SURE 2002 report

Characterization of Lung Surfactant Protein segments and its oxidation mutant peptide

Chun Keung, Lau

Chinese University of Hong Kong

Abstract

Langmuir monolayer of lipids and protein present in lung is known as Lung surfactant. Mechanically it decreases the work of breathing. One of peptides in lung surfactant, SP-B, is known to be important for increasing the fluidity of the layer. Mutant engineered segments of SP-B are used. They are the first 1-25 amino terminal peptide and first 11-25th amino terminal peptide and with all the sulfur-containing residues replaced with hydrocarbon residues. They shows similar action in Palmitic Acid monolayer relative to the natural sequenced version of same length. Yet the mutants are more effective in fluidizing and increasing the collapse pressure of the monolayer. Also the 11-25 segments lead to bigger shift of Pressure-Area curve per palmitic acid. Another 1-25\textsuperscript{th} peptide mutant with proline in SP-B removed also gives a stronger fluidization than the natural sequenced segments.
INTRODUCTION

The study of lipid monolayer (single molecule thick layer) at air/water interface is both of physical and biological interest. From a physical point of view, the system behaves as a two dimensional system and yet show interplay between 2D and 3D at low area available to each molecule, and its microscopic (in micron scale) behavior is studied both theoretically and experimentally. As biological interest, lipid monolayers exist in lung alveoli and also serves as modeling in some circumstances for membranes, which is a bilayer. In this study, we are interested in the monolayer system which was called Lung surfactant.

Lung surfactant is a complex mixture of proteins and lipids. It is adsorbed on the alveoli surface as a monolayer. In breathing cycles, alveoli expand and contract. Due to the alveolar lining’s surface tension, work done is required in the process. Lung surfactant acts two dimensionally on the air/water interface. It exerts pressure as a three dimensional system do; therefore lowers the surface tension and reduces the work of breathing. Among all of the components, surfactant protein B (SP-B) is known to increase the fluidity and the collapse pressure of the monolayer. Also it helps retention of material during cycles of compression.

Despite its importance, how SP-B structural features related to the function of the protein is not well understood. Our study tries to investigate the importance of several features—1. the importance of the sulfur containing residues; 2. the importance of proline at the insertion sequenc 3. the first ten residues’ importance — by using isotherm diagrams and Fluorescence Microscopy.
A number of previous studies have been done on the peptide functioning. Previous isotherm study on Palmitic acid, or phospholipid monolayers (DPPG:POPG binary monolayer) with first 1-25th N-terminus residues of SP-B (we call it SP-B1-25) shows that the segment captures the functioning of the full-length peptide. Addition of the segment in monolayers promotes change in morphology and collapse mechanism, and inhibits the squeeze-out of material at high pressure. Previous X-ray reflectivity study on palmitic acid-SP-B 1-25th segment system indicates that N-terminus is embedded in the hydrophobic part of monolayers while the other end protrude past the hydrophilic region into the fluid subphase. The NMR study of the peptide of 11-25th segment shows a helix with hydrophilic residues on one side is formed when dissolved in aqueous environment, so that the protein remains ‘floating’ on the surface layer.

In the study, palmitic acid monolayer serves as a model for lung surfactant systems, due to its simplicity in structure and its ease of handling. Also, the simplicity of the palmitic acid molecules enables future X-ray study.

METHOD
To study the morphology (the macroscopic arrangement of the film molecules) of the monolayer, the Langmuir trough is used. An Langmuir trough consist of 1.a teflon well which the subphase is contained; 2.teflon barriers in between the monolayer is deposited, and they can be translated for compression and expansion of the monolayer; 3. a surface tension sensor. In this case, the pressure sensor used is Wilhemy Plate(fig. 1)
Pressure-area diagrams at constant temperature, known as isotherms, can be recorded using the trough. A known quantity of molecules is spread drops by drops on the surface, forming a monolayer. Moving the barrier decreases the area available to each molecule, and the surface tension change is monitored. The pressure the monolayer exerts is given as the difference between the surface tension of the pure water subphase and the reading in the presence of the monolayer. Phase transitions and response to compression of the monolayer can be inferred from the diagrams.

