) are small hydrophobic apoproteins particularly important for maintaining the normal mechanical breathing cycle, facilitate the dynamic adsorption, spreading and the stability of the lung lining films in associated with lipids. [2] It is established that there are coordinated functionalities of SP-B and SP-C. Particularly, the deficiency of SP-B resulted in abnormal expression of SP-C and is associated with fatal Respiratory Distress Syndrome (RSD) for premature infants and acute RDS (ARDS) for older children and adults. [3-5]

Mature SP-C is a unique small (4.2kDa) hydrophobic lipoprotein exclusively expressed in pulmonary alveolar type II (ATII) cells. Human SP-C preprotein (proSP-C) (21kDa) is synthesized at the endoplasmic reticulum with 191-197 amino acids, and then translocated to Golgi apparatus. After post-translocation and proteolytic cleavage, matured SP-C is secreted into the alveolar linings together with SP-B and phospholipids. [3] The molecular structure of mature

SP-C is highly conserved through species. NMR study of mature SP-C in organic solvents reveals four structural motifs 1) two palmitoylated Cysteine residues (C5 and C6) surrounded by two Prolines at the N-terminus, 2) a positively charged Lys-Arg (K11 and R12) pair, 3) a very hydrophobic poly-valine segment, and 4) a heptapeptide C-terminus. [6] It has been determined that SP-C is a type II transmembrane protein. [7] NMR study of mature SP-C in apolar solvent indicates that it contains a rigid α -helix consisting of residues 9-34. The length of the α -helix is about 37Å which is in agreement with the length to span in the lipid bilayers. [8]

Extensive studies have been done focusing on the surface activities of SP-C in association with phospholipids to facilitate the adsorption, spreading and stabilization of the alveolar lining film. [2] Most recently, the advance in understanding of SP-C biosynthesis has indicated an emerging role of SP-C in conformational lung disease. Misfolding of SP-C presents a similar case to other chronic neurodegenerative diseases such as Alzheimer's and Huntington's disease, all are characterized by conformational conversion from α -helix to β -strands and formation of amyloid fibrils. Genetic abnormalities in SP-C are associated with idiopathic pulmonary fibrosis (IPF), pulmonary alveolar proteinosis (PAP), and less commonly, fatal interstitial lung disease. The majority of genetic mutations in the SP-C gene (*sftpc*) that lead to these diseases occur in the domains corresponding to pre-processed form of SP-C (proSP-C). [5] In the case of PAP, misfolding and formation of amyloid like fibrils of mature SP-C is possibly linked to partial or complete deacylated SP-C. [9] The question of what specific sequence domain elements determine the structural and surface active (functional) characteristics of SP-C still has yet to be thoroughly explored and is complicated further by its ability to transform between the two unique structures.

Work have been done in our lab in revealing a pH-dependent conformational change for bovine deacylated SP-C (dSP-C) [10] and recombinant deacylated human SP-C (rdhSP-C) respectively (reported elsewhere). In this study, we investigated the pH-dependent structural changes of the mutated recombinant deacylated human SP-C (rdhSP-C mutants) to elucidate the importance of certain structure motifs in the structural-functional relationship of SP-C. Particularly, site-directed mutagenesis at the N-terminal Cysteine residues, the positively charged Lys-Arg pairs, as well as in the poly-valine segment respectively revealed distinct patterns in conformational conversions upon pH perturbation.

FTIR transmission spectroscopy was employed to characterize the structural conversion of rdhSP-C mutants. Infrared spectroscopy is a powerful tool in protein structure determination. Spectral-structural correlation between the vibrational modes in the amide I region ($1700\text{-}1600\text{cm}^{-1}$) and the protein backbone secondary structure has been very well established in agreement with the theoretical predictions for both soluble proteins and membrane proteins [11] [12] FT-IR spectroscopy is particular valuable for studies of membrane proteins and their interactions with lipid matrix in terms of non-invasive, fast dynamics (10^{-13}s . compared to NMR 10^{-5}s .), and it does not require perfect crystallization as in X-ray diffraction. Characterization of protein aggregations using FT-IR spectroscopy involves the identification of a conformational α to β conversion, especially the formation of β -aggregation. *Ab initio* simulation of IR absorption indicated that twisted β -strands may be the key structure contributed to the formation of β -aggregation. [13]

Generalized 2D correlation analysis was developed by Noda in 1993 as a resolution enhancement technique. Arbitrarily chosen of parameters is avoid in generalized 2D correlation analysis in contrast to conventional resolution enhancement techniques such as self Fourier

deconvolution and curve fitting. The dynamic spectral features and sequential intensity variations are qualitatively evaluated in synchronous and asynchronous contour maps. [14] However, generalized 2D correlation is very sensitive to spectral noise, baseline fluctuations, frequency shifts and bandwidth changes in the experimental data set. Asynchronous correlation is especially prone to artifact peaks introduced by the abovementioned factors. *Model-based 2D asynchronous correlation* methods were developed by Dluhy et al. [15, 16] Generally, the waveform of the dynamic spectra can be modeled with certain analytical functions. In model-based 2D asynchronous correlation analysis, a set of experimental spectra were correlated to appropriate model functions, either sinusoidal ($\beta\nu$) or exponential ($k\nu$) in this case. Compared to the generalized 2D correlation, noise-induced artificial peaks are much reduced. More importantly, model-based 2D asynchronous analysis provides straightforward quantitative analysis of the *relative* rate changes in intensities of the interested spectral features. Model-based 2D asynchronous analysis has been successfully utilized in studies of pressure induced phase transition of phospholipids monolayer on the air/water interface, [17] as well as monitoring the interaction of surfactant proteins with phospholipids in model membrane systems. [10]

Due to the statistical nature of 2D correlation analysis, structural changes associated with crucial physical properties such as phase transition temperature or pH ranges are averaged and obscured. *Windowed* autocorrelation analysis by Richardson et al was developed to address this issue. [18, 19] In the moving window 2D correlation, autocorrelation was performed on a subset of the spectral matrix. A translating window containing a subset of spectra is moved along the external perturbation axis. External perturbation induced spectral-structural correlations, which are usually extracted by stacking a set of dynamic spectra, are revealed in windowed 2D auto

correlation map. This technique has also been applied to surface pressure induced structure changes of SP-B and SP-C in phospholipid monolayer. [10]

In the presenting study, model-based 2D asynchronous analysis and windowed autocorrelation provided unambiguous information on the pH-dependent mechanism for the structure changes of rdhSP-C mutants.

Material and Methods

Production and purification of rdhSP-C and its mutants has been detailed elsewhere. [20]. rdhSP-C mutants were received from our collaborator Dr. John E Baatz from Medical University of South Carolina. Mutants were accepted with concentration $\sim 150\text{-}400\mu\text{g/mL}$ and dialyzed against 80% methanol after reception to get rid of TFA from the purification procedure.

pH Titration and Transmission IR Experiments.

The protein mutants generated by above-mentioned methods were dialyzed against 80% ethanol to get rid of the spectral interference from TFA. An aliquot of dialyzed samples ($200\mu\text{L}$) were then subject to pH titration. The initial pH of the sample was usually in the range of 4-5. The spectrum of the initial pH value was collected first. The sample was then titrated using 0.01M HCl or 0.01M NaOH within the range of 2~9. After each titration, an aliquot of sample ($8\mu\text{L}$) was cast on the CaF_2 crystal window using a microliter syringe, and dried up to film by purging with dry air in a chamber. The transmission spectra were collected with a Bruker EQUINOX55 spectrometer in the mid-IR range ($3500\text{-}800\text{cm}^{-1}$) with a DLaTGS detector. Spectral resolution at 4cm^{-1} with 2-level zero filling and a Blackman-Harris 4 Terms apodization function were applied. Totally 256 scans were performed. Data analysis was carried out with Grams/32 AI software. Curve fitting was performed using Lorentzian lineshape in the region $1800\text{-}1560\text{cm}^{-1}$ on the average spectrum of each mutant.

2D correlation analysis

Numerical computation of 2D correlation analyses were performed following the algorithms in eq1-5. The dynamic spectra set $\tilde{y}(\nu, \mathbf{p})$ were acquired by subtracting the reference spectrum, typically the average spectrum $\bar{y}$. Calculation of the asynchronous correlation was simplified using the Hilbert-Noda transformation (eq3) in stead of the cumbersome fast Fourier transform.

$$\Phi(\nu_1, \nu_2) = \frac{1}{n-1} \sum_{j=1}^n \tilde{y}(\nu_1, \mathbf{p}_j) \cdot \tilde{y}(\nu_2, \mathbf{p}_j) \quad (1)$$

$$\Psi(\nu_1, \nu_2) = \frac{1}{n-1} \sum_{j=1}^n \tilde{y}(\nu_1, \mathbf{p}_j) \sum_{k=1}^n M_{jk} \cdot \tilde{y}(\nu_2, \mathbf{p}_j) \quad (2)$$

$$M_{jk} = \begin{cases} 0 & \text{if } j=k \\ 1/\pi(k-j) & \text{otherwise} \end{cases} \quad (3)$$

In the $\beta\nu$ asynchronous correlation (eq4), the experimental spectra were correlated to the sinusoidal function $\sin(k\phi+\beta)$, where β is the phase angle defined by $\beta = \tan^{-1}(\frac{\Psi}{\Phi})$ ($0^\circ \leq \beta < 360^\circ$). $k=10^\circ$, so that the correlation was performed with $\sim 1/4$ of a cycle of the sinusoid function. The temporal order of two signal features at ν_1 and ν_2 can be quantitatively measured by the effective angle $\beta_e = \beta + 90^\circ$ in the $\beta\nu$ correlation maps. [15]

$$\Psi(\nu, \beta) = \frac{1}{N-1} \sum_{j=0}^{N-1} \tilde{y}(\nu, \mathbf{n}_j) \sum_{k=0}^{N-1} M_{jk} \sin(k\phi + \beta) \quad (4)$$

In the $k\nu$ asynchronous correlation (eq4), the experimental spectra were correlated to the exponential function $\exp(-kt+R)$, where k is the rate describing the relative rate changes in intensity variations. The effective rate constant k_{eff} is defined as the point the maximum correlation intensity at particular frequencies in the $k\nu$ correlation map. Generally, the $k\nu$ map

consists of an upper $k_{\text{eff}+}$ and a lower $k_{\text{eff}-}$ panel, representing the positive and negative rate changes respectively compared to the initial rate in intensity. [16]

$$\Psi(\nu, k) = \frac{1}{N-1} \sum_{j=0}^{N-1} \tilde{y}(\nu, n_j) \sum_{k=0}^{N-1} M_{jk} \exp(-kt + R) \quad (5)$$

Windowed autocorrelation was calculated following the formalism by Richardson et al. [18] Each window contains 4 spectra translating along the pH axis.

All data set were baseline-corrected and normalized before 2D correlation analysis in the amide I region of 1570-1750 cm^{-1} . All the algorithms were carried out with MATLAB programs (v6.1, The MathWorks, Natick, MA) written in our lab. All 2D correlation maps were plotted with the same contour level.

Results and Discussion

Site-directed mutageneses of rdhSP-C were listed in **Table4.1**. Band assignment for the secondary structure was given in **Table4.2**. **Fig4.1**. illustrated the transmission IR spectra of rdhSP-C mutants in response to pH titration in overlaid format. All the mutants showed band intensity changes of major conformations: 1) α -helix at 1659 cm^{-1} 2) antiparallel β -sheet at 1633 cm^{-1} and 1687 cm^{-1} , 3) β -turns at 1679 cm^{-1} or β -strands at 1683 cm^{-1} and 4) random coils at 1645 cm^{-1} . The responses of the mutants to pH titration may be grouped in two patterns. For mutageneses at the N-terminus, Mut3 and mut4 (**Fig4.1.a**. and **Fig4.1.b**.) showed significant increase in β -structures in concomitant with the reducing in α -helix and β -sheet. A closer examination revealed subtle differences between Mut3 and Mut4. Mut3 showed a maximum absorbance at 1679 cm^{-1} , while it occurred at 1683 cm^{-1} for Mut4. Band at 1679 cm^{-1} could be assigned to type III β -turns based on the position potentials for each amino acid [21]. Band at 1683 cm^{-1} was assigned to short twisted β -strands. Mutageneses within the poly-valine segment

(Mut6 (L22P, **Fig4.1.c.**) and Mut7 (V21P, **Fig4.1.d.**), however, showed totally different patterns from that of Mut3 and Mut4 in response to pH titration. The increase in β -turn conformation were much limited compare to that of Mut3 and Mut4. Mut7 showed the least increase in β -turns. Instead, the increases in unstructured conformation as indicated by 1645 were much more significant for Mut6 and Mut7. Curve fitting was performed on the average spectrum (the reference spectrum for 2D correlation analysis) of each mutant to reveal the average fractions of each conformation for the mutants. As illustrated in **Fig4.2.**, as the site-directed mutagenesis moving from the N-terminus to the C-terminus of rdhSP-C, the content of β -turns reduced significantly, while the contents of β -sheets and α -helix increased. On the other hand, Mut3 and Mut4 were predominant with β -structures with least unstructured conformations (<15%). While β -sheet consist of ~30% for Mut6 and Mut7 respectively.

Model-based 2D asynchronous correlation analysis.

The dynamic conformational changes of the mutants in response to pH titration were quantitatively characterized using model-based 2D asynchronous correlation. **Fig4.3.** and **Fig4.4.** showed βv and $k v$ contour maps of rdhSP-C mutants respectively. The relative rate changes of intensity variations were quantitatively evaluated by β_{eff} and k_{eff} values as given in **Table4.3.a.** and **Table4.3.b.**.

Fig4.3.a and **Fig4.4.a** illustrated the results of the βv and the $k v$ correlations analysis on Mut3(C5S/C6S). The βv map was predominant with a broadened band centered at 1679cm^{-1} . In the $k v$ map however, a shoulder band at 1687cm^{-1} with about the same intensity was resolved together with two much weaker bands at 1645cm^{-1} and 1633cm^{-1} in the k_{eff+} panel. In addition, a fairly weak band at 1629cm^{-1} indicated that there were limited β -aggregations formed. The k_{eff-} panel presented only a broadened peak centered at 1679cm^{-1} . The relative rate changes in

intensity variations for the conformations were quantitatively determined from the k_{eff} values in the order $1629/k_{\text{eff}+2.3} > 1645/k_{\text{eff}+2.0} > 1687/k_{\text{eff}+1.9} = 1679/k_{\text{eff}+1.9}$. In addition, the band at 1679cm^{-1} had a value $k_{\text{eff}}=0.3$, indicating a two-step change in intensity, with the initial rate change increasing much faster.

Fig4.3.b. and Fig4.4.b. illustrated the results of the $\beta\nu$ and the $k\nu$ correlation on Mut4 (K11Q/R12Q). The $\beta\nu$ and the $k\nu$ contour maps were both predominant with a major peak at 1683cm^{-1} for Mut4. In the $\beta\nu$ map, the relative rate changes of intensities for the conformations were in the order $1645/\beta_{\text{e}354.3} > 1633/\beta_{\text{e}345.5} > 1659/\beta_{\text{e}322.8} > 1683/\beta_{\text{e}257.4}$. In the $k\nu$ correlation, a major band at 1683cm^{-1} and a weak band at 1633cm^{-1} were resolved in the $k_{\text{eff}+}$ panel. The band of α -helix was not apparent in the $k_{\text{eff}+}$ panel but appeared at a lower frequency at 1656cm^{-1} in the $k_{\text{eff}-}$ panel. The relative rate changes for the conformations are in the order $1645/k_{\text{eff}-0.2} > 1656/k_{\text{eff}-0.1} = 1633/k_{\text{eff}-0.1}$. The band at 1633cm^{-1} may also undergo a two-step change partially, as it also showed a weaker band in the $k_{\text{eff}+}$ panel with faster rate change in intensity. It was of note that the band at 1645cm^{-1} showed the fastest rate change in the $\beta\nu$ map. It was further resolved as a fast decrease in intensity in the $k\nu$ map (the $k_{\text{eff}-}$ panel).

Model-based 2D correlation analyses on Mut6 (L22P) (**Fig4.3.c. and Fig4.4.c.**) and Mut7 (V21P) (**Fig4.3.d. and Fig4.4.d.**) showed similar patterns. The $\beta\nu$ contour map of Mut6 showed a dominant band at 1645cm^{-1} followed by two other weaker bands of ordered structures. In addition, a weak band at $\sim 1687\text{cm}^{-1}$ was also resolved for Mut6. The relative rate changes in intensity variations for Mut6 were in the order $1645/\beta_{\text{e}225.9} > 1659/\beta_{\text{e}49.6} > 1630/\beta_{\text{e}43.5} > 1687/\beta_{\text{e}17.8} > 1679/\beta_{\text{e}10.4}$. In the $k\nu$ map, band 1645cm^{-1} were increased in intensity ($k_{\text{eff}+}=0.3$) while 1659cm^{-1} and 1630cm^{-1} were decreased in intensity. The latter could imply that small scale oligomerization may occur for Mut6. In accompany to 1630cm^{-1} , a fairly weak band was implied

at 1687cm^{-1} in $k_{\text{eff-}}$ panel. Band at 1679cm^{-1} were fairly weak in $k\nu$ map with a two-step change in intensity with the initial rate much faster. Mut7 showed somehow similar patterns in $\beta\nu$ and $k\nu$ maps to those of Mut6. However, a fairly weak increase at 1687cm^{-1} was observed in $k_{\text{eff+}}$ panel for Mut7. Furthermore, the formation of aggregations was relatively appreciable for Mut7 as indicated by a weak peak at 1620cm^{-1} ($k_{\text{eff-0.7}}$) in the $k\nu$ map, but with little β -turns formed.

Windowed auto-correlation analysis.

