Nano-Structuring and Molecular Domain Organizations in Lipid-Protein Membranous Interfaces

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Cell membranes, lung surfactants and other liquid crystalline systems have become an interesting area for structural studies, due to recent evidence suggesting that such supra-molecular assemblies have micro-nano scale organizations or domains. Such domains play a critical role in functioning of membrane proteins, as well as phase segregation and liquid ordering of soft-materials in two dimensions. These domains can be imaged dynamically from an air-water interface of Langmuir films of 17-25 Å thickness using a number of correlated physico-chemical methods. We have employed a series of correlated imaging techniques such as fluorescence, atomic force microscopy (AFM), time of flight-secondary ion mass (TOF-SIMS) and Raman-confocal spectroscopy to image micro and nano-scale organizations of specific components in lipid-rafts, bacterial and lung surfactant membranes. Using fluorescence, AFM and Raman the phase transition process and their respective domains in liquid-crystalline, gel and fluid-ordered states in monolayer films and bilayer liposomes could be dynamically observed. The lipid confirmations, tilt, specific lipid compositions of domains in some of these systems could also be imaged. Some recent evidence indicates that structural disorganization and anisotropy of domain structures detected using these imaging methods in membrane dysfunction, may allow for a better understanding of the molecular basis of some disease forms.

Keywords: Physico-Chemical Imaging, Monomolecular films, Lung surfactant, Model membranes, Lipid-rafts, Phase transitions.

1. Introduction

Most membranous, surfactant and liquid crystalline systems made of ampipathic molecules can be self assembled into a host of polymorphic structures such as micelles, monolayer, bilayer liposomes and nano-tubular phases. Due to the isotropic distribution of some specific component or the other in these systems into domains, they are also considered to be soft-materials [1]. Model membranes of one or more componential mixtures such systems can be easily prepared for imaging using either Langmuir-Blodgett films, or in bilayer (giant uni-lamellar) vesicles. Interfacial imaging of bilayers of cell membranes have shown that specific components such as sphingomyelin, cholesterol and fluid lipids can phase segregate in nano-scale domains termed "lipid-rafts" upon which membrane proteins dwell during functioning [2].

Lung surfactant (LS) is a secretory lipid-protein membranous complex which stabilizes the alveolar air-sacs of all mammalian species. The material is secreted in multiple bilayer containing lamellar bodies, which undergo transformation to tubular forms, and eventually coat the lung air-water interface with mono-molecular films and bilayer structures .These films reduce the surface tension of the alveolar interface during respiration and prevents lung collapse at low volumes [3]. However lack of secretion of the material or inactivation by leakage of serum proteins (among others) have been implicated in a variety of disease such as Respiratory distress syndromes (RDS) in neonates, acute-RDS (ARDS) in adults, cystic fibrosis and asthma among others. Due to the ubiquitous composition of LS, *in vitro* studies have shown that a proper functioning surfactant, the saturated lipid component 1, 2-dipalmiotyl-sn-phosphatidylcholine (DPPC) phase segregates in domains. Such domains due to their more solid-like

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tight intermolecular packing allow for reduction of surface tension to very low values at the lung air-water interface [3]. A recent study has also shown that surfactant extracted from diseased lungs showed a complete alteration of domain morphology compared to normal, suggesting specific molecular conformation of LS components may be altered in dysfunction and disease [3, 4].

Our laboratory has developed several structural methods in evaluating organizations of lipids and proteins in cell, bacterial and lung surfactant membrane models, using a set of correlated physico-chemical imaging methods [5-9]. Model membranes of the componential mixtures are prepared either in monolayer Langmuir films or bilayer vesicles [6]. These can be imaged using fluorescence microscopy during dynamic phase segregation process induced by surface pressure or thermal changes. Also by transferring the films by Langmuir-Blodgett technique on atomically flat mica substrates, exact molecular tilts, conformation and nano-scale ordering of lipids in films [10] can be observed using atomic force microscopy (AFM) and Time-of-Flight Secondary Ion Mass spectroscopy (TOF-SIMS) [9]. In some cases imaging of natural bovine lung surfactant extracts and bacterial membranes (*E. coli*) could also be performed using Raman-spectral microscopy.