However, the isotherms alone can provide some but not sufficient information on the morphology. To study the morphology of the monolayer, various imaging techniques like Brewster Angle Microscopy, Fluorescence Microscopy and Atomic Force Microscopy (with monolayer adsorbed on mica) is employed. The Fluorescence microscopy is employed in the present study, as images can be conveniently taken while recording isotherms.

Small amount (2% by wt) of fluorescently labeled molecules, which acts as ‘dye’, is added to the spreading solution for Fluorescence microscopy. The dye molecules will preferentially partition into more disordered region in the monolayer. When the monolayer is illuminated with absorption wavelength of the dye molecule, the different phase region will show contrast in the micron scale image.

Material

The palmitic acid (PA; Sigma Chemical, St. Louis, MO; >99% Pure) is dissolved in Chloroform as obtained. All subphase was prepared from Milli-Q system (Bedford, MA). Buffer subphase contained 0.1M of NaCl and 10mM each of sodium monobasic phosphate and sodium dibasic phosphate. The stock
solution is prepared with either pure chloroform or 1:1 methanol:chloroform solution (for peptides only). The dye molecule (NBD-DHA) is obtained from Molecular Probe and added in 2% wt in all of the experiments unless specified.

The peptide sequence I have studied are:

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-B 11-25WT</td>
<td>CRALI KRIQA MIPKG</td>
</tr>
<tr>
<td>SP-B 11-25Oxmut</td>
<td>ARALI KRIQA NleIPKG</td>
</tr>
<tr>
<td>SP-B1-25 WT</td>
<td>FPIPL PYCWL CRALI KRIQA MIPKG</td>
</tr>
<tr>
<td>SP-B 1-25Oxmut</td>
<td>FPIPL PYAWL ARALI KRIQA NleIPKG</td>
</tr>
<tr>
<td>SP-B 1-25 G-Flex</td>
<td>FGIGL GYCWL CRALI KRIQA MIPKG</td>
</tr>
</tbody>
</table>

Note: Nle structure, which is not a member of natural peptide, have the formulae-

![Norleucine](image)

Experiment:
All isotherms are conducted at a compression rate ranging from 0.08 to 0.18 mm/s. However, for all comparison, only isotherms compressing at the same rate are taken.

Five species of peptides are studied and they are listed in the above table. All peptide-PA mixture films are tested at two temperature 16 and 28°C. Pure water subphase is used in all but two series experiment. The 1-25 and 11-25 Oxmut and WT are tested using buffered saline and buffered saline with DTT.
Result:

**SP-B 11-25 WT and OXmut and PA**

1. PA/SP-B 11-25 WT and its oxidation mutant (OXmut) on Pure water at 16°C

The addition of both SP-B11-25 WT and OXmut altered the monolayer phase behavior. Their strength to change the monolayer showed up only at high concentration of peptide.

The monolayer is set at 16°C, which is well below the triple point of the monolayer on water. Below the triple point, any fluid-phase appeared due to the peptide addition is easily observed.

The PA layer (with fluorescence dye) change from gaseous phase (g) at high area per molecule to lc phase (lc) along compression beyond liftoff. Liftoff point is the area at which the pressure starts to rise appreciably in the scale of mN/m. In this case, the liftoff area is 24 Å²/molecule. Further compression will lead to a transformation to liquid crystalline phase then to solid crystalline (sc) phase, which appears as a kink in isotherm.

The collapse occurs at a pressure of ~50mN/m, with an area of ~17Å²/molecule. Discrepancy from the previous studies of pure palmitic acid is due to the higher weight % of dye molecules are added. The FM image shows that small fluid phase appear before the kink, indicating the phase behavior of the monolayer has slightly altered by the dye. However, as only the dye in the case does not change the qualitative behavior of the monolayer and the only dye available (NBD) had a low luminosity ratio, such weight % of the dye was used.
At collapse, a drastic fall of pressure is observed. Then the pressure stay at a certain value, and expanding the monolayer then will lead to sudden decrease of pressure. Some material is irreversible lost.