In the presenting study, we investigated the pH-conformation relationship using windowed auto-correlation, as shown in **Fig4.5**. Each window contained 4 spectra as a subset. The bands elongated along the pH axis reflected the dominant conformations for each rdhSP-C mutant within certain pH ranges, while those elongated along the frequency axis at a particular pH may indicate a fast intensity changes occurred. As illustrated in **Fig4.5.a.**, β turns were the dominant structure for Mut3 at $\text{pH}>5$. The intense peak at 1679 at $\text{pH}<4$ was associated to the increase of 1679cm^{-1} in the windowed pH range. As the correlation map were plotted using the averaged spectrum of each subset of spectra, the negative peak reflected an overall lower intensity of that peak within the pH windows compared to the average spectrum of that subset. It was also noticeable that the pattern of the windowed auto-correlation map was in accordance with a two-step change for the β structure for Mut3. For Mut4 (**Fig4.5.b.**), fast increase of the β structures occurred around $\text{pH}7$, while the unordered structure was predominant at $\text{pH}4-5$. It was interesting to notice the intensity change pattern between β -turns and unordered structures, which may imply a possible conversion between these two conformations. Mut6 and Mut7 showed total different responses from that of Mut3 and Mut4 upon pH titration. Mut6 was predominated with ordered structures throughout pH titration, particularly with α -helix and antiparallel β -sheet at $\text{pH}>5$. In contrast, windowed autocorrelation on Mut7 showed a single

band at 1645cm^{-1} started as earlier as at pH4.5. The intensity patterns in the windowed auto-correlation were most agreeable with the kv contour maps.

pH-induced structure conversions of rdhSP-C mutants overall exhibited a distinct trend as the site mutagenesis shifted from the N-terminus to the poly-valine segment. Mutagenesis at the N-terminus resulted in significant increase in β -structures. It was of note that Mut3 was mostly dominated by β -turn structure with the fastest increase in antiparallel β -sheet conformation. Meanwhile, the kv map of Mut3 clearly resolved concomitant increase of β -aggregation upon pH titration. As the Cys pairs were replaced by Ser pair, crosslink through disulfide bond was prevented, and could not be a factor contributed to the formation of β -aggregation for Mut3. Therefore, the formation of β -aggregation may be attributed to the intact poly-valine segment, which was predicated having intrinsic propensity for α to β conversion. For other mutants, the intact Cys pair would allow possible crosslink. The formation of antiparallel β -sheet or β -aggregations was little for Mut4. Instead, the band maximum at 1683cm^{-1} of Mut4 indicated the formation of short twisted β -strands, which was different from extended β -sheet, as predicated by *ab initio* calculations. [22] For mutagenesis in the poly-valine segment, the replacement with proline, a known “helix breaker”, resulted in fast denaturing for Mut6 and Mut7 upon pH titration. For mut6, the downshifted frequency of β -sheet may indicate the existence of some oligomers. Curve fitting on average spectra indicated that both Mut6 and Mut7 retained relatively higher level of β -sheet structure, which was indicative that an intact poly-valine segment was crucial for maintaining an ordered (hydrogen-bonding) structure. It was of note that Mut6 retained higher level of β -turn structure ($\sim 22\%$) while mut7 showed the little β -turn structure ($< 5\%$). In addition, fairly weak increases in anti-parallel β -sheet and β -aggregation structure were observed for Mut7 but not Mut6, as resolved in kv map. These discrepancies

could result from the different amino acids being replaced (Leu for Mut6, Val for Mut7), as well as the position of mutagenesis. It was possible that the mutagenesis of L22P for Mut6 may favor the formation of short edges of β -structure that preventing aggregations, [23] while replacement of Val with Pro in Mut7 were more effective in breaking down the helical structure.

Conclusions

The presenting study investigated the functional importance of certain structure motifs of rdhSP-C. As the mutageneses moving from the N-terminus to the C-terminus, the mutants unfolded into β -turn structure to unordered structure respectively. Limited β -aggregation was observed but not confined with mutagenesis at either N-terminal or in the poly-valine segment. It was implied that the poly-valine residues were of primary importance for maintaining the helical structure for rdhSP-C. In case of Mut3 (C5S/C6S), crosslink via disulfide bond formed by Cys pair was prevented. Therefore, it was implied that crosslink through the formation of disulfide bond at the Cys pair was not required for the formation of β -aggregation. Instead, an intrinsic α to β conformational conversion was favored. Mutagenesis with proline in the poly-valine segment resulted in breakdown of the helical structure as predicated by the nature of proline as a helical breaker. However, subtle difference between Mut6 and Mut7 were observed. For Mut7 but not Mut6, small but appreciable increase in β -aggregation was observed. The difference could be possibly attributed to the different amino acids being replaced, as well as the position where the mutagenesis occurred.

The presenting structure characterization of rdhSP-C mutants revealed important information with regard of the structural-functional relationship of certain motifs of rdhSP-C. Recent immunocytochemistry study indicated that palmitoylation at the Cys pair may facilitate the transfer of lipid monolayer into stacked multilayer structure (“reservoirs”) therefore promote

lipid recruiting and recycling. It has also been suggested that the Lys-Arg pair was important for trafficking of proSP-C out of the ER therefore may have influence on the aftermath post-ER palmitoylation at the Cysteine residues. [24] Our future work will conduct investigations on the interaction of these rdhSP-C mutants with phospholipids to elucidate the biological relevant structural-functional relationship of these motifs.

Acknowledgements

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Table4.1. Site-directed mutageneses of rdhSP-C.

rdhSP-C Mutants	Mutations
Mut3	C5S/C6S
Mut4	K11Q/R11Q
Mut6	L22P
Mut7	V21P

Table4.2. Peak assignment for the secondary structures of rdhSP-C mutants.

	Wavenumber (cm⁻¹)	Conformation assignment
Amide I region	1687	anti-parallel β -sheet
	1683/1679	β -strands/ β -turns
	1659	α -helix
	1645	Random coils
	1633	anti-parallel β -sheet

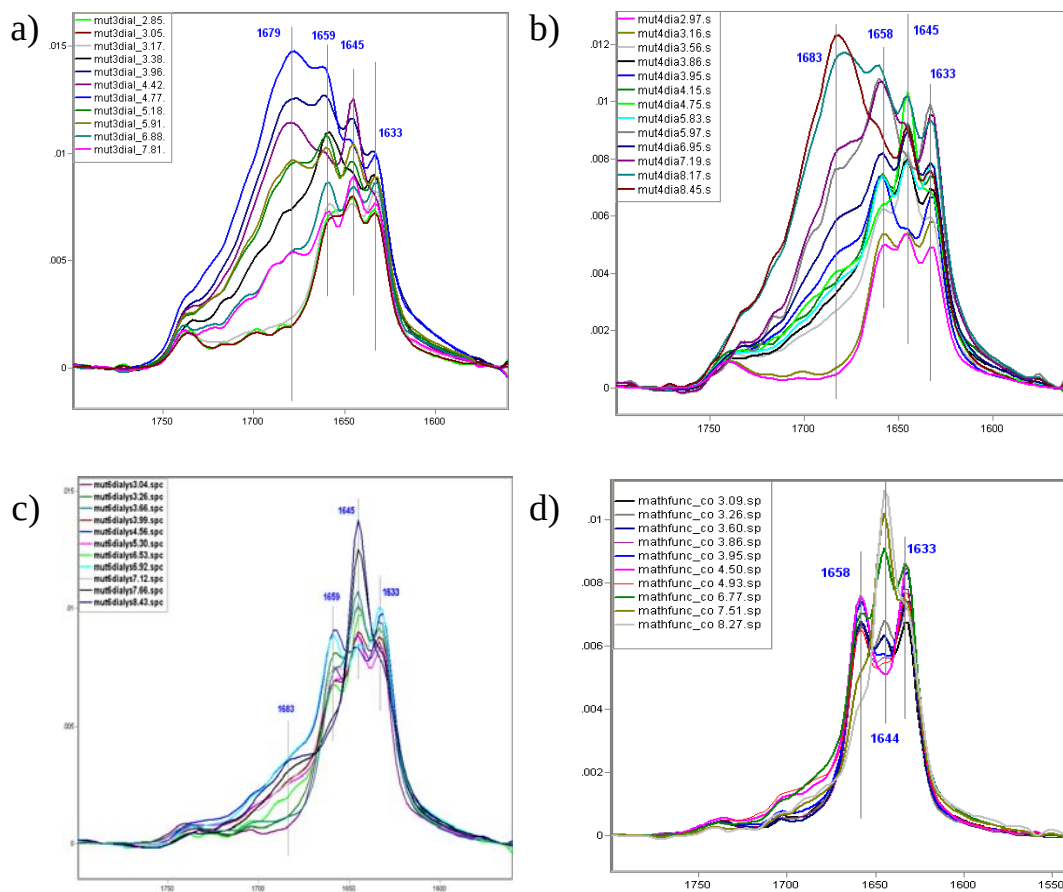


Fig4.1. Overlaid transmission IR spectra of the rdhSP-C mutants in response to pH titration. a) Mut3 (C5S/C6S), b) Mut4 (K11Q/R12Q), c) Mut6 (L22P), d) Mut7 (V21P).

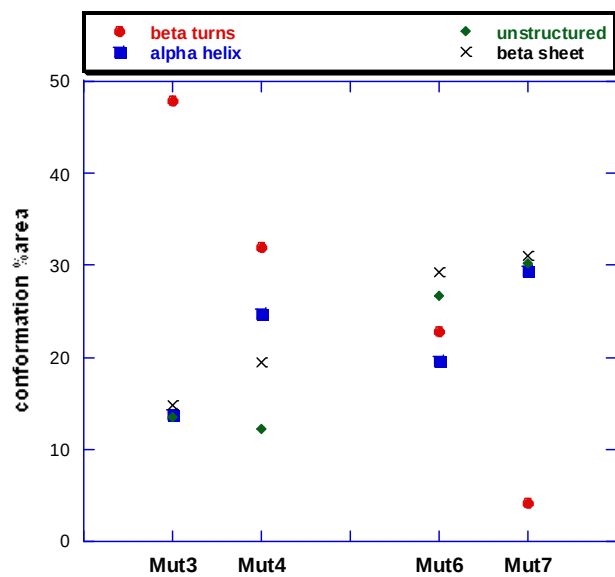


Fig4.2. Fractions of conformations of rdhSP-C mutants based on curve fitting on average spectra.

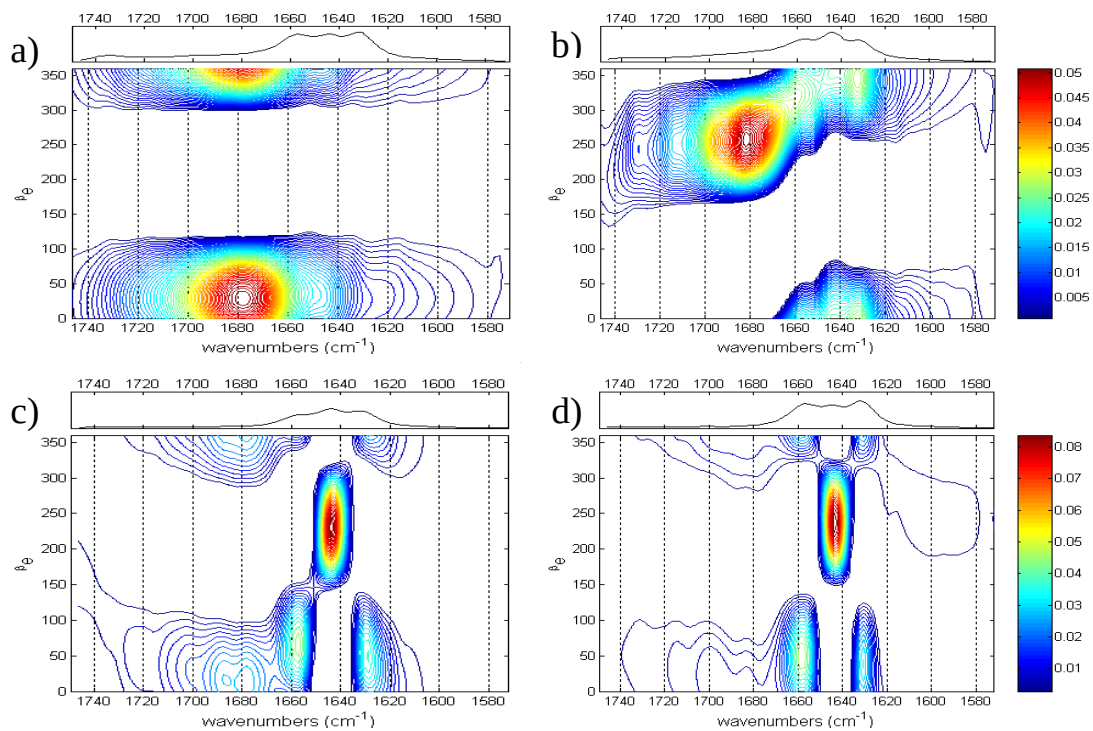


Fig4.3. βv asynchronous correlation analyses of the rdhSP-C mutants in response to pH titration.

a) Mut3 (C5S/C6S), b) Mut4 (K11Q/R12Q), c) Mut6 (L22P), d) Mut7 (V21P).

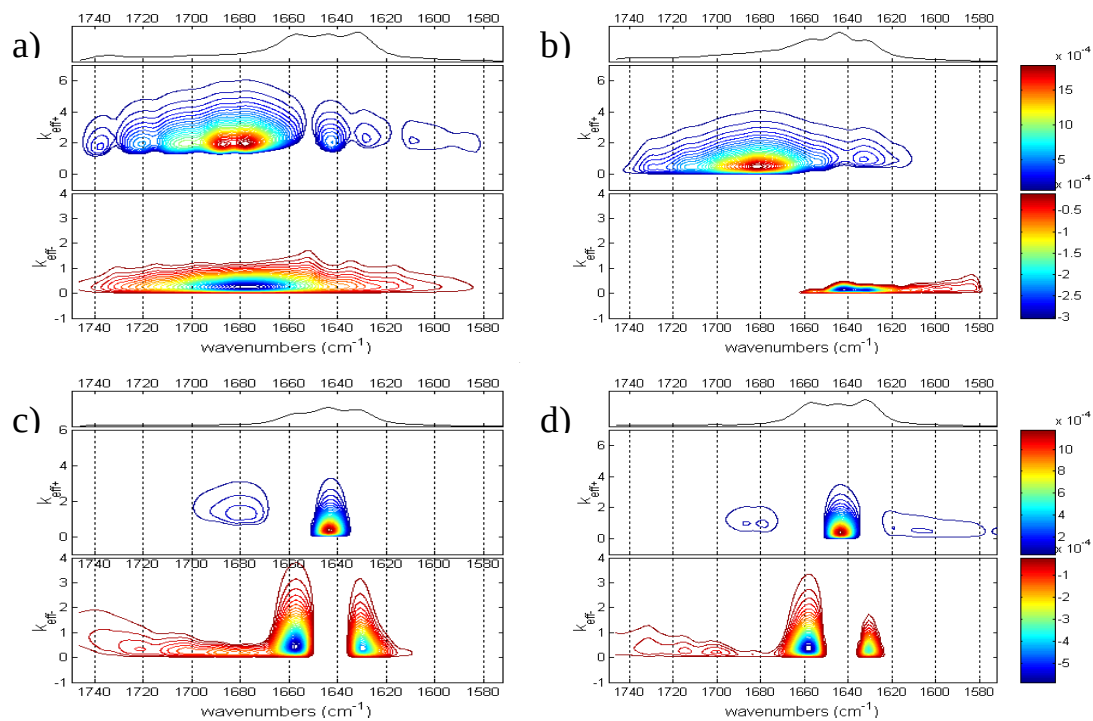


Fig4.4. kv asynchronous correlation analyses of the rdhSP-C mutants in response to pH titration.

a) Mut3 (C5S/C6S), b) Mut4 (K11Q/R12Q), c) Mut6 (L22P), d) Mut7 (V21P).

Table4.3.a. Quantitative $\beta\nu$ correlation analyses of rdhSP-C mutants.

Wavenumber (cm ⁻¹)	β_{eff}			
	Mut3	Mut4	Mut6	Mut7
1687			17.8	
1683		257.4		
1679	30		10.4	
1659		322.8	49.6	49.6
1645		354.3	225.9	236.9
1633,1630*		345.5	43.5*	37.3

Table4.3.b. Quantitative $k\nu$ correlation analyses of rdhSP-C mutants.

Wavenumber (cm ⁻¹)	Mut3		Mut4		Mut6		Mut7	
	<i>keff</i> +	<i>keff</i> -	<i>keff</i> +	<i>keff</i> -	<i>keff</i> +	<i>keff</i> -	<i>keff</i> +	<i>keff</i> -
1687	1.9					0.2	1.0	
1683			0.5					
1679	1.9	0.3			1.0	0.2	1.0	
1659, 1656 ^Δ				0.1 ^Δ		0.4		0.4
1645	2.0			0.2	0.3		0.4	
1633,1630*			1.0	0.1		0.3*		0.3
1629,1620 [#]	2.3						0.7 [#]	

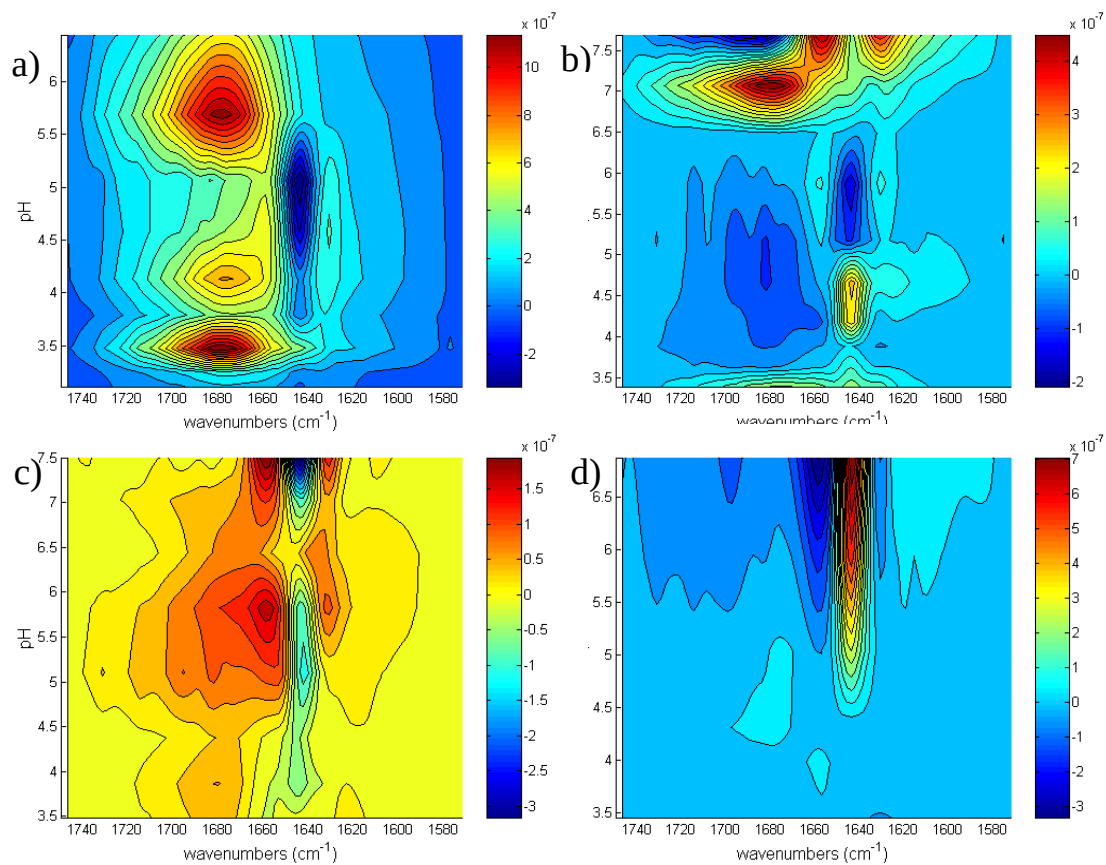


Fig4.5. Windowed auto-correlation analyses of rdhSP-C mutants.

a) Mut3 (C5S/C6S), b) Mut4 (K11Q/R12Q), c) Mut6 (L22P), d) Mut7 (V21P).