2. Phase Transitions in Films and Bilayers

Figure 1 shows the lateral surface pressure induced phase transition occurring in monomolecular films of a lipid-raft mixture (A) and bovine lung surfactant extract (B). The films were formed by solvent spreading of the dissolved lipid-protein materials to a clean air-buffer interface, after doping the materials with a small amount of fluorescent probe 1-palmitoyl-2-nitrobenzodioxo-docanoyl phosphatidylcholine (NBD-PC). During surface pressure induced phase transition gel or ordered lipids phase segregate out into circular (black) domains from the more isotropic fluid phase and the NBD-PC preferentially partitions in the less ordered fluid phase. The films can be imaged using epifluorescent microscopy either from the air or from the sub-phase water, and the design and construction of such Langmuir-surface balance with microscopic attachment has been detailed elsewhere [10]. Such films can also be deposited at a selected surface pressure on solid substrate and imaged using atomic force microscopy (AFM) [Fig.1C]. The dark gel-like domains seen in the lung surfactant films by fluorescence (Fig. 1B) also have higher height profile as shown in the AFM (Fig 1C) due to the more perpendicular orientation of the fatty acyl chains of DPPC which are induced by increasing packing densities or surface pressure. The gel or ordered domains have been considered to be tilt-condensed due to the specific molecular orientation of the lipid being visualized in the domains [11]. The raft lipid mixture (Fig 1A) containing sphingomyelin, cholesterol and fluid phospholipids however show a different form of ordering than gel domains. Cholesterol orders the fluid lipids into a liquid-ordered (dark-large circular domains) phase while fluidizing the sphingomyelin in coexistence with an anisotropic fluid lipid only phase upon which the probe NBD-PC partitions. These two models suggest that fluid-gel phase separation (B) as well as fluid-fluid immiscibility (A) can be dynamically imaged using a simple probe partitioning technique, as well as specific molecular tilts and orientations of the lipids can be physically mapped using AFM [5].

