Physicochemical Mapping of Phase Heterogeneity in Biomembrane Films

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ABSTRACT
Langmuir-Blodgett (L-B) films have long served as models of cell membranes and lung surfactant systems. However, these monomolecular films are difficult to image due to their fragile and dynamic nature. Raman-spectral microscopy and TEM suggest the presence of phase heterogeneity in such systems. Using correlated atomic force and fluorescence microscopy with secondary ion mass mapping of L-B films, snapshots of the heterogeneity were imaged as gel-fluid domains or lipid rafts in phase coexistence. The molecular tilt of lipid chains, chemical composition, rigidity and dynamics of these phase transitory structures could be monitored from an air-water interface. The physicochemical organization of such structures suggests dynamic lipid-protein organization in membranes.

INTRODUCTION
Pulmonary surfactant (PS), a lipid-protein complex, is secreted at the lung air-water interface. PS contains significant amounts of dipalmitoylphosphatidylcholine (DPPC), phosphatidylglycerol (PG), and cholesterol among a host of other lipids, and small amounts (10%) of specific surfactant proteins SP-A, SP-B, SP-C and SP-D [1]. The material coats the interface with membranous layers (more and more bilayers), which reduce the surface tension of the interface to prevent alveolar collapse during normal respiration. The material is packaged as multimamellate vesicular structures or lamellar bodies (LB) which undergo transformations to a nanotubular complex called tubular myelin (TM) (Fig 1). TM is presumed to be the precursor of the surfactant active films or layers. Despite intensive effort over the last four decades, the layers, ranging in thickness from 1.7 to 2.0 nm (monomolecular film) or its underlying bilayers (4-10 nm) have not yet been defined by imaging in situ. The fragile nature of these structures [1]. However extracting the PS complex from lungs, and spreading them at an air-water interface using Langmuir-Blodgett methods [2], is considered one strategy which allows imaging of surfactant.

Langmuir-Blodgett (L-B) films have long served as standard models for studying biological membranes since the films represent a monolayer or half the typical thickness of the bilayer cell membrane [2]. By solvent spreading or by adsorption of lipid-protein complexes to a clean air-water interface, LB films are formed, and by altering surface packing of such films, characteristic phase transitions can be studied [2]. However, due to the inherently complex nature of cell membranes and/or PS, individual components in these systems require discrimination by chemical imaging [3]. Studies on models of PS suggest that the lipids undergo a two-dimensional phase separation or segregation into fluid-fluid or gel-fluid domains, suggesting the systems are dynamic [2-4]. Similar domain structures have also been demonstrated in biological membranes as specific sphingomyelin-cholesterol enriched ‘lipid rafts’. However the demonstration and imaging of domains have proved difficult due to the factors of ultrathin (~2 nm), dynamic (thermal lipid-chain motions), hydration (requires an air-water interface) and compositional complexity of the films [2]. Such factors do not allow for standard TEM or STEM due to embedding, freezing and staining causing alteration or destruction of thin film morphology. A series of correlated methods of epifluorescence microscopy (EpM), atomic force microscopy (AFM), Raman spectroscopy and secondary ion mass spectrometry (SIMS) were utilized to study domains in these systems.

METHODS
The images of surfactant dispersions were obtained using standard transmission electron microscopy (Phillips 410 TEM) from Lowicryl embedded section after staining with uranyl acetate [5]. These dispersions were also imaged using a Renishaw Raman-imaging microscope with WIRE V 1.2 (Renishaw, Gloucestershire, UK) on a heat-cool microscope stage, and imaging using Raman vibrational shifts of C-H stretching in DPPC acyl chains [6].