CHAPTER 5

SURFACE PRESSURE INDUCED CONFORMATIONAL CHANGES OF RECOMBINANT DEACYLATED HUMAN SURFACTANT PROTEIN C AND A MUTANT IN PHOSPHOLIPID MONOLAYER AT THE AIR/WATER INTERFACE¹

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Abstract

Polarization Modulation External Reflectance IR spectroscopy (PM-IRRAS) was employed in the investigation of recombinant deacylated human pulmonary surfactant protein C (rdhSP-C) and its mutant (Mut4, K11Q/R12Q) of the secondary structure conversion upon surface pressure perturbation in deuterated dipalmitoyl phosphatidylcholine (DPPCd62) monolayer at the air/water interface. Model-based 2D correlation analysis provided much improved resolution for protein structure determination in amide I region. Compared to the wild type rdhSP-C in DPPCd62 monolayer, a pronounced structure conversion from α -helix to β -sheet was observed for Mut4. However, no significant formation of β -aggregation was observed for Mut4. DPPCd62 showed pronounced dehydration and increased order in acyl chain upon monolayer compression.

Keywords: recombinant deacylated human surfactant protein C, site-directed mutagenesis, conformational change, DPPCd62, PM-IRRAS, Langmuir film, air/water interface, surface pressure perturbation, model-based 2D correlation analysis.

Introduction

Human pulmonary surfactants consist of 85% phospholipids, 4-8% neutral lipids and about 10% surfactant proteins. Phosphatidylcholines (PC~46%) and anionic phosphatidylglycerol (PG ~10%) constitute the bulk of phospholipids. Disaturated PCs such as 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) are essential to resist lung collapse at the end of exhalation attributed to its high gel-to-liquid-crystal phase transition temperature ($T_c \sim 41^\circ\text{C}$) of the acyl chains. Anionic PGs and unsaturated phospholipids are essential to retain fluidity of the aqueous alveolar linings for re-adsorption and fast re-spreading of the surfactant materials upon inhalation. [1, 2] There are four pulmonary surfactant apoproteins. Surfactant protein A and D (SP-A and SP-D) are hydrophilic and mainly responsible for the innate host defense against the invading pathogens such as viruses and bacteria, as well as particulates such as pollen. Surfactant protein B and C (SP-B and SP-C) are small hydrophobic apoproteins particularly important for maintaining the normal mechanical breathing cycle, facilitate the dynamic adsorption, spreading and the stability of the lung lining films in associated with lipids. [3] It is established that there are coordinated functionalities of SP-B and SP-C. Particularly, the deficiency of SP-B resulted in abnormal expression of SP-C and is associated with fatal Respiratory Distress Syndrome (RSD) for premature infants and acute RDS (ARDS) for older children and adults. [4-6]

Mature SP-C is a unique small (4.2kDa) hydrophobic lipoprotein exclusively expressed in pulmonary alveolar type II (ATII) cells. Human SP-C proprotein (proSP-C) (21kDa) is synthesized at the endoplasmic reticulum with 191-197 amino acids, and then translocated to Golgi apparatus. After post-translocation and proteolytic cleavage, matured SP-C is secreted into the alveolar linings together with SP-B and phospholipids. [4] NMR study reveals four structure

motifs of mature SP-C in organic solvents: 1) two palmitoylated Cysteine residues (C5 and C6) surrounded by two Prolines at the N-terminus, 2) a positively charged Lys-Arg (K11 and R12) pair, 3) a very hydrophobic poly-valine segment, and 4) a heptapeptide C-terminus. [7] It has been determined that SP-C is a type II transmembrane protein. [8] NMR study of mature SP-C in apolar solvent indicates that it contains a rigid α -helix consisting of residues 9-34. The length of the α -helix is about 37Å which is in agreement with the length to span in the lipid bilayers. [9] Palmitoylation was proposed in stabilization of the helical structure. [10]

Extensive studies have been done focusing on the surface activities of SP-C in association with phospholipids to facilitate the adsorption, spreading and stabilization of the alveolar lining film. [3] Most recently, the advance in understanding of SP-C biosynthesis has indicated an emerging role of SP-C in conformational lung disease. [4] Misfolding of SP-C presents a similar case to other chronic neurodegenerative diseases such as Alzheimer's and Huntington's disease, all are characterized by conformational conversion from α -helix to β -strands and formation of amyloid fibrils. Genetic abnormalities in SP-C are associated with idiopathic pulmonary fibrosis (IPF), pulmonary alveolar proteinosis (PAP), and less commonly, fatal interstitial lung disease. In the case of PAP, misfolding and formation of amyloid like fibrils of mature SP-C is possibly linked to partial or complete deacylation of SP-C. [11] The majority of genetic mutations in the SP-C gene (*sftpc*) that lead to these diseases occur in the domains corresponding to pre-processed form of SP-C (proSP-C). [6] Recently, some immunocytochemistry studies investigated the mutageneses in proSP-C. [12-15] According to the "positive inside rule", [16] the positively charged Lys-Arg pair plays a role in determination of the orientation of proSP-C as a type II transmembrane protein, [8] i.e. the N-terminus remains in the cytoplasm while the C-terminus in the ER lumen. Immunocytochemistry study indicated that

mutagenesis of the Lys-Arg pair may influence palmitoylation of proSP-C and the following trafficking events. [15] Collectively, the proper insertion of proSP-C influences the normal folding of SP-C. It is therefore of interesting to investigate biophysically the structural-functional relationship of certain motifs in SP-C. The presented study is focused on the structural influence of mutagenesis at the Lys-Arg pair.

External reflection IR spectroscopy (IRRAS) at the air/water interface was first demonstrated by Dluhy et al in the middle of 1980's. Quantification analysis of the thin film at the air/water interface showed significant properties different from that at the air/metal surface. It was demonstrated that good signal to noise ratio could be obtained at the air/water interface despite the fact that water has much weaker reflectance compared to metals. [17] IRRAS has been proved a very suitable tool for in situ investigation of Langmuir films and model pulmonary surfactant system at the air/water interface. [18-20] *Polarization modulation external reflection IR spectroscopy* (PM-IRRAS) was developed to discriminate the anisotropic IR absorption of sample molecules from the isotropic sample environment such as water vapor. [21, 22] The output signal from the detector has a double modulation with the frequency modulation from the FTIR interferometer and the polarization modulation from a photo-elastic modulator (PEM). The PEM generates a fast modulation between the parallel (p-) and the perpendicular (s-) polarizations from the polarizer. It is therefore largely compensates water vapor absorption in mid-IR region, which overlaps with the amide I region (1600-1700cm⁻¹). Instrumental setup of PM-IRRAS usually adopts the two-channel electronics with a lock-in amplifier. The signal generated is expressed by:

$$S = \frac{I_-}{I_+} = C \frac{J_2(\phi_0)(R_p - R_s)}{(R_p + R_s) + J_0(\phi_0)(R_p - R_s)}$$

where I_+ is from the FTIR interferometer modulation, I_- is from PEM modulation. J_2 and J_0 are the second- and zero-order Bessel functions of the maximum dephasing ϕ_0 introduced by the photoelastic modulator.

PM-IRRAS has also been applied to investigate the structure and orientation of biological Langmuir films. [23] Recently, PM-IRRAS has been applied in investigation of the structures of a synthetic monomeric human surfactant protein B (mSP-B₁₋₂₅) and the deacylated bovine surfactant protein C (dSP-C) in binary phospholipid monolayers respectively. [24, 25]. The presenting study compared the surface pressure induced response of rdhSP-C Mut4 (K11Q/R12Q) to that of wild type rdhSP-C.

Generalized 2D correlation analysis was developed by Noda in 1993 as a resolution enhancement technique. The dynamic spectral features in response to external perturbation such as temperature, pH and pressure can be monitored for linearly correlated intensity variations in the synchronous analysis, while the non-linearly correlated intensity variations are monitored in asynchronous analysis. As a result, sequential information at two different wavelengths can be *qualitatively* extracted based on the Noda's rules. [26] *Model-based 2D asynchronous correlation* methods were developed by Dluhy et al. [27, 28] Generally, the waveform of the dynamic spectra can be modeled with certain analytical functions. In the model-based 2D asynchronous correlation analysis, a set of experimental spectra were orthogonally correlated to a model function, being either sinusoidal (βv) or exponential ($k v$). Compared to the generalized 2D correlation, noise-induced artificial peaks are much reduced. More importantly, model-based 2D asynchronous analysis provides straightforward *quantitative* analysis of the relative rate changes in intensities of the interested spectral features. Application of model-based 2D

correlation analysis to studies at the air/water interface demonstrated the above mentioned advantages.

Materials and Methods

DPPCd62 are from Avantilipids (Alabaster, AL) and used as purchased. Bulk DPPCd62 solutions are prepared by dilute the original concentration (10mg/mL in chloroform) into 1mg/mL (Chloroform: Methanol = 4:1). rdhSP-C Mut4 (K11Q/R12Q) were provided by our collaborator Dr. John E Baatz at Medical University of South Carolina. The production and purification methods were detailed elsewhere. [29] rdhSP-C wild type (referred as WT in the following) and Mut4 were accepted with concentration in 150-400 μ g/mL and were dialyzed against 80% methanol after reception to get rid of TFA from the purification procedure. An aliquot of WT or Mut4 was withdraw to mix with DPPCd62 to yield a 10%(w/w) WT/Mut4 + DPPCd62 mixture and vortex to homogeneous solution before experiment.

Langmuir film compression. A mono-molecular film was formed spontaneously by spreading an aliquot of $\sim$ 30-40 μ L solution of the above mentioned mixture on NaCl subphase (120mM) in the customized Langmuir trough (Riegler & Kirstein GmbH, Germany). The monolayer was left for 10min for solvent evaporation and equilibrium. The surface pressure was monitored with a Wilhelmy plate. Step-wise compression of the film was performed during IR data collection. In between each target pressure, the film was left for 5min for equilibrium before data collection of $\sim$ 10-15min. The monolayer appeared to be stable and reproducible for all experiments.

PM-IRRAS experiments. IR spectra were collected using a Bruker EQUINOX55 IR spectrometer with customized external reflectance accessory in couple to the Langmuir trough. A high sensitivity narrow-band MCT detector was used for signal detection. A Zn wire polarizer was placed before the PEM. The PEM has a frequency of 50KHz changing between the linear p- and

s- polarization and therefore resulted in 100KHz for each cycle of modulation. Sampling frequency of the double modulated PM-IRRAS was limited by the time constant of the Lock-in amplifier. The time constant of the Lock-in amplifier in the presenting study was set at 30 μ s (33kHz) to allow a normal IR interferometer frequency at 14kHz. A background spectrum was first collected with the sample-free subphase. Then the monolayer was spread on the subphase and data were collected at each target surface pressure.

Data acquisition and preprocess. Data were collected at resolution of 8 cm^{-1} , zerofilling at level 2, as well as with triangle apodization function and power spectrum phase correction. Data were preprocessed with Grams32/AI(6.0) software. Data were first smoothed using Savitzky-Golay algorithm (2 degree, 15pts). Care was taken to make sure that band width of C=O was not changed after smooth to avoid band distortion. Baseline correction was performed with sextic function for the truncated regions including the CD₂ stretch mode, the amide I region, and the polar group region of DPPCd62 (2200-1000 cm^{-1}).

Model-based 2D correlation analysis. The detail of the algorithms for model-based 2D correlation can be found in Ref. [27, 28]. Calculations of correlation were performed using a program written with MATLAB in our lab (MATLAB 6.1 or newer version). Dynamic spectra were obtained by mean centering to the average spectrum. For pressure-dependent PM-IRRAS experiment at the air/water interface, surface density normalization was carried out to adjust intensity variations due to the changes in molecular density during compression. [30] In addition, the negative band centered at 1645 cm^{-1} due to abnormal dispersion of water bending mode was compensated for each spectrum using a Lorentzian lineshape with various intensity (negative) and band width (FWHH) to yield a flat baseline.

Results and Discussion

Fig5.1. showed the isotherms 10%WT+DPPCd62 and 10%mut4+DPPCd62 monolayers respectively. As expected, both WT and Mut4 induced fluidity into the monolayers, as no distinct phase transition from LC-LE to LC were observed. However, the fluidization effect of both WT and Mut4 were small that a surface pressure of up to $\sim 70\text{mN/m}$ of the monolayer was achieved. In addition, monolayer with rdhSP-C WT exhibited higher compressibility compared to monolayer with Mut4.

Fig5.2. and **Fig5.3.** illustrated the PM-IRRAS spectra of 10%WT+DPPCd62 and 10%mut4+DPPCd62 respectively upon monolayer compression. The most pronounced bands were from DPPCd62. IR bands centered at 2195 and 2090 represented the asymmetric and symmetric CD_2 stretching modes of the DPPCd62 acyl chains respectively. Both bands were observed down shifted $2\sim 3\text{cm}^{-1}$ upon monolayer compression, indicating an increased order of the acyl chains. [31] The band centered at 1735cm^{-1} was due to the $\text{C}=\text{O}$ stretching of the ester groups of DPPCd62 that bridging the phosphate headgroup and the acyl chains. A very well characterized dehydration of DPPCd62 was observed upon monolayer compression, as indicated by the band shift from low frequency $\sim 1715\text{cm}^{-1}$ to high 1738cm^{-1} . [32] Two other pronounced bands centered at 1225cm^{-1} and 1090cm^{-1} were due to the asymmetric and symmetric stretching of the phosphate headgroup of DPPCd62. A characteristic negative dip occurred in PM-IRRAS spectrum was due to the abnormal dispersion of the bending mode of water, which exactly overlapped with the amide I band of protein secondary structure. As demonstrated in **Fig5.2**, the amide I band centered around 1653cm^{-1} for WT and 1654cm^{-1} for Mut4 were still fairly well resolved in the PM-IRRAS spectra. It was of note that up to high surface pressure $\sim 65\text{mN/m}$ for the WT, the amide I band was no longer very much appreciable. A possible explanation was that

wild type rdhSP-C was excluded from highly packed DPPCd62 monolayer. The amide I band of Mut4 existed throughout monolayer compression.

As shown in **Fig5.2** and **Fig5.3**, PM-IRRAS was able to resolve the amide I band in one single experiment. Further resolution of the secondary structure was assisted by employment of 2D correlation analysis, particularly model-base 2D correlation analysis.

Fig5.4. illustrated the generalized 2D correlation analysis for 10%WT+DPPCd62 dynamic spectra. **Fig5.4.a.** showed the synchronous contour map of 10%WT+DPPCd62. Along the diagonal axis, peaks were most resolved at 1715cm^{-1} and 1648cm^{-1} , as well as an elongated peak centered at 1690cm^{-1} , which were corresponding to the hydrated C=O band, the unordered structures and a combination of antiparallel β -sheet and β -turns respectively. The positive cross peak of 1694 and 1648 indicated the intensities of those two bands were linearly changing during monolayer compression. **Fig5.4.b.** showed the asynchronous contour map of 10%WT+DPPCd62. As expected, a cross peak of 1694 and 1648 could not be determined, i.e., the linear changes are not presented in the asynchronous map. Only non-linear changes of peak intensity were resolved in the asynchronous map, e.g. positive cross peaks of $1687 \rightarrow 1725$ and $1648 \rightarrow 1737$. (“ $\rightarrow$ ” denoted that the intensity changes occurring one ahead of the other.) It was apparent that asynchronous map was very much complicated with possible artifact peaks resulted from spectral noises and absorptions of water vapor residues. Interpretation of the asynchronous correlation became a daunting task in this case. However, model-based 2D correlation analysis presented much more clear pictures of dynamic structure conversion of both WT and Mut4 as discussed in the following.

Fig5.5. presented the results from model-base 2D asynchronous correlation analysis of 10%WT+DPPCd62. **Table5.1.** quantitatively summarized the relative intensity changes of

resolved peaks. The sequential out-of-phase changes in peak intensities were in the order: 1737→1621→1612→1725→1672→1648→1711→1705. Bands in the region from 1700-1750 were collectively assigned to C=O of different degree of protonation or hydration/dehydration. [33] Bands at 1621 and 1612 clearly indicated the formation of β -aggregation of wild type rdhSP-C upon monolayer compression. As indicated by β_{eff} values, the relative intensity change of β -aggregation was faster than that of both β -turn and unordered structures. At the same time, the C=O of DPPCd62 experienced step by step dehydration. No peak of helical structure was resolved in $\beta\nu$ map. It was possible that the overall intensity changes of the helical structure were not appreciable by $\beta\nu$ correlation. Comparatively, no appreciable α -helix was resolved in the $k\nu$ map. However, a peak centered around 1662cm^{-1} , which was assigned to 3_{10} -helix, was resolved in the $k_{\text{eff-}}$ panel of $k\nu$ map (**Fig5.5.b.**), indicating that there could be small decrease in helical structure. As the $k_{\text{eff+}}$ values represents the relative increase rate in intensity change and $k_{\text{eff-}}$ the relative decrease rate, $k\nu$ map further illustrated increases in β -aggregation of wild type rdhSP-C, as indicated by relative rate changes of intensities for strong bands at 1621cm^{-1} and 1612cm^{-1} . Concomitant increases were also resolved in the $k\nu$ contour map for weak bands at 1669cm^{-1} (β -turns) and 1639cm^{-1} (overlap of unordered structure and β -sheet). The unordered structure at 1648cm^{-1} were resolved with both $k_{\text{eff+}}$ and $k_{\text{eff-}}$ values, which implied a two-step change in the relative rate changes for 1648cm^{-1} , with a faster decrease rate than increase rate. In addition, dehydration of DPPCd62 was clearly indicated by decrease of 1715cm^{-1} but increase of 1738cm^{-1} .