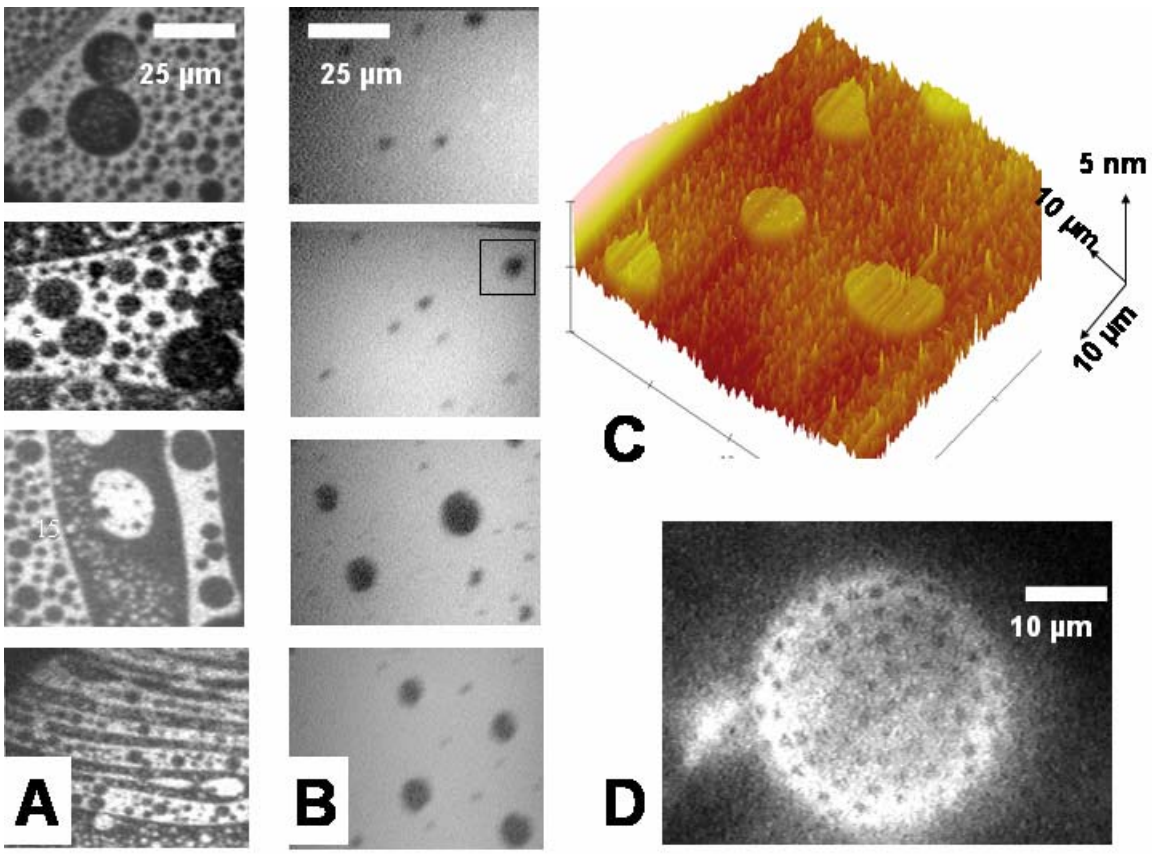

Fig. 1. Epifluorescence microscopy images of natural membranous materials in films (A-C) and bilayers (D). The films were laterally compressed between 20-40 mN/m surface pressure. These model membranes were prepared with a lipid-raft mixture (A), bovine lung surfactant extract (B, D) and the dark areas represent the gel-ordered (rafts) phase domains, and the bright areas the fluid phase in which the probe NBD-PC partitioned. In (C) a Langmuir-Blodgett deposit of the surfactant film as imaged using AFM is shown, with higher topographical height profile of the gel domains.

Although monolayer films have long served as good models for membrane structural studies, it is not clear to date what the lateral surface pressure of these lipid-protein systems is in bilayer [12-14]. Studies over the last decade by have established that the domains in films can also be observed using fluorescence microscopy of giant uni-lamellar vesicles (Fig 1D) [15]. These 20-50 µm bilayers in giant uni-lamellar vesicles (GUV) are formed on platinum electrodes lined with dried membranous lipids by passing a small AC current between the electrodes in buffer [8, 14]. The vesicles can then be imaged using similar probe partitioning techniques as in films, however here the phase transitions are induced by thermal cooling or heating cycles. The saturated hydrocarbon chains of the phospholipids melt upon heating or freeze upon cooling (black domains in Fig 1D), thus allowing for the process to be dynamically imaged . However due to the curvature of the uni-lamellar vesicles or liposome, confocal and two-photon fluorescence microscopy are better imaging methods since these allow for imaging the surface as well as cross-section of the bilayers where layer by layer analysis from the surface to the middle of the spherical GUV can be performed. The vesicle studies have shown that the gel-domains of the lung surfactant bilayer (Fig 1D) are coupled across each hemi-layer of the bilayers [8, 15].

3. Nanoscale Organization of Domains

Scanning probe microscopy (SPM) have allowed for high resolution imaging of various biological and organic system structures under fluid or in air. However molecular-atomic resolution imaging can only be performed using scanning tunnelling modes in air or vacuum and only if the samples are good conductors of the tunnelling currents. The AFM modes of the SPM can however be applied to image films of organic molecules in air, using surface contact mode when the films are deposited on an atomically flat mica or highly organized pyrolitic graphite [14,16]. The nano-scale organization of the gel phospholipids in the lung surfactant film are shown in Figure 2. The gel domain (Fig 2A) has a normal higher height (0.8 nm) profile than the surrounding fluid isotropic phase and can easily be observed at a lower resolution (10 x 10 μm) by AFM in the contact mode. After zooming into a selected area of the gel domain a molecular resolution image may be obtained using a thin tip (10 nm) and nano-scale organization of the phospholipids at high packing density can be obtained (Fig 2B). The small bumpy areas indicate the hydrocarbon terminal methyl (CH_3) groups, and the nano-scale organization is measured and further contrast enhanced using fast Fourier transforms as displayed as a three dimensional (somewhat) ordered lattice network (Fig 2C). The AFM imaged DPPC phospholipid lattice spacing and single molecule dimensions in films and bilayer have been recently substantiated by us [17] and others [11, 16] using X-ray and neutron diffraction methods.