Imaging films of surfactant directly from the air-water interface was achieved using a custom designed EpM attached to a Langmuir-Blodgett surface balance [4]. In this technique the solvent spread or adsorbed films of a lung surfactant preparation BLES (bovine lipid extract surfactant) and DPPC were imaged using small amount (1 mol %) of a fluorescent probe 1-palmitoyl, 2-nitro-bezoxy-diaze- docanoyl phosphatidylinsole (NBD-PC) (Avanti Polar Lipids, Birmingham, AB), incorporated in the lipids before spreading [5]. BLES (BLES Pharmaceutical, London, Ontario, Canada) is a clinical surfactant preparation containing all surfactant lipids and hydrophobic proteins SP-B and SP-C, without the neutral lipids such as cholesterol [1]. In some studies components of surfactant such as DPPC and DPCC + cholesterol (3 mol %) films were also imaged. The films were compressed at a desirable surface pressure, and the domain evolution at various lipid packing density was monitored with the EpM directly from the air-water interface. The domain morphology was observed from the property of the probe to partition preferentially in the fluid or disordered phase of the films (the probe excluded areas are seen as the dark domains as in Fig 2) [4]. Some of these films were deposited onto a solid substrate for AFM and TOF-SIMS using the L-B technique either on mica, glass or gold coated mica [2,3]. Contact mode AFM of films was performed using a DI Nanoscope IIIa, SPM (Veeco Instruments, Santa Barbara, CA) in the height and friction modes using 20 nm diameter SiN cantilever-tips [5]. The secondary ion or TOF-SIMS imaging (ION-TOF IV SIMS, ION- TOF GmbH, Münster, Germany) was performed by exposing the gold-substrate deposited films to a pulsed gallium-ion beam at incident currents of 0.85 pA at a pressure below 10^-6 Pa [3]. The secondary ion fragments were detected for specific mass/charge

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BIOGRAPHY
Dr Nag obtained his BSc (Hons) and MSc (physiology) from Calcutta University, India, and his MSc and PhD (biochemistry) at Memorial University. He was an MRC-Canada Lung Association postdoctoral fellow at Univ. of Western Ontario. At present he is an assistant professor and CIHR-New Investigator at Memorial, studying the structural biochemistry of membranes in lung disease.

KEYWORDS
domains, Langmuir-Blodgett technique, phase transition, AFM, fluorescence, TOF-SIMS.
ratios from the time of flight spectra [3].

RESULTS AND DISCUSSION

Figure 1A suggests the typical structures observed using TEM of bovine pulmonary surfactant dispersions. The TM structures are seen as networks of lipid-protein tubules organized in a square lattice network, as has been previously observed at the lung air-water interface [1, 5]. These TM structures are formed from the lamellar bodies by post secretory transformations and specific lipid-protein interactions [1]. The dark lines or walls of the TM are of standard bilayer thickness (~4 nm) and the lattice of the squares ~40 nm x 40 nm. The image in Fig 1 suggests structures observed in BLES dispersions using optical (B) and Raman (C) imaging [6], with the selected Raman spectra of CH\textsubscript{2} stretching mode shown in (D). The images suggest that PS dispersions are highly organized into nanoscale structures (A) and possibly phase segregated into specific lipidic or lipid-protein domains (B and C).

The structures of DPPC (A) and DPPC + cholesterol (B) films as observed directly from the air-water interface using EpM are shown in Fig 2. The dark areas represent the probe-excluded phase which comprises gel-like or liquid condensed (LC) domains. The LC domains increase with increasing lipid packing or surface pressure \(\pi\) of the film (top to bottom of the panels A and B). The LC domains evolve into kidney-bean shaped structures (A, top) to more flower-shaped forms (A, bottom) and grow in amount with an increase in \(\pi\). Addition of cholesterol alters the LC domains into more spiral shapes (B), and this occurs due to alteration of line tension at the gel-fluid domain boundaries. The images of a LC domain as observed using EpM (top), AFM (middle) and TOF-SIMS (bottom) in panel (C) suggests that such correlated methods can reveal similar physicochemical morphology of the film. The EpM images clearly indicate the dynamic nature of phase segregation of lipids at an air-water interface.