Fig5.6.a. showed the synchronous contour map of 10%mut4+DPPCd62. Two peaks were resolved in amide I region along the diagonal axis: 1698 and 1654cm^{-1} , representing anti-parallel

β -sheet and α -helix respectively. A cross peak was also resolved for these two peaks, indicating simultaneous changes in peak intensities. In the asynchronous map (**Fig5.6.b.**), three pairs of cross peaks were very much pronounced: 1705 $\rightarrow$ 1694, 1656 $\rightarrow$ 1696, 1695 $\rightarrow$ 1738. The band at 1705 were assigned to C=O of doubly hydrated forms. [34] Clearly, asynchronous correlation analysis was very much sensitive to small changes in band shift and band overlapping, as demonstrated here by the resolves cross peaks. In addition, the cross peak pair of 1656 $\rightarrow$ 1696 were not expected if it was assumed that they were of the same conformational origins as the cross peaks resolved in the synchronous map (1654 and 1698). It was possible this pair of cross peak was resulted from artifacts due to spectral noise or water vapor absorption. Therefore, application of generalized 2D correlation analysis on PM-IRRAS spectra presented more complications due to instrumentation noise and residue peaks of water vapor.

Fig5.7. showed the results of $\beta\nu$ and $k\nu$ correlation analyses on 10%mut4+DPPCd62 dynamic spectra respectively. A strong peak at 1695cm⁻¹ was resolved in $\beta\nu$ map for anti-parallel β -sheet. Other weak peaks were resolved for helical structure (overlap of α -helix and 3_{10} -helix) at 1660cm⁻¹, β -turn at 1678cm⁻¹, and overlapped unordered structure with β -sheet at 1639cm⁻¹ as well. The α to β structural conversion was very much pronounced in the $k\nu$ map, as indicated by a relative increase of 1695cm⁻¹ in k_{eff+} panel and a relative decrease of 1660cm⁻¹ in the k_{eff-} panel. A fairly weak but relatively faster increase ($k_{eff+}=0.7$) at $\sim$ 1622cm⁻¹ could be assigned in the $k\nu$ map, which was indicative of limited formation of β -aggregations. The relative rate changes of the intensity variations were summarized in **Table5.2.**

In the presenting study, surface pressure perturbation induced pronounced formation of β -aggregation for wild type rdhSP-C in DPPCd62 monolayer. The result was in line with the

observation for deacylated SP-C and for PAP patients. [10, 11, 35] In comparison, Mut4 with mutagenesis at the Lys-Arg pair showed pronounced increase in β -sheet conformation concomitant with relative decrease in helical structures. However, the formation of β -aggregation was very much limited for Mut4. Recent studies indicated that amyloid like fibrils has distinctive β -structure rather than extended β -sheet. [36, 37] In case of Mut4 in DPPCd62 monolayer, IR signals of extended β -sheet were pronounced. The ability of SP-C in forming amyloid like fibrils was considered due to the intrinsic propensity of the poly-valine segment to undergo α to β conversion. [38] As shown in the presenting study, Mut4 with intact poly-valine segment formed extended β -sheet structure with very limited β -aggregation formation in DPPCd62 monolayer. In a previous study of a pH-dependent structure conversions of pure rdhSP-C and mutants dissolved in 80% ethanol, increase in β -aggregation was indicated for the wild type rdhSP-C to some degree, while no appreciable β -aggregation was observed for Mut4 either. Collectively, it was indicated that mutagenesis of K11Q/R12Q limited the formation of β -aggregation, which seemed independent of the molecular environment for Mut4. It was therefore concluded that other than dictated by the intrinsic propensity of poly-valine segment, the formation of β -aggregation could also involve different structure motifs of rdhSP-C. It was also implied that the formation of extended β -sheet may not eventually result in the formation of β -aggregations, which was in agreement with the prediction by *ab initio* calculations that these two β -structures may be of different origin.

Conclusions

The presenting study investigated the structure conversion of one mutant of rdhSP-C, i.e. Mut4 (K11Q/R12Q) in DPPCd62 monolayer at the air/water interface. Model-based 2D

correlation analysis provided advantages for data interpretation of PM-IRRAS spectrum in terms of less susceptible to instrument noises and signals from water vapor residues. In addition, it provided important information on dynamic structure changes of the monolayer upon surface pressure perturbation. Specifically, Surface pressure induced α to β conformational conversion for Mu4 in DPPCd62 monolayer was unambiguously resolved by $k\nu$ correlation analysis. It was demonstrated that PM-IRRAS combined with model-based 2D correlation analysis was particularly suitable for investigation of model biological systems at the air/water interface. The presenting study also indicated that the formation of β -aggregation may have different structural origin other than extended β -sheet. Different structural motifs may be involved in the formation of β -aggregations of rhdSP-C.

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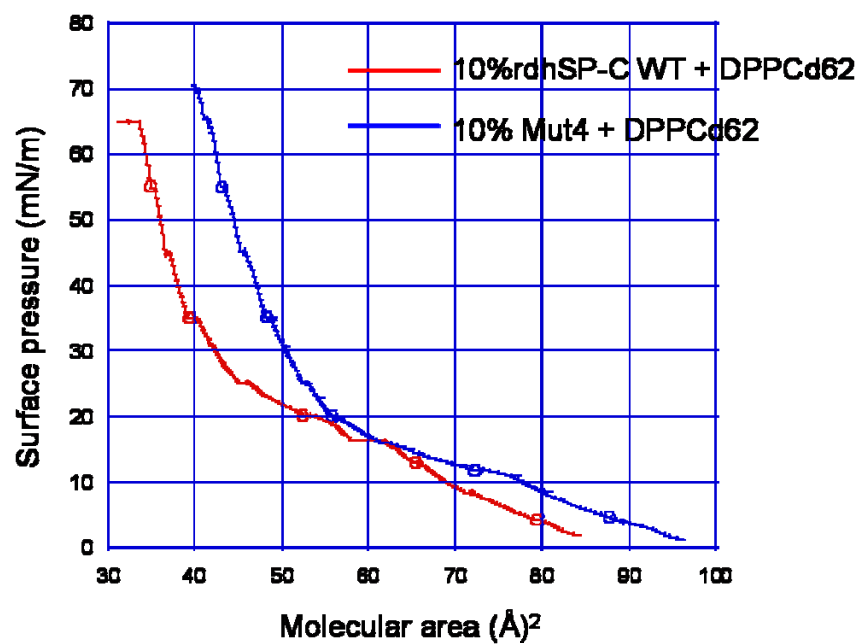


Fig5.1. Isotherms of 10% rdhSP-C WT +DPPCd62 and 10%mut4+DPPCd62 monolayers respectively.

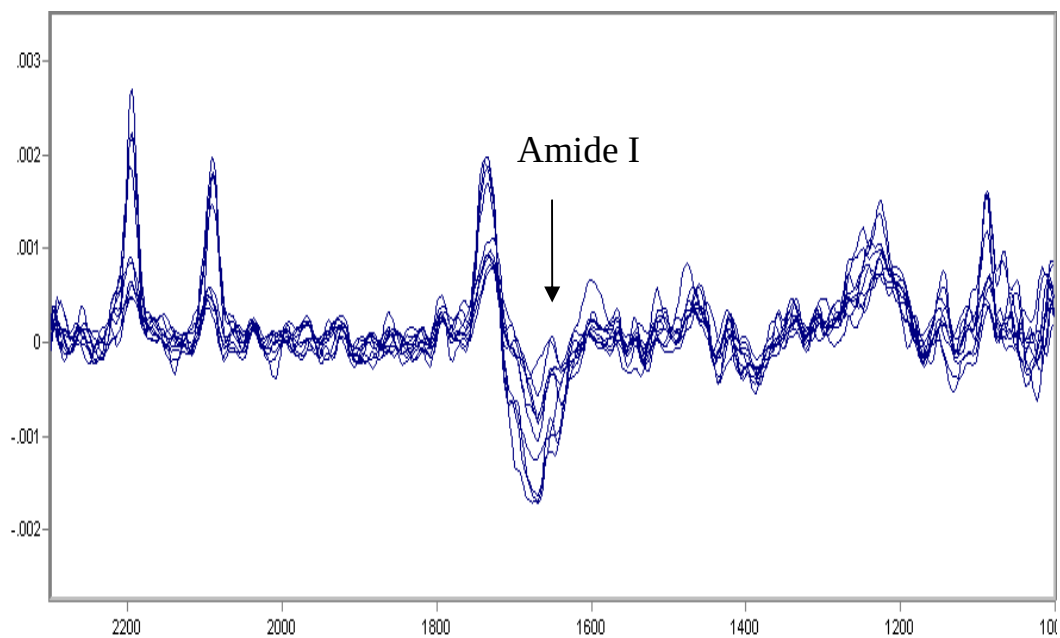


Fig5.2. PM-IRRAS spectra of 10%rdhSP-C WT+DPPCd62 monolayer at the air/water interface. Surface pressure changes from 0-65mN/m.

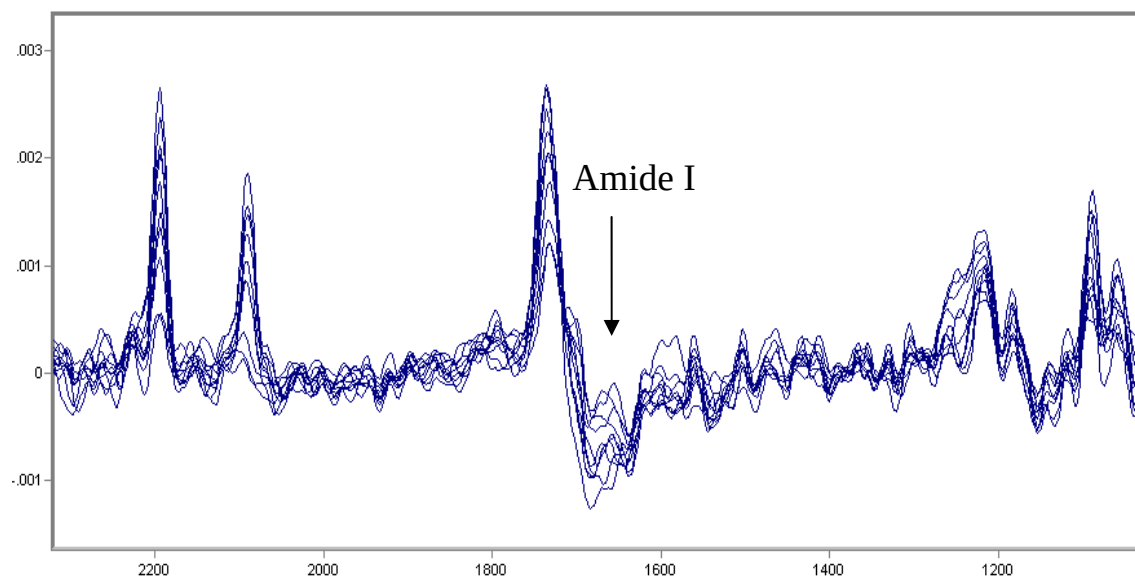


Fig5.3. PM-IRRAS spectra of 10%mut4+DPPCd62 monolayer at the air/water interface. Surface pressure changes from 0-60mN/m.

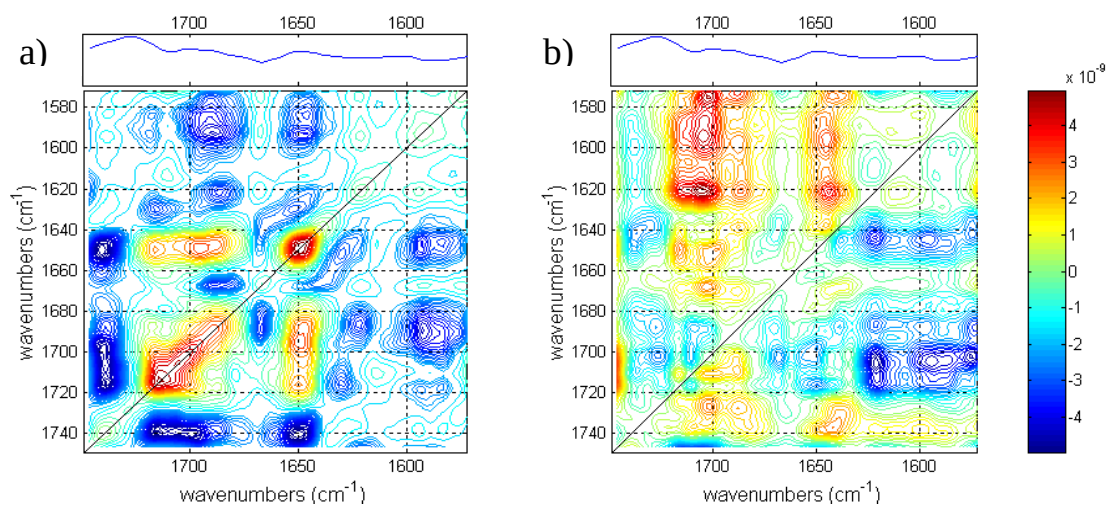


Fig5.4. Generalized 2D correlation analysis of 10%mut2+DPPCd62 monolayer at the air/water interface. a) Synchronous map, b) Asynchronous map.

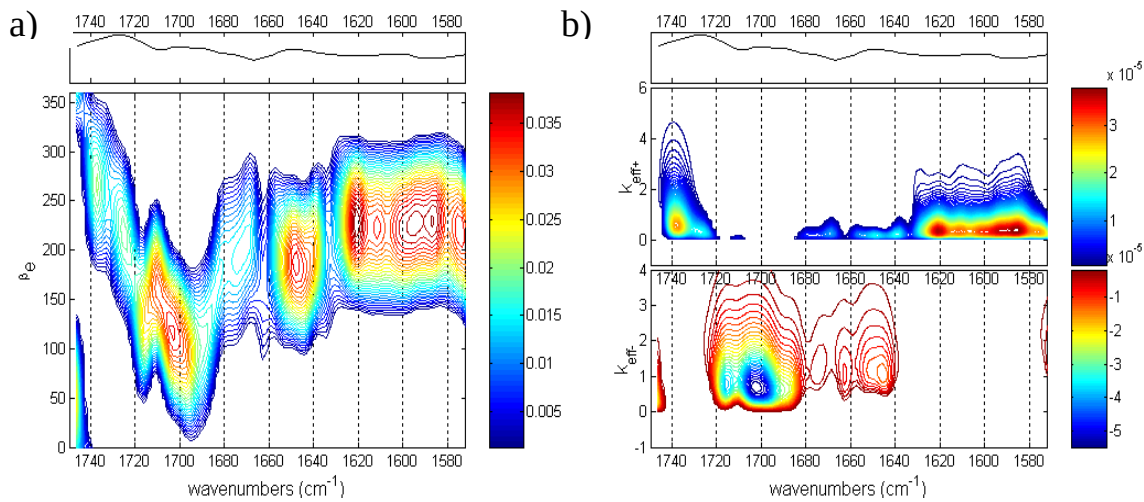


Fig5.5. Model-based 2D correlation analysis of 10%rdhSPC WT+DPPCd62 monolayer at the air/water interface. a) βv correlation analysis, b) $k v$ correlation analysis.

Table5.1. Quantitative evaluations of the relative rate changes of the intensity variations of 10%WT+DPPCd62 monolayer at the air/water interface with β_v and k_v correlation analysis.

	Frequency (cm^{-1})	β_{eff}	$k_{\text{eff}+}$	$k_{\text{eff}-}$
C=O	1738	236.9	0.5	
	1725	217.3	-	
	1715	-	-	0.8
	1703	118.2	-	0.7
Amide I	1674	191.6	-	1.2
	1669	-	0.2	
	1662			1.1
	1648	186.7	0.2	1.1
	1639		0.3	-
	1621	228.4	0.3	-
	1612	224.7	0.3	-

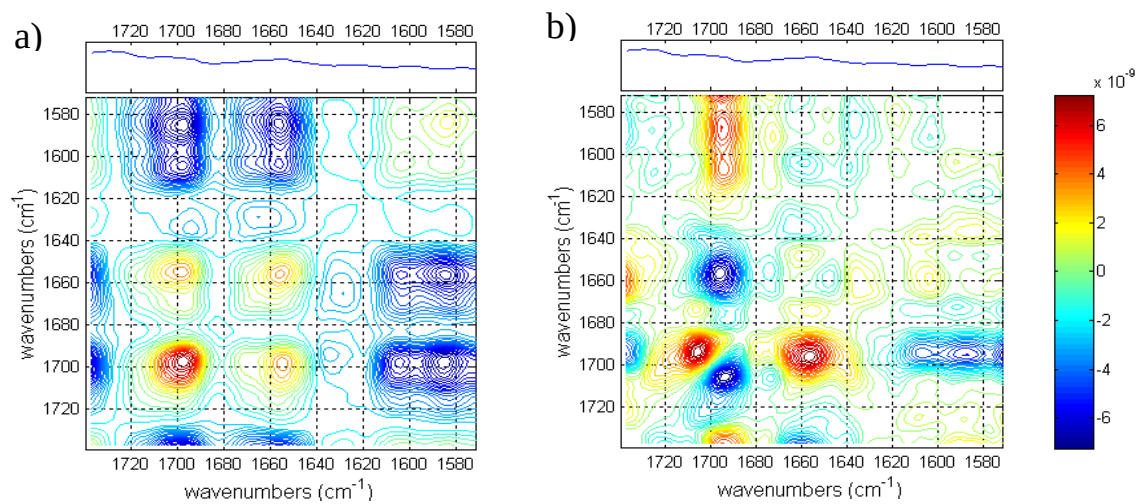


Fig5.6. Generalized 2D correlation analysis of 10%mut4+DPPCd62 monolayer at the air/water interface. a) Synchronous map b) Asynchronous map.