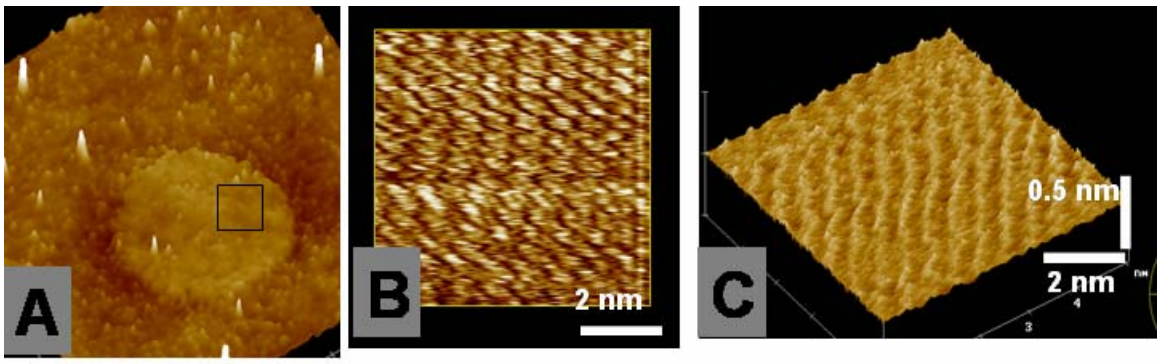

Fig. 2. Nano-scale organization of a gel domain (Boxed area in A) of lung surfactant film, imaged using contact mode AFM. The image in (B) was obtained by high resolution (5nm x 5 nm x 0.5 nm) scanning of the domain internal area and the small bumps represent the phospholipid acyl chain terminal methyl groups. The Fourier-transformed 2D image in (B) as shown in (C), suggests the existence of 40-50 $Å^2$ areas organized into nano-structured ordered lattice network as previously substantiated by other methods [11,16].

4. Chemical Imaging of Model and Native Membranes

Although AFM is a versatile tool in observing membranous structures, its ability is limited in providing surface topographical information of mostly single component model membranes. In case of most cellular or native membranous complex such as LS, the multiple components or complex composition does not allow for detailed studies to be performed using AFM, or discriminate component specific domain organizations as in the LS [3-9]. Bovine lung surfactant contains about more than twenty varieties of lipids and four surfactant lipid associated proteins. In a film formed with such complex materials the identity of specific lipids and proteins are important to understand the functioning of each individual component and their role in LS surface tension lowering properties [3]. In a recent study we have observed that surfactant from diseased lungs do not have any significant amount of gel-phase or domains structures and such films are unable to lower surface tension to low values [4]. Such structure-function relations are also important in case of cell membrane lipid-rafts since specific proteins and enzymes are know to reside in such rafts. The standard chemical or elemental identifier methods such as scanning electron microscopy cannot be used to image ultra-thin (20-30 Å) films due to their fragile

nature, as well as these methods require sample coating. Recently methods using either mass spectral imaging or using bond vibrational detection spectroscopy such as Raman and Fourier transform infra red (FTIR) microscopy was able to identify specific membrane components in model systems [14, 18, 19].