Figure 3 shows the images of deposited Langmuir-Blodgett (L-B) films of DPPC imaged by contact mode AFM in air. The images were obtained by depositing such films on mica by the L-B method and imaging such films within 1 hour of deposit to avoid any appreciable aging effects. The typical kidney shape (A) of the LC domain is observed to be maintained in this time period. However, beyond 2-5 h such domains change and alter shapes, suggesting the effect of the dehydration over time on the film morphology. A line section analysis (B) of the domain morphology (in A) suggests a height difference of 0.8 \pm 0.2 nm between the LC and fluid phase. This difference of vertical height between the LC and fluid domains is due to the tilt of the phospholipid chains being more perpendicular to the air-water interface in the LC compared to those in the more disordered fluid phase [2]. These differences of height are in close agreement with previous studies done on similar films using X-ray diffraction and ellipsometry [2]. Imaging films in contact mode AFM also allows for determination of the material properties of the domains using the friction mode. Simulta-
Smaller liposomes, in the process of adsorbing these layers are of typical bilayer thickness. Obtained (data not shown), suggesting that height difference of around 4.5 nm was of small cavities or holes in the planar layer, under water, and imaged during the planar domains) compared to those of the chains in the fluid phase. Formed using liposomal suspension of BLES to be adsorbed directly to a clean mica slide (Figs 4 and 5A). The planar bilayers were domains are possibly made of lipids other than those in the LC domains. This difference of chain length in the domains is possibly due to micro-domains inside the LC domains. The height difference between a LC and a micro-domain (peculiarly also kidney-shaped) is in the order of a few tenths of nanometers (0.1 - 0.3 nm). This small difference cannot be explained by chain tilts of phospholipids (as in Figs 3 and 4) and suggested that the micro-domains are possibly made of lipids other than those in the LC domains. This difference in the LC domains is possibly due to 2 or 3 carbon extensions of the fatty acyl chains of the non-DPPC phospholipids. However, an exact chemical signature from these lipids is required for any certain conclusions to be made.

The AFM of planar adsorbed bilayers of BLES shown in Fig 5B suggests that the head-group regions of the phospholipids do not have any appreciable packing difference (or domains) compared to those of the chains (Figs 4 and 5A). The planar bilayers were formed using liposomal suspension of BLES to be adsorbed directly to a clean mica slide under water, and imaged during the planar bilayer formation. From AFM measurements of small cavities or holes in the planar layer, a height difference of around 4.5 nm was obtained (data not shown), suggesting that these layers are of typical bilayer thickness. Smaller liposomes, in the process of adsorbing (height in 50 -100 nm range), are seen as the circular shaped structures which eventually dissipate onto the surrounding bilayers over time (Fig 5B). Thus the AFM method allows not only for measuring chain tilts and material properties of membrane domains, but also suggests events occurring at the bilayer interface such as vesicular absorption and formation. However, as suggested in Fig 5A, a multicomponent system such as complex cell membranes and PS requires other strategies in deciphering the exact chemical composition of domains, such as those using TOF-SIMS.

Although SIMS is an invasive technique, which destroys the film while generating secondary ions (SI) due to surface sputtering by a primary ion beam, chemical composition of multicomponent domains as in Fig 5 can be mapped (Fig 6). The time-of-flight SI spectra of a DPPC film and typical images (inset) obtained from such films (left inserts) and those from a BLES film (right inserts) are shown in Fig 6. The phospholipid LC domain morphology can be imaged as secondary ion distribution of the head-group fragment choline (86 Da) and phosphocholine (184 Da), which are quite distinct SI. Due to such chemical signatures from DPPC, the composition of BLES domains can be mapped. However, the image obtained from the parent ion DPPC (734 Da) does not suggest any clear cut domain morphology, and only the total ion images (top right panel) could be analyzed from specific ion fragments. Also, high intensities of secondary ion signals (>10^3) are required to map any specific morphology. The dark area of the images suggests that the minor or smaller peaks in the spectra do not generate enough SI for any appreciable image contrast to be obtained [3]. This would clearly suggest that the domains in the gel phase of BLES films are mainly made of DPPC, a major component of PS. Since these films are made on a saline sub-phase, specific images obtained from the distribution of inorganic ions (image at 23 Da) such as sodium or calcium in such domains are mapped. Considering the complex composition of BLES, it is conceivable that such positively charged ions (Na+) are mapped in specific regions of the LC domain, where this ion may interact with negatively charged PS lipids such as PG.

These physicochemical methods lead to micro- and nanoscale imaging of phase heterogeneity in soft materials such as biomembranes and pulmonary surfactant. With rapid enhancement of such imaging methods to work under conditions of physiologic or ambient conditions of pH, temperature and ionic environment, imaging the molecular architectures of complex biological systems is possible. A clear understanding of the molecular distribution of such systems is required for determining the functions of its individual components.

REFERENCES