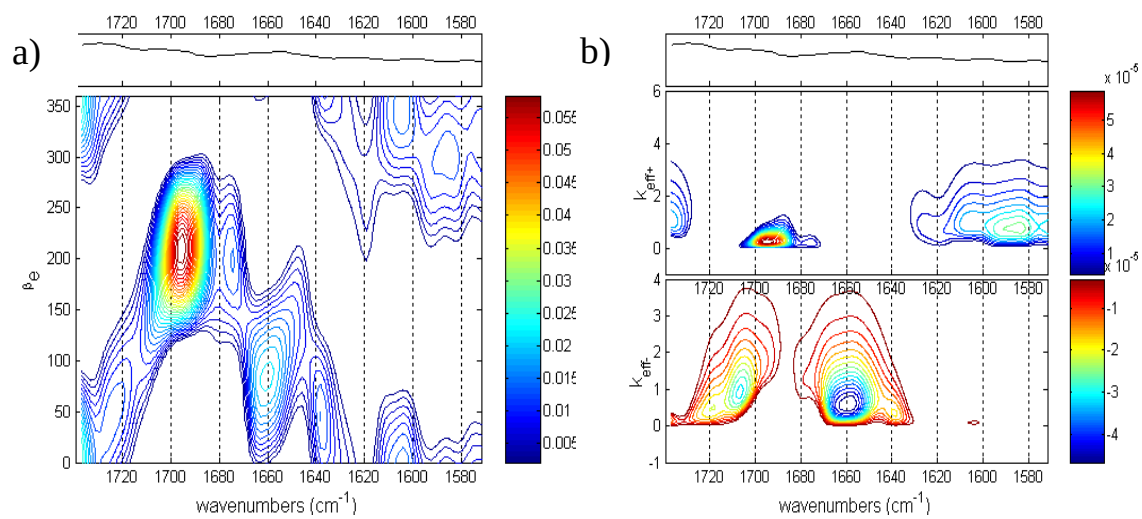


Fig5.7. Model-based 2D correlation analysis of 10%mut4+DPPCd62 monolayer at the air/water interface. a) βv correlation analysis, b) kv correlation analysis.

Table5.2. Quantitative evaluations of the relative rate changes of the intensity variations of 10%mut4+DPPCd62 monolayer at the air/water interface with β_v and k_v correlation analysis.

	Frequency (cm^{-1})	β_{eff}	$k_{\text{eff}+}$	$k_{\text{eff}-}$
C=O	1742	14.1	-	
	1737	56.9	1.1	
	1718	-	-	05
	1707	-	-	1.0
Amide I	1695	210.0	0.3	
	1678	200.2	-	1.2
	1660	82.7		0.6
	1648	-	-	1.1
	1639	45.9	-	0.3
	1622	-	0.7	-

CHAPTER 6

THE CONFORMATION AND ORIENTATION OF A RECOMBINANT DEACYLATED HUMAN SURFACTANT PROTEIN C MUTANT (K11Q/R12Q) IN PLANAR SUPPORTED BILAYERS: AN ATR-FTIR INVESTIGATION¹

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Abstract

A mutant (Mut4) of recombinant human deacylated pulmonary surfactant protein C (rhDSP-C) with mutagenesis at the positively charged Lys-Arg pair (K11Q/R12Q) was investigated on its structure and orientation in phosphatidylcholine (PC) bilayers upon thermal perturbation. Structure conversion of Mut4 in planar PC bilayers was demonstrated by model-based 2D correlation analysis on ATR-FTIR dynamic spectra. A significant increase in β -structures in concomitant with appreciable β -aggregation formation was observed for Mut4 in supported bilayers upon thermal perturbation. In addition, the orientations of the acyl chains of saturated PC component and that of β -sheet of Mut4 were determined. The acyl chains of DPPC₆₂ had an average tilt angle of $\sim 20^\circ$ to the membrane normal. The β -strands of Mut4 were found tilt $\sim 23^\circ$ to the membrane normal. The angular parameters defining the orientation of antiparallel sheet were found at $\sim 45^\circ$ and $\sim 43^\circ$ respectively. It was implied that the β -structure may not be of large extended forms, but probably consisted of short twist β -strands.

Keywords: ATR-FTIR, polarization, planar supported bilayer, vesicle self-assembling, vesicle fusion, conformational changes, molecular orientation, recombinant human deacylated surfactant protein C, site-directed mutagenesis.

Introduction

Human pulmonary surfactants consist of 85% phospholipids, 4-8% neutral lipids and about 10% surfactant proteins. Phosphatidylcholines (PC~46%) and anionic phosphatidylglycerol (PG ~10%) constitute the bulk of phospholipids. Disaturated PCs such as 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) are essential to resist lung collapse at the end of exhalation attributed to its high gel-to-liquid-crystal phase transition temperature ($T_c \sim 41^\circ\text{C}$) of the acyl chains. Anionic PGs and unsaturated phospholipids are essential to retain fluidity of the aqueous alveolar linings for re-adsorption and fast re-spreading of the surfactant materials upon inhalation. [1, 2] There are four pulmonary surfactant apoproteins. Surfactant protein A and D (SP-A and SP-D) are hydrophilic and mainly responsible for the innate host defense against the invading pathogens such as viruses and bacteria, as well as particulates such as pollen. Surfactant protein B and C (SP-B and SP-C) are small hydrophobic apoproteins particularly important for maintaining the normal mechanical breathing cycle, facilitate the dynamic adsorption, spreading and the stability of the lung lining films in associated with lipids. [3] It is established that there are coordinated functionalities of SP-B and SP-C. Particularly, the deficiency of SP-B resulted in abnormal expression of SP-C and is associated with fatal Respiratory Distress Syndrome (RSD) for premature infants and acute RDS (ARDS) for older children and adults. [4-6]

Mature SP-C is a unique small (4.2kDa) hydrophobic lipoprotein exclusively expressed in pulmonary alveolar type II (ATII) cells. Mature SP-C has been characterized with four structural motifs 1) two palmitoylated Cysteine residues (C5 and C6) surrounded by two Prolines at the N-terminus, 2) a positively charged Lys-Arg (K11 and R12) pair, 3) a very hydrophobic poly-valine segment, and 4) a heptapeptide C-terminus. [4] It has been determined that SP-C is a

type II transmembrane protein. [5] NMR study of mature SP-C in apolar solvent indicates that it contains a rigid α -helix consisting of residues 9-34. The length of the α -helix is about 37Å which is in agreement with the length to span in the lipid bilayers. [6]

Extensive studies have been done on the surface activities of SP-C in association with phospholipids to facilitate the adsorption, spreading and stabilization of the alveolar lining film. [3] Most recently, the advance in understanding of SP-C biosynthesis has indicated an emerging role of SP-C in conformational lung disease. Misfolding of SP-C presents a similar case to other chronic neurodegenerative diseases such as Alzheimer's and Huntington's disease, all are characterized by conformational conversion from α -helix to β -strands and formation of amyloid fibrils. Genetic abnormalities in SP-C are associated with idiopathic pulmonary fibrosis (IPF), pulmonary alveolar proteinosis (PAP), and less commonly, fatal interstitial lung disease. In the case of PAP, misfolding and formation of amyloid like fibrils of mature SP-C is possibly linked to partially or complete deacylated SP-C. The majority of genetic mutations in the SP-C gene (*sftpc*) that lead to these diseases occur in the domains corresponding to pre-processed form of SP-C (proSP-C). [7] Several immunocytochemistry studies on proSP-C have focused on the functionality of SP-C N-terminus.[8, 9] It was revealed that the Lys-Arg pair may have influence on trafficking SP-C out of ER membrane in properly folded structure. [10] Our previous work has investigated the pH-dependent unfolding of rhdSP-C mutants. [11] It is of interesting to investigate how these mutants would interact with phospholipid bilayers. In the presenting study, Mut4 with the positively charged Lys-Arg pair replaced by Gln pair (K11Q/R12Q) was investigated of its structure conversion upon thermal perturbation and its orientation in supported planar PC bilayers.

ATR-FTIR has been widely using in biophysical studies of biomembrane system. Polarization ATR provided advantages in determination of molecular orientations in biomembrane system. With the knowledge of empirically determined dichroic ratio, the proper approximation of amplitudes of the electric fields at surface boundary, as well as the refractive indices of the stratified surface system, the average distribution of the molecular orientation in a fixed laboratory frame can be acquired.

$$R = \frac{A_{\parallel}}{A_{\perp}} = \frac{A_x + A_z}{A_y} = \frac{\langle E_x^2 \rangle \langle \sin^2 \theta \rangle + 2 \langle E_z^2 \rangle \langle \cos^2 \theta \rangle}{\langle E_y^2 \rangle \langle \sin^2 \theta \rangle}$$

where θ is the angle between the transition moments and the molecular axis. To determine the orientation of non-symmetric biaxial system such as β -sheet, a combination of dichroic ratios of the orthogonal transition moments of amide I and amide II provided the solution for discrete orientation parameters which define the orientation of β -sheet plane to the membrane normal, as well as the orientation of the β -strand within the sheet. [12]

Generalized 2D correlation analysis was developed by Noda in 1993 as a resolution enhancement technique. In addition, sequential occurrence of the intensity variations at different wavenumbers can be *qualitatively* extracted based on the Noda's rules. [13] *Model-based 2D asynchronous correlation* methods were developed by Dluhy et al. [14] [15] Generally, the waveform of the dynamic spectra can be modeled with suitable analytical functions. In the model-based 2D asynchronous correlation analysis, the experimental spectra were correlated to a model function, being either sinusoidal ($\beta\nu$) or exponential ($k\nu$). Compared to the generalized 2D correlation, noise-induced artificial peaks are much reduced. More importantly, model-based 2D asynchronous analysis provides straightforward *quantitative* analysis of the relative rate changes in intensities of the interested spectral features.

Materials and Methods

DPPCd62 and POPC are from AvantiLipids (Alabaster, AL) and used as purchased. Sample solutions are prepared by dilute the original concentration (10mg/mL in chloroform) into 1mg/mL (Chloroform: Methanol = 4:1). POPC and DPPCd62 were mixed in a ratio 1:2. Mut4 (K11Q/R12Q) were provided by our collaborator Dr. John E Baatz at Medical University of South Carolina. The production and purification methods were detailed elsewhere. [16] Mutants were accepted with concentration $\sim 150\text{-}400\mu\text{g/mL}$ and dialyzed against 80% methanol after reception to get rid of TFA from the purification procedure. An aliquot of mutant sample was then withdraw to mixed with PC mixture to yield a 10%(w/w) Mut4 + PC mixture and vortex to homogeneous solution. Sample mixture with/without mutants were purged under N_2 to dry in glass vial and left in vacuum overnight to get rid of trace solvent. Hydration was carried on by freeze-thaw the sample in 120mM NaCl for three times to yield a milky cloudy solution. Hydrated sample were extruded through polycarbon membrane ($0.1\mu\text{m}$) to yield a clear homogeneous LUV solution and finally diluted with 120mM NaCl.

The Ge crystal ($50\times 10\times 2$) was cleaned by first soaked into 0.1% *Hellmanex*[®] II solution (Hellma GmbH & Co. KG) for one hour then rinsed with DI water thoroughly. Aftermath, the crystal was put into DI water bath sonicated for one hour with four changes of DI water. At last, the crystal was rinsed with boiled DI water. Compared to the cleaning with methanol:chloroform mixture, the treatment with alkaline detergent yielded a more hydrophilic crystal surface which was preferable for monolayer deposition and vesicle adsorption.

Planar supported bilayers were prepared using both vesicle self-assembling (VSA) and vesicle fusion methods (VF). [17]

Langmuir film transfer and vesicle fusion on Ge crystal.

Cleaned Ge crystal was first immersed into NaCl subphase (120mM). Then an aliquot of ~ 120 μ L solution of the above mentioned mixture were spread on NaCl subphase (120mM) on the Langmuir trough (Nima, England) to form a mono-molecular film. The monolayer was left for 5min for solvent evaporation and equilibrium before compression. The transfer of the monolayer was performed at 30mN/m. The surface pressure was monitored using a Wilhelmy plate. After the Ge crystal was completely withdrawn from the subphase, it was left in position for about 5min for dried up. The Ge crystal was then dismounted from trough dipper and assembled with the ATR liquid cell plate. The side of monolayer facing down to the accessory was wiped off using methanol. Data was collected for the single side of transferred monolayer for quality control and vesicle fusion verification.

The LUV solution was spread onto either the clean Ge crystal (for vesicle self-assembling method) or the monolayer-transferred Ge crystal (for vesicle fusion method), and incubated at 55°C for 45min in the sealed liquid cell. After incubation, extra vesicle solution was rinsed off with large amount of 60mM NaCl. The liquid cell with crystal was then placed into the desiccator and kept in dark for 15min to get rid of water residues before data collection.

ATR-FTIR experiment and Data preprocess.

ATR spectra were collected using a DigLab FTS7000 IR spectrometer with Harrick ATR liquid cell accessory and thermal control. A high sensitivity narrow-band MCT detector was used for signal detection. For polarization experiments, a KR5 polarizer was placed before the beam entry to the ATR accessory. Polarization switch between the parallel (p-) and the perpendicular (s-) polarization was fulfilled using an ORIEL motor control. Background was collected for each temperature in the range of 25-65°C. 512 scans were performed in total at resolution of 4cm⁻¹. Triangle apodization function and auto-zero filling were used.

Data were preprocessed with Grams32/AI software. Savitzky-Golay smoothing (2 degree, 15pts) was performed first. Care was taken to make sure that band width of C=O was not changed after smooth to avoid band distortion. Baseline correction was performed for each truncated spectral regions: 3050-2750 for CH₂ region, 2300-2000 for CD₂ region, 1810-910 for C=O, amide I and II, and phospholipid headgroup region together.

Model-based 2D correlation analysis.

The details of the algorithms for model-based 2D correlation can be found in Ref. [14, 15]. Pretreated data were mean centered to the average spectrum to construct the dynamic spectra. Normalization of amide I region was performed against the region of OH stretching centering $\sim 3433\text{cm}^{-1}$ to account for the influence of hydration. Calculations of correlation were performed using a program written with MATLAB (v6.1 above) in our lab.

Determination of molecular orientation.

The amplitudes of the electric fields at surface were calculated using Harrick thin film (two phases) approximation. The values of refractive indices for the three phase system, i.e. Ge crystal, bilayer films, and air, were 4.0, 1.46 and 1.0 respectively. Peak intensities of height were used for calculation of dichroic ratios.

Results and Discussion

1. Conformational changes of Mut4 in PC bilayers upon thermal perturbation.

Fig6.1. showed the *non-polarization* spectra of PC monolayer by Langmuir transfer and 10%mut4+PC bilayers prepared with VF method respectively. As measured from CD₂ bands (2195cm^{-1} and 2094cm^{-1}), the intensity ratios of protein-incorporated bilayers to PC monolayer were ranging from 1-2 (data not shown). Assuming the refractive indices for PC monolayer and that of protein-incorporated bilayers were the same, it was indicative that inhomogeneous

coverage of bilayers was formed on Ge crystal. It also implied that dynamic exchange between PC monolayer and protein-incorporated PC vesicles occurred during incubation. Model-based 2D correlation analysis was performed to elucidate structure conversion of Mu4 in PC bilayers upon thermal perturbation. **Fig6.2.** showed the $\beta\nu$ and the $k\nu$ correlation analyses on non-polarization spectra. **Table6.1.** presented quantitative comparison of the relative intensity changes of each conformation. For PC components, bands were resolved for dehydrated (1737cm^{-1}), monohydrated (1715cm^{-1}) and doubly hydrated (1705cm^{-1}) C=O respectively. [18] In amide I region, peaks were resolved in $\beta\nu$ map for β -sheet/ β -strands (1689cm^{-1}), β -turns (1673cm^{-1}), 3_{10} -helix (1665cm^{-1}), unordered structure (1649cm^{-1}), antiparallel β -sheet (1632cm^{-1}) and β -aggregation (1625cm^{-1} and 1612cm^{-1}) respectively. The weak peaks at 1625cm^{-1} and 1612cm^{-1} indicated that only limited β -aggregations were formed. The relative intensity changes for the conformations were quantitatively determined from β_{eff} values, which indicated the conformations changes in the order: $1689 \rightarrow 1673 \rightarrow 1612 \rightarrow 1649 \rightarrow 1632 \rightarrow 1625 \rightarrow 1665$. In $k\nu$ map, the same conformations were resolved as in $\beta\nu$ map. $k\nu$ analysis further distinguished relative increases in rate changes of intensities from those decreased upon thermal perturbation. It was clearly indicated that β -sheet/ β -strands (1689cm^{-1}), β -turns (1673cm^{-1}) were increased while the peak at 1665cm^{-1} , 1649cm^{-1} and 1632cm^{-1} decreased. Limited increase in β -aggregation was also indicated in the k_{eff} panel at 1625cm^{-1} and 1612cm^{-1} . Model-based 2D correlation analysis clearly indicated dominant β -structures of Mut4 with limited amount of β -aggregates in PC bilayers.

Fig6.3. showed the *polarization* ATR spectra of 10%mut4+PC bilayer prepared with VSA method in the amide I and the CD_2 regions respectively. It was of note that the resultant

bilayers with VSA preparation were still of high degree of hydration. A peak centered at $\sim 1645\text{cm}^{-1}$ dominated in the amide I region for both p- and s- polarization spectra. This peak in couple with a broad peak at $\sim 3423\text{cm}^{-1}$ (not shown) indicated the existing of water molecules. As temperature increased, the peak at 1645 diminished, indicating a loss of water content. The intensity ratios of the CD_2 bands of the protein-incorporated sample to that of PC monolayer were about 2, indicating a single bilayer was formed. However, the intensity ratio of the headgroup region was less than 2. It could be possibly due to the interaction between PC head group and the protein.