Time of flight-secondary ion mass spectrometry (TOF-SIMS) have become a standard tool for detecting chemical distribution of specific ions in inorganic systems, organic films, polymer coatings and lipid-protein model membranes. The technique uses an inert gallium or other ion beams as a primary ion source which when focussed-sputtered on a sample surface allows for fragmentation and desorption of the surface molecules as secondary ions. These generated secondary ions are then analyzed using their time of flight patterns or molecular mass/charge ratios in a mass analyzer. Any secondary parent or fragment ion peak in the mass spectra can be individually or collectively mapped and pixel distribution of the intensities can give a relatively good image of chemical distribution over a 50-10 μm area of the surface [18]. Figure 3 shows the distribution of a specific ion (C) from the gel-domain region (boxed in A) of a lung surfactant film and the distribution of the secondary ions plotted in the pixel maps shown in (B). The surfactant film was deposited on a gold coated mica substrate and imaged first using AFM (Fig 3A) and later imaged using TOF-SIMS (3B). The complete positive secondary ion spectra and a small enhanced region (inset) are shown in Fig 3C. The mass by charge ratios (m/z) for typical phospholipids are stated as absolute atomic units or in Daltons, since in these hydrophobic lipid film systems generate mainly ions with a single charge of +1 (9,18). The image shown in Fig 3B were mapped for either total ions (from all parent and fragments shown in the spectra in C), for calcium ions [m/z=40] and for the DPPC parent ion at 734 [m/z=734]. For observing specific chemical distribution the film was prepared on a sub-phase containing 2 mM calcium chloride, since this cation binds to an acidic phosphatidylglycerol (PG) component of the LS [3]. The binding of the cation to the PG leads to specific aggregates of the phospholipid into the center of gel domain, which shows higher height profile in the AFM image (A), as well as a relatively brighter spot in the TOF-SIMS image (center) detected at 40Da for calcium (B). However these images also suggest that some calcium seems also to interact or bind to the other phospholipids such as DPPC in the gel phase which suggests that the ion can have interactions with specifically oriented lipid head-groups in such domains. The spectral intensities obtained from the parent DPPC ion were very weak (inset of 3C), and the image had to be contrast enhanced to observe a complete domain. The study confirms that indeed LS phospholipids DPPC and PG are found in the gel domains, and divalent ions bind strongly to such domains.