Model-based 2D analysis was also applied to polarization ATR spectra of bilayers prepared with VSA method, as shown in **Fig6.4.** and **Table6.2.**. In $\beta\nu$ map for p-polarization spectra (**Fig6.4.A** left), bands were resolved for β -sheet/ β -strands (1687cm^{-1}), 3_{10} -helix (1667cm^{-1}), unordered structure (1642cm^{-1}) and β -aggregations (1625cm^{-1} and 1615cm^{-1}). The relative changes in intensities were in the order: $1667 \rightarrow 1642 \rightarrow 1687 \rightarrow 1615 \rightarrow 1625$. For s-polarization spectra (**Fig6.4.B** left), bands were resolved for β -turns (1681cm^{-1} and 1677cm^{-1}), unordered structure (1646cm^{-1}) and β -aggregations (1614cm^{-1}). The relative changes in intensity were in the order: $1681=1677 \rightarrow 1646 \rightarrow 1614$. In the $k\nu$ correlation analysis, the decrease in water content resulted in pronounced decrease in most bands as indicated in the k_{eff} - panel for both p- and s-polarization spectra. The increases of 1687cm^{-1} , 1667cm^{-1} , 1632cm^{-1} were resolved for p-polarization spectra (**Fig6.4.A** right). Comparatively, increase in β -turns (1681cm^{-1} and 1677cm^{-1}) as well as unordered structures (1642cm^{-1}) were pronounced for s-polarization spectra (**Fig6.4.B** right). For both p- and s-polarization, the relative decrease of bands at 1625cm^{-1} and 1612cm^{-1} however indicated that β -aggregations were originally existed in Mut4 in bilayers,

which implied that preparation of bilayers may have induced the formation of β -aggregation. For both β_v and β_v maps, the secondary structure of Mut4 resolved in p-polarization spectra were different from those resolved in s-polarization spectra, which indicated that there was thermal induced reorientations of the secondary structure of Mut4. Nevertheless, the dominant increases in β -structures were distinguishable and pronounced for Mut4 with VSA bilayer preparation. In comparison to 10%mut4 in PC bilayers prepared by VF method, VSA bilayer preparation may have induced the formation of β -aggregation as indicated by the appreciable decrease of 1625cm^{-1} and 1615cm^{-1} concomitant with the decrease in water content.

It was of note that β -structures resolved for Mut4 in different PC bilayer preparations were different. For VF preparation (**Table6.1.** non-polarization spectra), the β -structure was with higher frequency $\sim 1689\text{cm}^{-1}$ of β -sheet/ β strands, as well as pronounced β -aggregation formation. For VSA preparation (**Table6.2.** polarization spectra), β -turn structure was more pronounced. Although this difference may be of direct results from different bilayer preparation, it was also indicative that environmental changes, i.e. transferring from monolayer to bilayer may also contribute to the mechanisms for structural conversion of rdhSP-C Mut4 as well as the formation of β -aggregations. The PC bilayers, on the other hand, stabilized the structure of rdhSP-C Mut4, which was the same as for synthetic SP-C analog in PC micelles. [19]

2. Orientation of Mut4 in PC bilayers.

It was indicated above in polarization ATR spectra of Mut4 with VSA bilayer preparation that β -structures may undergo orientation changes in response to thermal perturbation. Orientation of the secondary structure of Mut4 in the same VSA bilayer preparation was further determined from the dichroic ratios of certain secondary structure vibrational modes.

Orientations of both the acyl chains of saturated PC component and that of Mut4 in the bilayers were calculated. The values of dichroic ratios and the calculated angles were given in **Table6.3.** The orientations of the acyl chains of DPPCd62 were calculated based on empirical dichroic ratio measured with CD₂ bands upon thermal perturbation. **Fig6.5.** showed the tilt angle the acyl chains with respect to the normal of the membrane bilayers changed upon thermal perturbation. As the temperature increased, the tilt angle of the acyl chains ranged from $\sim 19^\circ$ to 23° . The results were in good agreement with reported acyl chain orientation in hydrated bilayers. [20, 21] It was shown that at lower temperature, the acyl chains of DPPCd62 were aligned closer to the normal of the bilayers, which may due to a tightly packed conformation of the acyl chains in gel phase. The maximum tilt angle of acyl chains was found a little bit above the phase transition temperature of DPPC (41°C). The deviation was attributed to deuteration of the acyl chains. The orientation of the acyl chains of POPC was not calculated in considering the involvement of a C=C unsaturated bond, which introduces a kink in the acyl chain and therefore a change of the orientation of the transition dipole of the CH₂ bond with respect to the main axis would be expected.

The orientation of the Mut4 in PC bilayers was of the most interest. As already shown, model-based 2D correlation indicated decrease in α -helix. Meanwhile, curve fitting failed to resolve the helix band around 1655cm^{-1} . Rather, the increase in anti-parallel β -sheet was pronounced. Therefore, orientation calculation was performed for antiparallel β -sheet. In comparison, the polarization spectra at 25°C of a single bilayer prepared by VF method were analyzed too (**Fig6.6.**). For both VF and VSA methods, polarization spectra resolved a predominant peak at $\sim 1637\text{cm}^{-1}$ for antiparallel β -sheet, which has a transition moment $\nu_\perp(\pi, 0)$ [22, 23] perpendicular to β -strand axis. This mode in combination with the amide II band at

1530cm^{-1} of $\nu_{||}(0,\pi)$ was proposed to be used for the calculation of the orientation of β -sheets. [12] However, no strong peak was resolved for amide II band around 1530cm^{-1} for Mut4 in PC bilayers for both bilayer preparations in the presenting study. Instead, the weak component of antiparallel β -sheet at $\sim 1687\text{cm}^{-1}$ was resolved with appreciable intensity, though distinct band shape was obscured under the broad amide I envelop. The weak component of antiparallel β -sheet IR absorption has the same transition moment orientation as amide II band at 1530cm^{-1} . [22, 23] In principle it may also be used in the orientation calculation of β -sheet. [12] Assuming antiparallel β -sheet was the dominant structure of Mut4 in PC bilayers, and there was no overlapping with other conformations, the dichroic ratios at 1687cm^{-1} of $\nu_{||}(0,\pi)$ mode was therefore adopted in the orientation calculation for Mut4 in PC bilayers. The resultant angular parameters were given in **Table6.2.** In the temperature range from $45\text{-}65^\circ\text{C}$, the tilt angle of β -strands was in the range of $20\text{-}23^\circ$ relative to the membrane normal. Subsequent calculations revealed the two angular parameters that defining the orientation of β -sheet. The angle of β , which defines the tilt of the β -strand in the sheet plane, was determined with a median value of $\sim 45^\circ$. The angle of α defining the tilt of the sheet plane to the membrane normal was determined $\sim 43^\circ$. The increase in temperature up to 65°C only resulted in up to 1° fluctuations in the angular parameters. Consistently, the angular parameters calculated for Mut4 at 25°C in PC single bilayer prepared by VF method was about 1.5° different from the results by VSA methods.

The inclination of β -strands to the membrane normal was 3° away from the tilt angle of PC acyl chains with respect to the membrane normal. This result was favored for a β -structure orientation in the PC bilayers. As pointed out by Marsh, this angle however is not identical to the strand orientation within the plane of the sheet. The angular parameters describing the orientation

of β -sheet instead suggested a larger tilt of the sheet from membrane normal at $\sim 45^\circ$. Since the orientation of the unsaturated PC acyl chains were not directly available. It was therefore not known if this tilt angle of sheet would be comparable to the orientation of the unsaturated PC acyl chains. In conjecture, the tilt of the β -sheet would be accommodated by the kink in the unsaturated PC acyl chains. In addition, the angular difference between the tilt angle of β -strands and that of the sheet plane could imply a twist or bending existing for Mut4 β -structure, in notice that Marsh's formalism was successfully applied to proteins such as OmpA with β -barrel structure. However, SP-C is strictly a small polypeptide with only one transmembrane domain. Collectively, it was not likely that antiparallel β -sheet existed in large extended scale for Mut4 in PC vesicles. Instead, it was more plausible that short twist β -strands or β -turns were formed for Mut4 in PC bilayers. This conclusion in turn was in agreement with the interpretation of the IR frequencies observed. A recent molecular model depicted the twist-hairpin could probably applied to the β -structure of Mut4 as well. [24]

Conclusions:

The structure of one mutant of rhdSP-C, i.e., Mut4 (K11Q/R12Q) were incorporated into phosphatidylcholine vesicles. Planar supported bilayers were further prepared on Ge crystal with two different methods, i.e. vesicle self-assembling (VSA) and vesicle fusion (VF) methods respectively. Dynamic structural changes of Mut4 in bilayers prepared in both methods were analyzed using model-based 2D correlation analysis. Dominant increase in β -structures was pronounced for Mut4 in PC bilayers with both preparation methods. However, the resultant β -structures were different for Mut4 in different PC bilayer preparations. Meanwhile, the existence of β -aggregation was pronounced for both preparations. The increase in β -aggregation was

observed with Mut4 in bilayers of VF preparation. In contrast, β -aggregation was decreased for Mut4 in VSA bilayer preparation, which may be resulted from the preparation method itself. Collectively, the differences in resultant β -structures of Mut4 in different PC bilayer preparation indicated that changes of phospholipid environments, i.e. transferring from monolayer to bilayer, may play an import role in mediating the structural changes of Mut4, as well as the formation of β -aggregations. As the transform between monolayer and multilayer in the alveolar lining film has been established, [3] it may also be inferred from the presenting study that structure conversion and formation of β -aggregations of SP-C may also be resulted from environmental interferences.

Orientation of the acyl chains of saturated PC component and that of Mut4 were investigated using polarization ATR-FTIR spectroscopy. Of the two preparations, no significant difference of the molecular orientation was observed. Thermal perturbation had more pronounced effect on the orientation of PC acyl chains, which was attributed to the induced phase change of the acyl chains. Mut4 retained almost same orientation upon thermal perturbation. It was indicated that β -strands of the sheet had the same tilt angles as that of PC acyl chains, i.e. almost perpendicular to the membrane normal. In addition, angular parameters defining β -sheet orientation could also imply a twist of the sheet plane in agreement with the spectral-structural correlation for Mut4 in PC bilayers.

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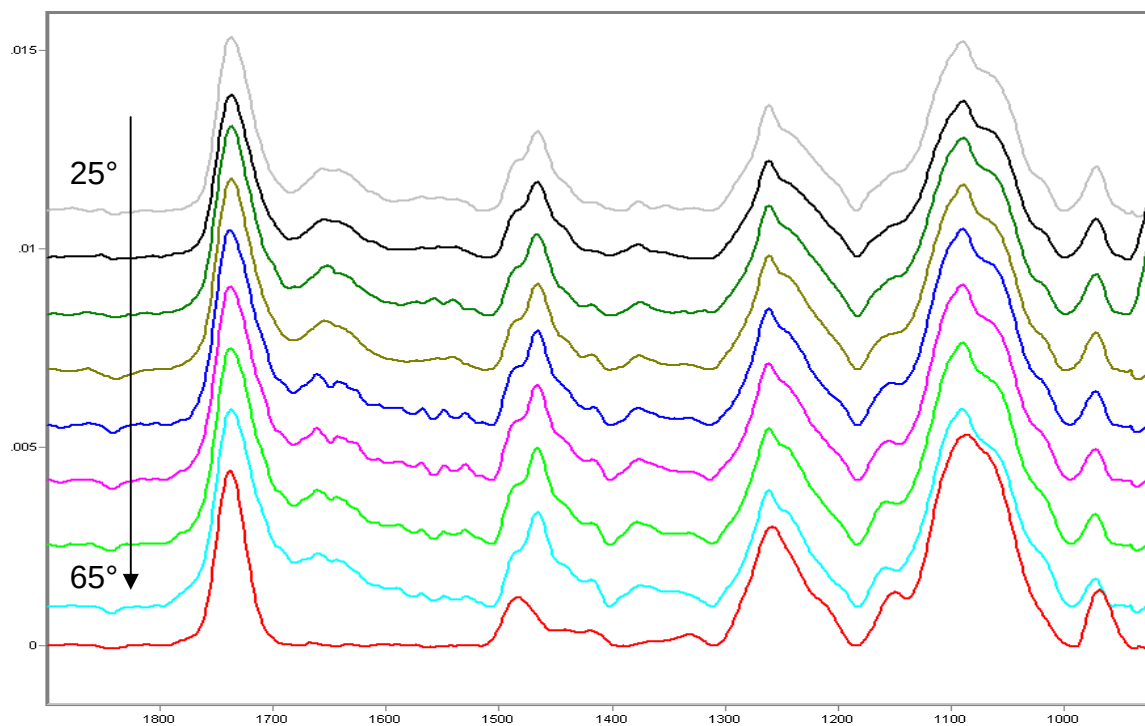


Fig6.1. *Non-polarization* spectra for 10%mut4+DPPCd62:POPC (2:1) prepared by vesicle fusion (VF) method. The bottom spectrum was of DPPCd62:POPC monolayer prepared by Langmuir transfer at 25°C.

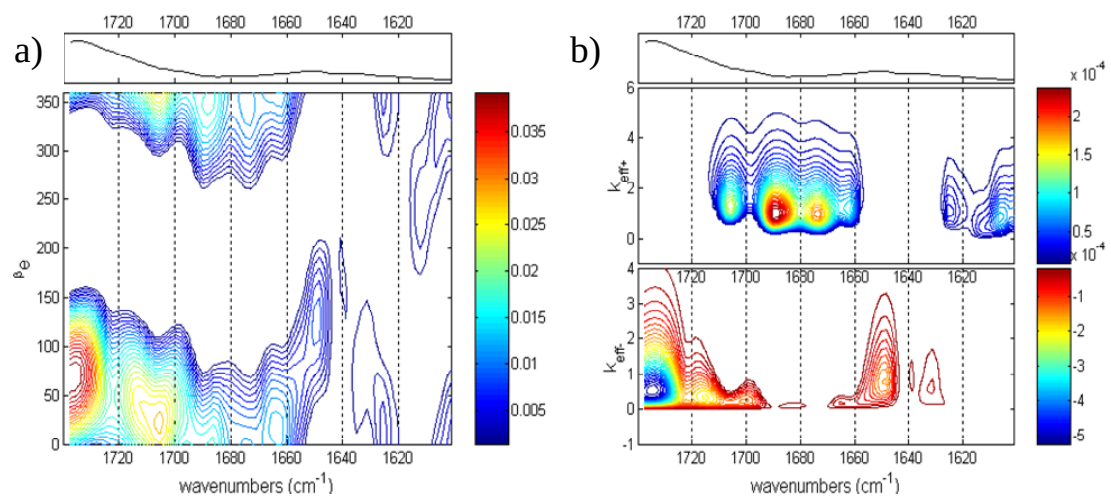


Fig6.2. a) β_v and b) k_v correlation maps of 10%mut4+PC prepared by VF methods.

Table6.1. Quantitative evaluation of the relative intensity changes of the secondary structures by $\beta\nu$ and $k\nu$ correlation analysis on 10%mut4+PC in planar supported bilayers prepared by VF method.

	Frequency (cm^{-1})	β_{eff}	$k_{\text{eff}+}$	$k_{\text{eff}-}$
C=O	1737	69.2	-	0.6
	1715	-	-	0.4
	1705	21.4	1.3	-
Amide I	1689	349.6	1.0	-
	1673	348.4	1.0	-
	1665	16.5	-	0.2
	1649	128.0	-	0.8
	1632	81.4		0.6
	1625	19.0	1.1	-
	1612	244.3	0.5	-

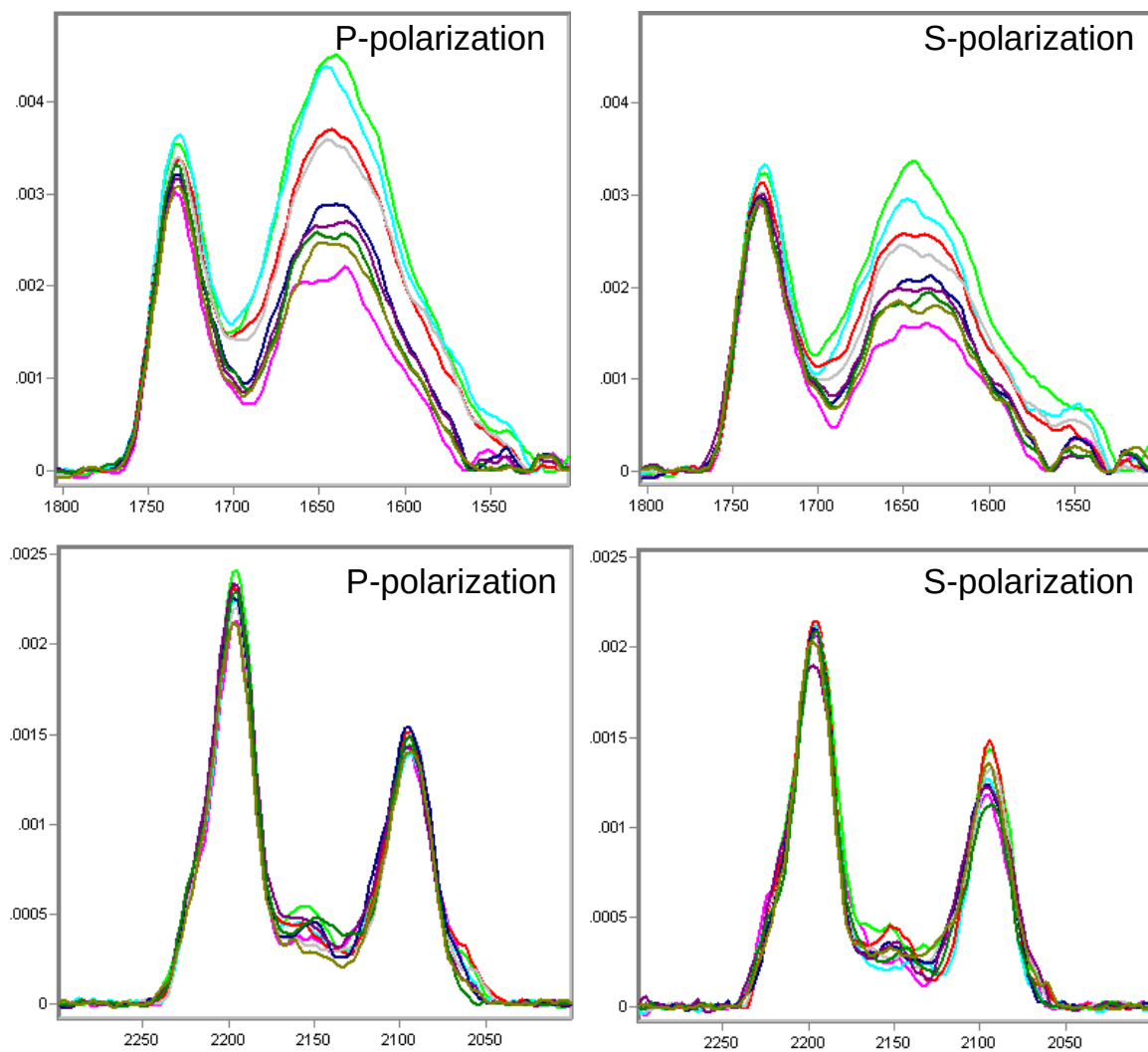


Fig6.3. Polarization ATR-FTIR spectra of 10%mut4+PC prepared by vesicle self-assembling (VSA) method.

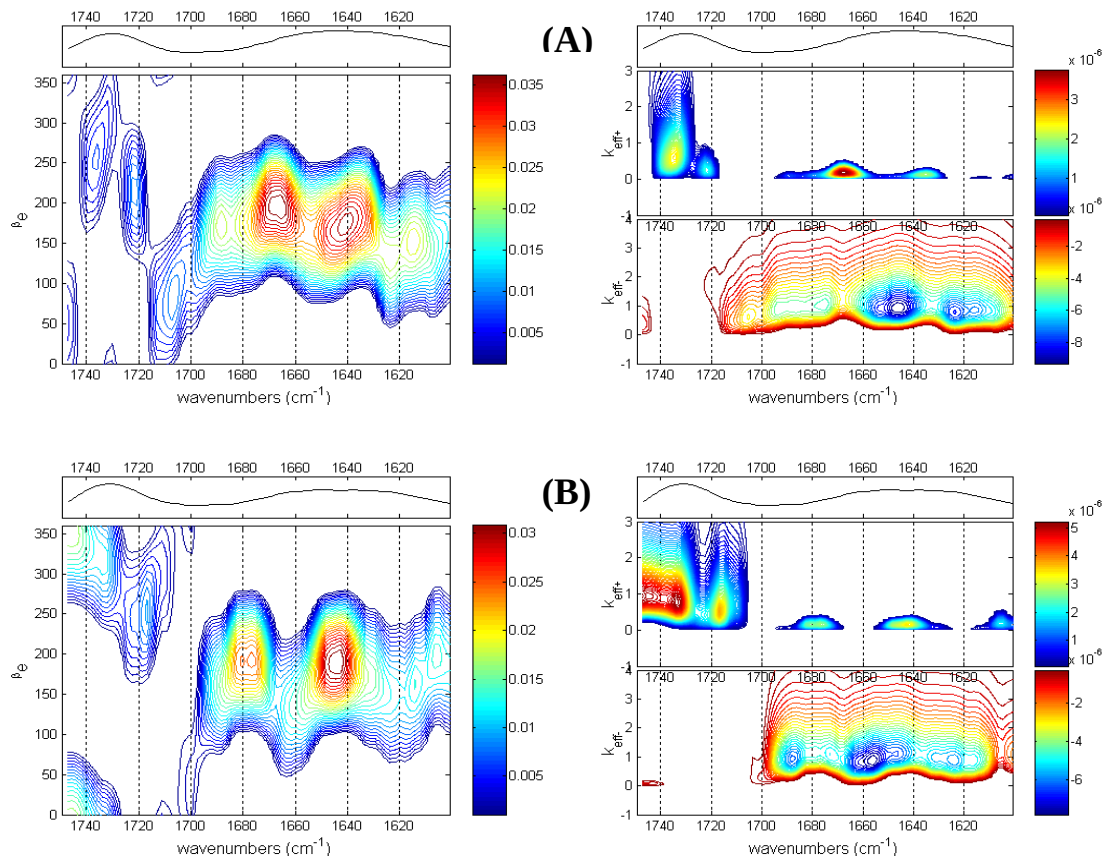


Fig6.4. $\beta\nu$ and $k\nu$ correlations of polarization spectra of 10%mut4+PC bilayer prepared by VAS method. (A) p-polarization. (B) s-polarization.

Table6.2. β_{eff} and k_{eff} values of polarization spectra of 10%mut4+PC bilayer prepared by VAS method.

p-polarization				s-polarization			
Frequency (cm ⁻¹)	β_{eff}	$k_{\text{eff}+}$	$k_{\text{eff}-}$	Frequency (cm ⁻¹)	β_{eff}	$k_{\text{eff}+}$	$k_{\text{eff}-}$
1687	172.0	0.1	0.9	1687	-	-	0.9
	-	-	-	1681	190.4	0.2	-
1677	-	-	1.0	1677	190.4	0.2	-
1667	195.3	0.2	-	1673	-	-	1.1
1653	-	-	0.9	1657	-	-	0.9
1646	-	-	0.9	1646	188.0	-	1.1
1642	173.3	-	-	1642	-	0.1	-
1632	-	0.1	-	1632	-		1.0
1625	131.6	-	0.8	1624	-	-	0.9
1615	151.2	-	0.8	1614	161.0	-	0.9

Table6.3. Orientation parameter for Mut4 and DPPCd62 acyl chains in planar supported bilayers prepared by VSA method.

Temp. (°C)	Wavenumber (cm ⁻¹)	R ^{ATR}	S	θ	β	α
25-65	2195	1.03-1.24** 1.07±0.06@	0.79-0.83 0.82±0.01	19.73-21.86 20.08±0.66		
	2094	1.01-1.33 1.132±0.11	0.77-0.83 0.81±0.02	19.53-23.1 20.69±1.20		
45-65	1687	1.14-1.37 1.33±0.09		20.73-23.63 23.06 ±1.12	44.45-45.04	43-44.03
	1635	1.32-1.38 1.36±0.02			44.89±0.23	43.4±0.38
25	Amide I (VF method)	1.271687 1.611635		22.25	43.55	41.72

** Referred to the range of the orientation parameters.

@ Referred to the median value ± standard deviation.

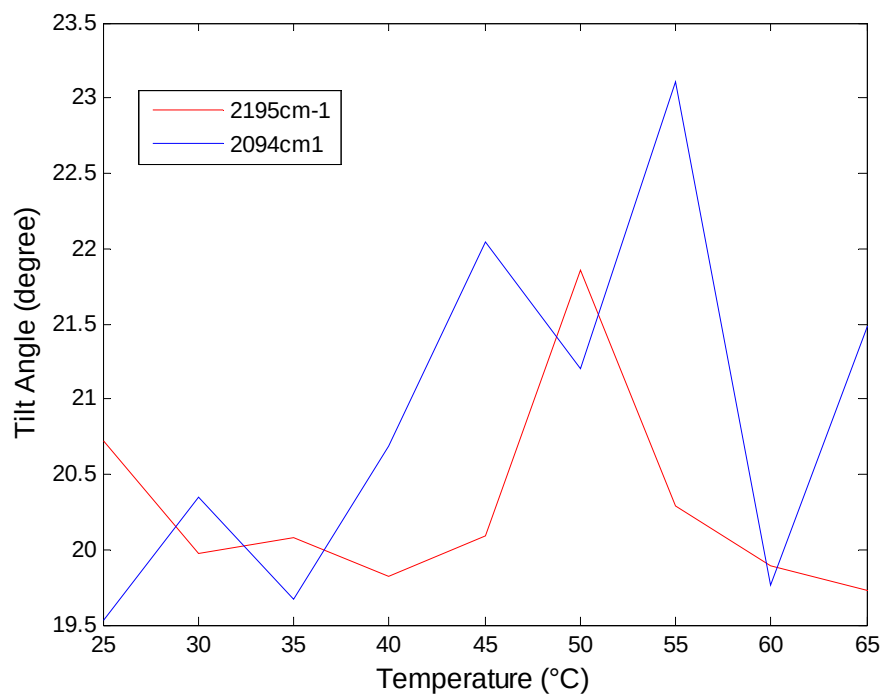


Fig6.5. Orientation of the hydrocarbon chains of DPPCd62 upon thermal perturbation.

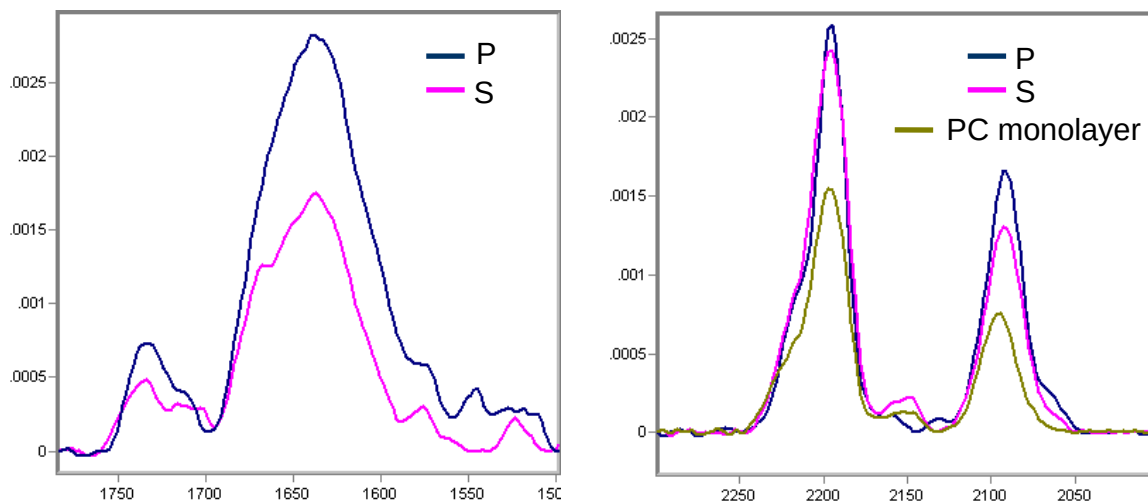


Fig6.6. Polarization ATR-FTIR spectra of 10%mut4+PC prepared by vesicle fusion (VF) method. Amide I band was obtained by subtraction of PC monolayer spectra from 10%mut4+PC spectra of different polarizations.

CHAPTER 7

AN TEST REPORT OF INTRINSIC SERS USING SILVER NANORODS BASED PLATFORM FOR THE DIFFERENTIATION OF PROTAVIURS STRAINS¹

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Abstract

An intrinsic SERS technique based on Silver nanorods (AgNRs) substrates in combination with chemometrics was applied to the differentiation of Rotavirus strains. The presenting study described the methodology of fabrication of AgNRs using e-beam evaporation, Raman spectroscopy on data collection, as well as data interpretation with chemometrics models. The effectiveness and efficiency of the SERS AgNRs platform for bioanalysis was demonstrated.

Keywords: SERS, Silver nanorods (AgNRs) substrates, chemometrics, cluster analysis PLS-DA, rotavirus strain.

Introduction

The phenomenon of surface enhancement is considered mainly resulted from the increased surface plasmon resonance due to the metallic morphology at nanoscale. The enhancement factor is found to be at the order more than 10^6 . [1] Surface enhanced Raman spectroscopy (SERS) are currently with high popularity for bioanalytical applications due to its high sensitivity and simple versatile sampling method. Up to date, the highest SERS sensitivity has been reported for single molecule detection. [2] Signature Raman vibrational modes of most of the biological samples also provided abundant structural information. A large body of applications using SERS as bioassay platform is considered as *extrinsic* SERS, which involves chemical modifications of metallic nanostructures with Raman reporter molecules or labeling with functional molecules such as antigen for immunoassays. One example is given by Porter *et al* in a new released tutorial review. [3] A second line of application of SERS has employed *intrinsic* SERS in bioanalysis. In this category, no chemical modification of the metallic nanostructure is involved. SERS signal comes directly from the analytes themselves. SERS spectra therefore acquired are subject to chemometrics analysis to provide information such as concentration quantification and sample classification. The presenting study demonstrated the application with intrinsic SERS in differentiation of rotavirus strains from clinic samples.

Rotavirus is the most common cause of diarrhea for children. According to CDC, it causes approximately 55,000 children hospitalized each year in the US, and a death total for over 600,000 children annually worldwide. The incubation period for rotavirus disease is about 2 days. As outlined by CDC official website, diagnosis of rotavirus infection may be made by rapid antigen detection of rotavirus in stool specimens. Characterization of strains may be further done with enzyme immunoassay or reverse transcriptase polymerase chain reaction (r-PCR). However,

the following tests are not commonly employed. As a matter of fact, enzyme immunoassay or PCR, though being the standard way in clinic diagnosis, are both time consuming and costly. This is held true in general and also accounts for the motivation for the wide interests in exploration of SERS as platform for rapid biodiagnosis.

In quantitative analysis of spectra, differences in spectra may be extracted by univariate analysis, such as using intensity ratios of certain vibrational modes. However, with a large body of data, it would be expected to be a daunting task to perform univariate analysis. In addition, objective criteria for univariate analysis would also be difficult to establish for samples prepared in different way. Chemometrics has been very well established for multivariate analysis in analytical spectroscopy. [4, 5] In the presenting study, two chemometric models i.e. cluster analysis and PLSDA were applied to SERS spectra for differentiation of rotavirus strains.

Methods and Materials

Ag nanorods substrates preparation.

E-beam OAD fabrication of Ag nanorods (AgNRs) substrates was carried out as described previously. [6] Briefly, a piece of glass slide was first wiped with methanol and then pre-cut into $1 \times 1 \text{ cm}^2$ chips (substrates). The substrates were then cleaned by immersed in hot piranha solution ($\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2$ in 4:1) for 30 min. Aftermath, the glass substrates were rinsed with DI water thoroughly. The substrates were immediately dried with N_2 stream after rinsing. Cleaned substrates were loaded into a customized electron beam/sputtering evaporation system. A thin Ti film of 20nm was first coated onto the substrates as adhesive, following a film of Ag of 500nm. Both were performed with the evaporation beam axis normal to the substrates surfaces. Deposition was controlled at nominal rate 2 and $3 \text{ \AA/s}$ respectively. A third layer, i.e. AgNRs was deposited with the substrates held at an incident angle of 86° . The nominal deposition rate was

3.2Å/s for AgNRs. Throughout deposition, the thicknesses of the films as well as AgNRs were monitored with a quartz crystal film thickness monitor (QCM). The nominal thickness/length of AgNRs was 2000nm. However, the resultant thickness/length of AgNRs was about the half of the nominal value. The optimal AgNRs substrates were with average rod diameter ~100nm, and density of ~13rods/ μm^2 . For quality control, a representative number of AgNRs substrates were tested with BPE methanol solution of 10^{-5}M . Generally, the intensity of the characteristic doublet of BPE at 1639cm^{-1} and 1611cm^{-1} were with saturated signal with CCD detector.

Rotavirus sample preparation.

Clinic rotavirus samples were provided by the Royal Children's Hospital and Monash Medical Center (Melbourne, Australia). Samples were obtained with cell culture media and eight human rotavirus strains in the same cell culture media. The cell culture media as a negative control and five rotavirus strains were applied to SERS test in the presenting study. The concentration of all sample solution were not known to us, but estimated to be similar. In SERS experiments, a 1:10 dilution into DI water of the original concentration as accepted was first applied to test. Based on the resultant SERS signal quality, further dilutions may be applied.

SERS spectra acquisition.

Each rotavirus strain of a dilution was applied to three individual AgNRs substrates respectively using DNArase/RNArase free pipette tips (Fisher Scientific) with a sample volume of 1 μL . The same strain of different dilution may have applied to the same substrate as long as there was no overlap between the sample spots. By using different substrates in testing the same sample (strains or cell culture media), spectral difference arose from different substrates was monitored. Raman spectra were acquired with Renishaw inVia Raman microscope system (Renishaw, England) with diode laser source at 785nm. A nominal power of 10% of the source

power, which was about ~28mW at the sample were applied. The 5x objective was used to collect Raman scattering. 10s exposure with 3 accumulation were applied throughout. For each sample spot on the substrate, five spectra were collected at five different local points within the sample spot.

Data preprocess.

All data were transferred into Excel worksheet to construct the data matrix for further analysis. The constructed data matrix were then imported to MATLAB (MATLAB v7.0 above), where chemometrics were executed with the PLS-toolbox (v.4.0, Eigen Vector Research Inc.). Preprocess of data for x-block was performed with Savitzky-Golay 1st derivative (2degree, 15pts), vector normalization, and mean centering in sequence. For PLS-DA analysis, a y-block training set was constructed in Excel worksheet and imported to MATLAB. Only mean centering were performed for y-block.

Results and Discussion

In total 75 spectra were collected for the six samples, i.e. the cell culture and five rotavirus strains. **Fig7.1.** showed the baseline-corrected average spectra of each sample. Up to 15 spectra were collected for one sample on three different substrates. Spectra collected from different substrates showed high reproducibility. Therefore, variations contributed by different substrates may be ignored. Raman shift were assigned to amide I region (1700-1600cm⁻¹), CH₂ scissoring overlapped with COO⁻ v_s mode (1460-1300cm⁻¹). The significant peaks at 1594cm⁻¹ together with 1002cm⁻¹ was assigned to Phe residues. [7, 8] The strong Raman shift at 1045cm⁻¹ was attributed the CN stretching mode. It was of note that the spectrum of YO was different from the other strains and the control media with a sharp band at 722cm⁻¹, this band was generally assigned to the backbone COO⁻ deformation. However, in the presenting study, other structural

difference may also contribute to the Raman shift at 722cm^{-1} . In addition, samples were in cell culture media complex. Therefore, caution was taken not to over-interpret the data.

Differentiation of rotavirus strains with chemometrics

Fig7.2. showed the cluster analysis using K nearest neighbor algorithm with PCA of PC5 for the five rotavirus strains and the cell culture media. Samples were fairly well classified. The cell culture media MA104 was also correctly differentiated from the rotavirus strains. It was of note that the dendrogram showed hierarchical clustering of MA104 as a subgroup of ST3 and YO group, which was against the expectation for MA104 as the cell culture media to be at the highest hierarchical level that governed all the strains. The resultant error in hierarchical orders could be attributed to the variations in Raman intensity levels for different samples resulted from different dilutions applied. Therefore cluster analysis resulted in reasonably good classification for rotavirus samples. Cluster analysis is defined as unsupervised analysis, which predicates sample variations without the knowledge of the similarity between the samples. In the case of rotavirus samples, the strains were known. PLSDA was therefore applied for supervised classification of rotavirus.