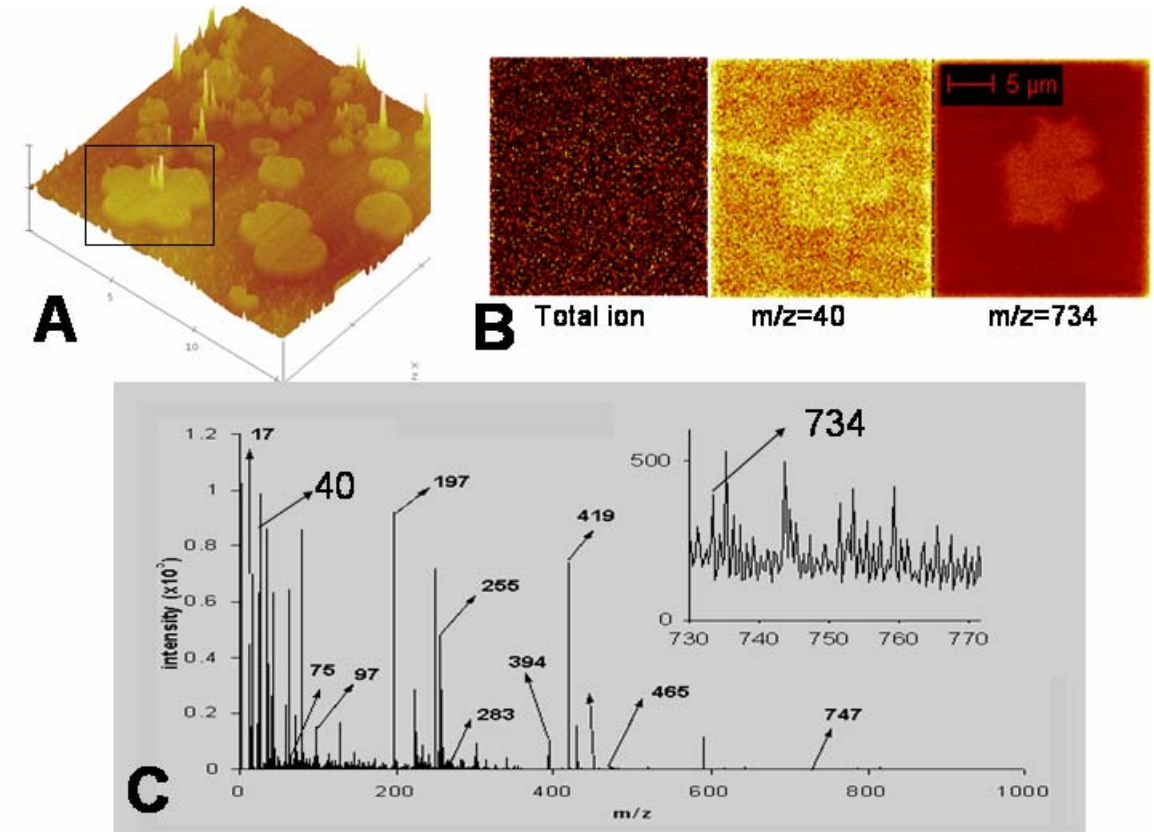

Fig. 3. TOF-SIMS image (B) and the complete mass-spectra (C) from a surfactant film deposited on gold-coated mica and initially imaged using AFM (A). The flower like shape of the domain (boxed in A) is due to interaction of sub-phase cations with the PG component of surfactant, and the shapes are maintained in the secondary ion images of the cation (m/z-40) as well as a parent DPPC phospholipid ion (m/z=734), which phase segregate into the gel domains. The enhanced mass spectra from the parent ion is shown inset of (C), and is close to the background spectra, since most of the parent molecules are fragmented to generate the secondary ions.

The TOF-SIMS method for chemical imaging of native cell membranes is limited since the method requires robust samples, specialized substrates (gold coating); spectra can only be obtained in vacuum and from dehydrated samples, as well as destruction of the samples during imaging by fragmentation of the parent ions. Although lipid monolayer films are quite stable under such conditions for TOF-SIMS, since phospholipids at the air-water interface are locked in specific tight conformation and freezing of minimum head-group hydration levels, cell membranes and bilayer model membranes (such as GUV) has to be studied under fluid and are not very stable or robust when dehydrated. Here Raman or FTIR-spectral microscopy comes to better use although the vibrational spectral resolution of currently available microscopes are limited by the sensitivity of the detectors as well as sample concentration [20, 21]. The advantage however is that vibrational spectral- microscopies require minimum sample preparation, can be performed under fluids as well as spectra can be collected within a very short time [14, 21]. Specific conformations of the molecule (trans/gauche for lipids) in membranes or distribution of some bonds such as Amide-I/II for proteins in a sample has allowed for imaging complex samples such as membranes in gel or fluid phase, or the surfaces of native cell membranes and membrane protein distribution in such lipid environment. In Raman or FTIR microscopy a confocal objective-lens combination is used to collect frequency shifted spectra from the sample excited either via visible or IR laser beam [20-22]. FTIR-spectral imaging however is limited since, water (H-O-H) has a large and broad vibrational adsorption band around 1700 cm^{-1}which submerges signals from various other bonds found in this broad

band area for most biological samples (Amide-I, Amide-II from proteins, and methyl and methylene skeletal vibrations of phospholipids of membranes).