In the PLSDA analysis, raw data set was constructed into an x-block (75×1491 , i.e. each spectrum was collected with 1491 data points.), which determined the descriptive statistics, i.e. the mean and the standard deviation for each variable of the known groups. A “y-block” variable matrix corresponding to the x-block was established based on the knowledge of the known classes of the spectra. An individual spectrum either belonged to the class, i.e. “true”=1, or not belonged to the class (“false”=0). In case of the five rotavirus sample and the cell culture media, six classes were defined in total, therefore the y-block was with a dimension of 75×6 . PLSDA analysis determines the best fit of the descriptive x-block against the y-block. As a result, within-

class variations were minimized, while variations between classes were maximized. A venetian-blinds cross validation method with 8 splits was simultaneously applied to the fitting procedure to determine how good the fit was. About every one out of nine spectra was withheld as unknown to be tested against the training set (the rest of spectra). Cross validation were such performed to the whole data set. **Fig7.3.** showed the resultant Y cross validation predicated plot. The dashed red line in the plot defined the threshold for predicating the y-values in the future. Variables above the threshold belonged to the class, while those below the threshold did not. It was demonstrated that each sample strain was very well discriminated against the cell culture media and from each other. The effectiveness and validity of the applied PLSDA model was summarized in **Table7.1.** The statistics showed 100% sensitivity and specificity for all y-values predication, which indicated perfect fitting of the x-block against y-block. Therefore, the six samples were effectively differentiated with their SERS spectra.

Conclusions

The presenting study demonstrated that intrinsic SERS based on AgNRs platform may serve a robust method for bioanalysis of rotavirus. The same platform has been applied to respiratory virus detection. [9] It should be pointed out that AgNRs substrates provided good control of nanostructure morphology, high sensitivity SERS signals and considerably good reproducibility. All of these advantages are crucial for development of fast reliable economic biosensors. Chemometrics analysis may not only be applied for differentiation of virus strains, but could be further used for quantification of concentration-induced variations in the future.

Acknowledgement

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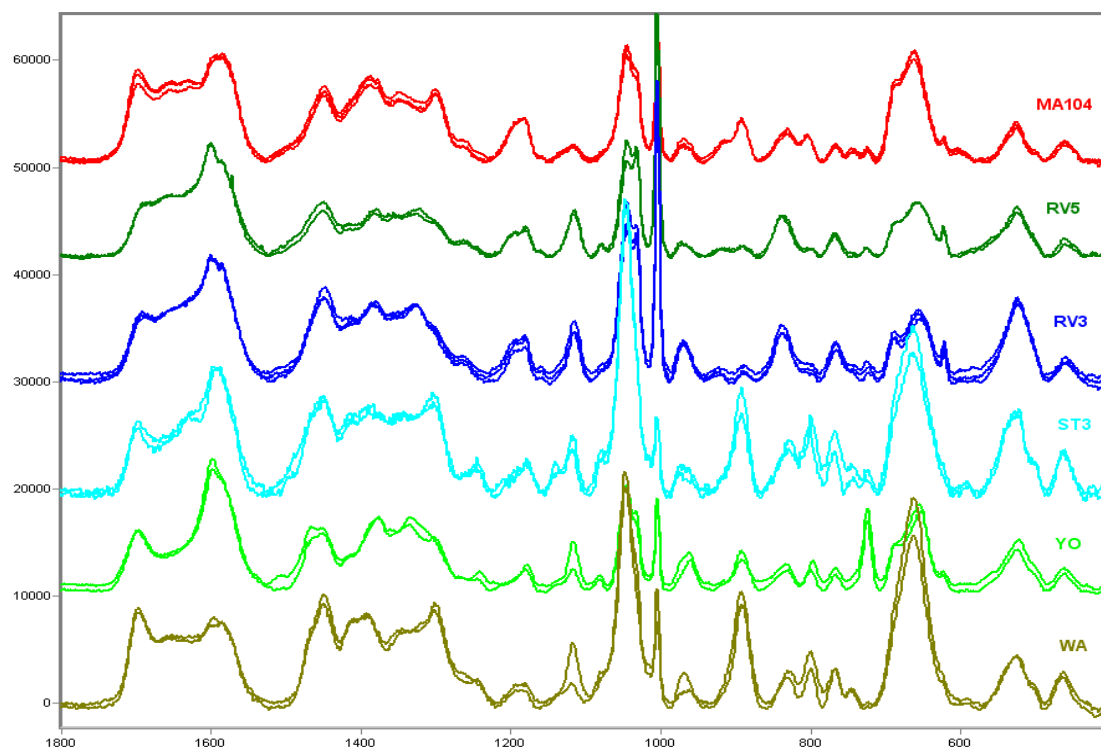


Fig7.1. SERS spectra of rotavirus strains and cell culture media.

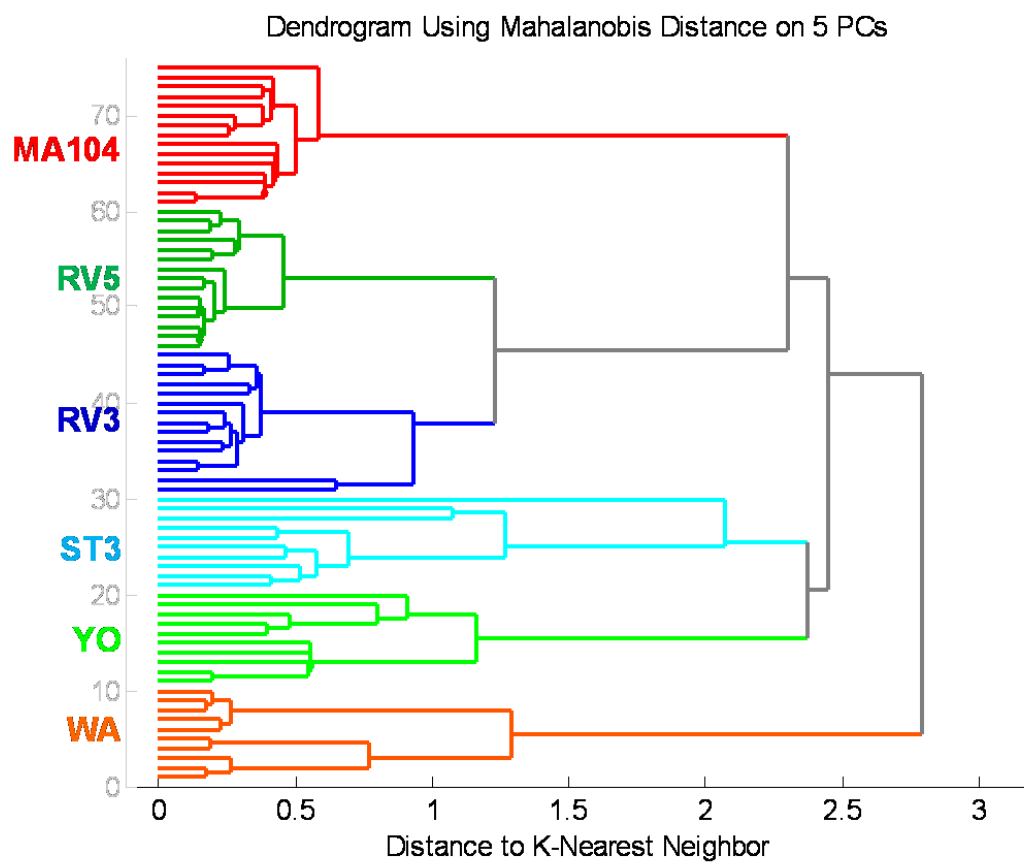


Fig7.2. Cluster analysis of rotavirus strains and cell culture media.

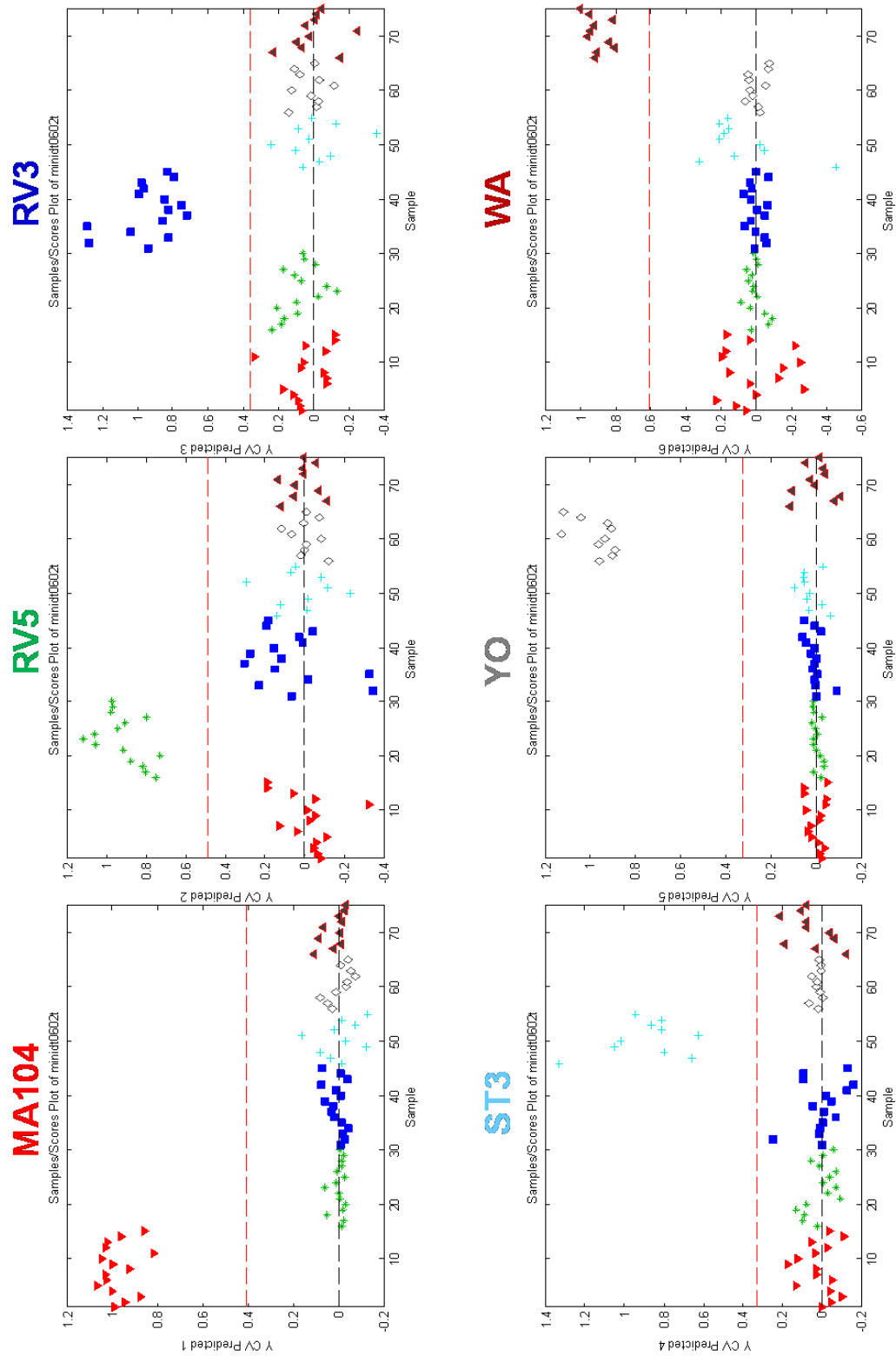


Fig7.3. Y cross validation predicted plot for rotavirus and cell culture media.

Table7.1. Statistics for PLSDA modeled class:

Cross validation: venetian blinds with 8 splits						
Sensitivity (Cal)	1.000	1.000	1.000	1.000	1.000	1.000
Selectivity (Cal)	1.000	1.000	1.000	1.000	1.000	1.000
Sensitivity (CV)	1.000	1.000	1.000	1.000	1.000	1.000
Selectivity (CV)	1.000	1.000	1.000	1.000	1.000	1.000
Class. Err (Cal)	0	0	0	0	0	0
Class. Err (CV)	0	0	0	0	0	0
RMSEC	0.0442971	0.108732	0.0955825	0.0879073	0.0438047	0.0860676

CHAPTER 8

CONCLUSIONS

In Chapter 3, combination of various 2D correlation techniques on FTIR transmission spectra was explored to determine pH-induced structure conversion of wild type rdhSP-C. A pH-dependent α to β structural change was manifested as previously observed with bovine SP-C. The relative changes of intensity variations were further quantitatively extracted from model-based 2D correlation analysis. As a specific case, heterospectral correlation between the NHs region and the amide I region indicated different spectral origins for α -helix at 1659cm^{-1} and unordered structure at 1645cm^{-1} . These two bands were usually distinguished by H-D exchange experiments. Windowed auto-correlation analysis revealed the pH-conformation relationship. It was indicated that β -structures were predominant at pH above 6. It was demonstrated that 2D correlation analysis provided a powerful tool for spectral resolution enhancement and structure determination.

In Chapter 4, a set of site-directed mutageneses of rdhSP-C were investigated with a pH-dependent unfolding mechanism. Mutageneses at the N-terminus for Mut3 (C5S/C6S) and Mut4 (K11Q/R12Q), as well as in the poly-valine segment for Mut6 (L22P) and Mut7(V21P), resulted in distinct patterns of structural changes upon pH titration. The following observations and conclusions were made from the study.

First, the structure changes for mutageneses at the N-terminus and that in the poly-valine segment were in total different patterns. As the mutagenesis moving from the N-terminus to the

poly-valine segment, the mutants unfolded into β -turn structure and unordered structure respectively. Mut3 and Mut4 were predominant with β -structures, while Mut6 and Mut7 exhibited fast denaturation upon pH titration. It was concluded that the poly-valine residues were of primary importance to maintain the stability of the helical structure of rdhSP-C. Furthermore, for mutagenesis in the polyvaline segment, the amino acid residues being replaced as well as the position of the mutagenesis occurred resulted in subtle difference in Mut6 and Mut7. Although replacement with proline basically broke the helical structure, no formation of β -aggregation was observed for Mut6 (L22P). Recent study has indicated that native β -sheet proteins adopted strategies, such as positive charged residues, short twisted β -strands and loops, to prevent the formation of aggregations. It was possible that mutagenesis of L22P could result in short edges for Mut6 therefore prevented the formation of β -aggregations. For Mut7, the break-down of the helical structure were accompanied by fairly small but appreciable amount of β -aggregation.

Second, the possibility that the formation of β -aggregation through disulfide bonds of the Cys pair was excluded for Mut3. However, Mut3 showed the most pronounced increase in antiparallel β -sheet and β -aggregation. The ability of Mut3 in forming β -aggregation was therefore attributed to the intrinsic propensity of the intact poly-valine segment to undergo α to β conversion, which was considered to be the main driving force for SP-C to form amyloid like fibrils. For Mut4, the predominant β -structure was attributed to short twist β -strands based on *ab initio* predications of β -sheet. However, no appreciable β -aggregations were observed for Mut4.

In Chapter 5, surface pressure induced structure changes of wild type rdhSP-C and Mut4 (K11Q/R12Q) in DPPCd62 monolayers were investigated at the air/water interface using PM-IRRAS. 2D correlation analysis further provided important information on dynamic structural

conversion of the polypeptides. Model-based 2D correlation analysis was demonstrated with advantages for resolving PMIRRAS spectra, in terms of its insensitiveness to spectral noises and interfering signals from water vapor residues. Wild type rdhSP-C showed pronounced formation of β -aggregations in response to surface pressure changes. In contrast, Mut4 exhibited pronounced increase in β -sheet conformation. However, no appreciable β -aggregation was observed. It was concluded that the formation of β -structure could be mediated by the mutagenesis of K11Q/R12Q for Mut4. It was also implied that the formation of β -aggregation could also involve different structure motifs other than dictated by the intrinsic propensity of the poly-valine segment. More importantly, it was concluded that β -aggregation may have a different origin from the extended β -sheet structure.

In Chapter 6, thermal induced structural changes of Mut4 were investigated in planar supported PC bilayers prepared with two methods, i.e. vesicle self-assembling (VSA) and vesicle fusion (VF) method. The latter coupled PC vesicles to the Langmuir transferred PC monolayer to yield a single bilayer on Ge crystal. In addition, the orientation of β -sheet of Mut4 in PC bilayers was revealed. The investigations resulted in the following conclusions.

First, structural changes of Mut4 in bilayers prepared with both methods were determined by model-based correlation analysis. Regardless of the external perturbation applied to the systems, dominant increase in β -structure was observed. On the other hand, the resultant different β -structures by different bilayer preparations may implied that transfer from monolayer to bilayers could mediate the structure conversion of Mut4, as well as induce the formation of β -aggregations. These conclusions were of importance to understand the mechanism of β -aggregation formation of native SP-C in biological environment.

Second, orientation investigation indicated that β -strands of the sheet had the same orientation as the acyl chains of DPPCd62 in the bilayers (the results were independent of bilayer preparation methods). However, orientation parameters of β -sheet of Mut4 in bilayers indicated large tilt angle from the membrane normal. The orientation parameters collectively may imply the existing of twisted β -strands, rather than extended β -sheet for Mut4. The conclusion was in line with *ab initio* predications that these two β -structures may be of different structural origins.

In summary, systematic biophysical investigation of the conformational changes of rdhSP-C wild type as well as its mutant(s) under external perturbations revealed the functional importance of certain structure motifs of SP-C. It was also indicated that the mechanisms for the formation of β -aggregation of SP-C may involve different functional motifs of SP-C, as well as the changes in phospholipid environments.

Chapter 7 described the methodology using intrinsic SERS based on silver nanorods (AgNRs) platform and chemometrics to differentiate rotavirus strains. The method was proved to be fast, effective with high reproducibility for classification of rotavirus strains. Two chemometrics models were employed, i.e. cluster analysis and PLSDA. Cluster analysis resulted in reasonably good classification of rotavirus strains. Cross validation indicated that PLSDA was a robust model for discrimination of rotavirus strains. The results were of importance for further development of the AgNRs based intrinsic SERS technique.