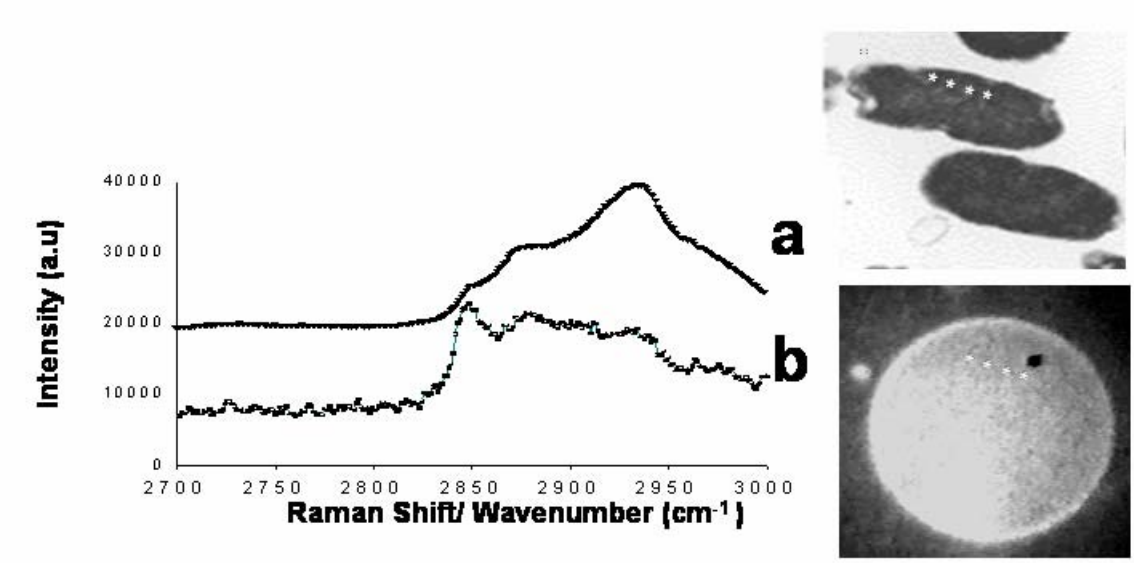

Fig. 4. Raman spectra obtained from the surface of a native bacterial (a) and lung surfactant bilayer (b) membranes, using line section (*) scanning of the surface.. The images on the right panels were were obtained from the optical confocal objective attached to the Raman microscope. The Raman frequency shift of the methylene (CH_2) symmetric vibrations showed the highest peaks, noted at ~ 2940 cm^{-1} for the bacterial membrane glyco-lipids (a) and the surfactant at ~ 2850 cm^{-1} (b). By altering temperature or by introducing additives in the sample, the change peak-shifts can indicate phase transition, however Raman signals from single bilayers are too weak to pixel-map as images.

The Raman-spectra from a native bacterial membrane surface (a) and a lung surfactant bilayer or GUV surface (b) is shown in Figure 4. The membrane surface of both systems was sampled directly using a line scan analysis along the line marked by asterisks. The images shown in the left are obtained using the confocal optical microscope, as the Raman signals were too weak to give any demonstrable image map as well as the domains were possibly too small to be identified in the membranes. By using such line by line scans and selecting a specific peak of one or more spectra, a digital map of the peak intensity has can be plotted. The highest intensity peak between wavenumber 2800-2900 cm-1were obtained from the phospholipid CH2 (methylene) symmetric vibrations for the bacteria and the surfactants. Since bacterial membranes do not contain any specific phospholipids as found in lung surfactant, the peak spectra around this range are very different for the two different membranes. Although we have just began to apply this technique to our model membrane systems, there has been consistent resolution problems due to extremely weak signals being obtained to observe any appreciably visually distinguishing features form single mono or bilayers of lung surfactant, and also possibly the averaging of signals from beam areas smaller than the domains. Studies by others have however been able to detect protein distribution in model membranes containing bacterial proteins, by surface enhancing the Raman signals [20-22], and others have observed distribution of a specific phospholipid domain distribution in bilayer dispersions of skin-membrane lipids [19], suggesting the feasibility of these vibrational spectral-microscopy for membrane studies.

In summary, these correlated physico-chemical imaging methods allow for observing the molecular conformations and component distribution of membranous interfaces. Although each method is limited by its optical and spectral resolution (Raman-microscopy), sample preparation or invasiveness (TOF-SIMS), probe induced artefacts (fluorescence microscopy), surface topography only (AFM), a mix and match of one or more method allows us to comprehend molecular details of complex and nano-structured membranous systems. Recently such structural studies in our and other laboratories have suggested that

in disease and dysfunction the supra-molecular organizations of such membranous systems may be disrupted. Thus such structural methods may yield specific information to more complex biological and biochemical processes pertaining to membranes, and study the molecular-structural basis of some membrane disease states.

Acknowledgements: The studies performed in this project were supported by grants from the Canadian Institute for Health Research-New Investigator Awards, Janeway Children Health Advisory Committee, National Scientific and Engineering Council of Canada and Canadian Foundation for Innovation Infrastructure (AFM & Raman-Confocal microscope) to KN. The authors would also like to thank Dr. Nils O. Petersen, CEO, National Institute for Nano Technology, Alberta, Canada for the use of TOF-SIMS facility.

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