The FM shows the change from gas to lc then to sc phase visually. The initial image before liftoff shows the coexisting dark gas phase and gray lc phase. Beyond liftoff, a homogenous gray layer is formed, with some small white region staying even beyond collapse that was not reported in pervious study. (fig 2,3A). This accounts to the different amount of dye added. At collapse, sc phase showed up on the layer as growing crystalline structure.(fig3B).

The addition of peptides showed dramatic change of phase behavior. When the 11.6% of SP-B11-25 WT by weight is added, the FM image shows difference. A fluid phase appeared and coexisted with gas and lc phase(fig 2A). In all cases, the fluid phase diminished beyond the kink location. The fluid phase cannot be observed, but the image was not bright enough to judge if it is so.(fig 3B)

Fig 3. A. The gas-lc phase coexistence before liftoff. B The formation of crystalline structures (arrowed).
Fig 4. FM images of PA+SPB11-25 WT at 16°C. A): the appearance of the network like structure near the kink. B) the disappearance of contrast at high pressure.

Yet the isotherms are more or less similar. The shape of the curve was similar, with the area of the kink and the collapse area being the same. This indicates that the peptide did not take up the space of palmitic acid. Yet the peptide remains on the layer up to expansion, as there is an increase of liftoff area, also of the collapse pressure and of the stable pressure value on compression beyond collapse.

The isotherm of PA with 11.2% of SP-B11-25 Oxmut by weight (same molar % as 11-25WT) was similar to its wild type counterpart. In addition, the two peptides gave rise to little difference in the FM images.

The isotherms are similar too, but different features were noticed. 1. The liftoff area of PA monolayer with Oxmut (we label it Oxmut+PA) is smaller than the PA monolayer with WildType sequenced peptide (we label it WT+PA); 2. The stable pressure value on compression beyond collapse is lower in Oxmut+PA case.
The comparison suggests that the Oxmut may be less effective in retention of material in the monolayer at low concentration. (fig 5)

Doubling the weight percentage of peptides added, however leads to different observation. The images were similar in the low concentration case, yet the isotherms are quite different (fig 6). The liftoff area of Oxmut+PA is lower than the WT+PA. Furthemore, the collapse pressure of Oxmut+PA is higher (~68mN/m) than the WT+PA (62mN/m). Also note that the collapse is much less drastic in the case of the Oxmut+PA. The Oxmut seems to be more effective.

2. PA/SP-B 11-25 WT and its oxidation mutant at pure water at 28°C

The monolayers were set at 28°C, several degree above the triple point of palmitic acid(25°C). Increasing the surface pressure, the phase behavior of the monolayer now follows the sequence: if initially it is gas, then it will go from gas to le(liquid expanded) to lc then to sc phase. The liquid expanded phase is different from the lc phase in microscopic sense the lipid
molecule tails are not aligning although raised up from the surface. A monolayer under observation would be a homogenous phase with no particular features. In the present experimental setting, the monolayer appears as le-g coexistence initially. In the Fluorescence Microscopy image, fluid phase appears as bright and the gas phase appears as some small ‘dots’. On compression it will become a homogenous bright le phase. Then further compressing, a le- lc phase coexists. The lc phase domains nucleated. At the end of the coexistence region, the domains merged and the pressure shot up at a kink (up to ~22mN/m at area per molecule 20 Å²). The sc phase formed into a continuous sheet. Collapse followed and then a drastic drop of pressure was observed. The addition of peptides, even at low concentration (11.2% by weight) again changed the behavior of the monolayer. The presence of the peptides did not change the liftoff area much.

WT(11.2% by weight)+PA traced a similar curve as the pure PA case, but the collapse area and pressure rise(to ~49mN/m at an area of 23 Å²). Such rise indicate the wild type peptide segment have some effect on the behavior of the monolayer.

Oxmut (11.6% by weight, the same molar % as WT) +Pa has a liftoff area even lower than the PA-only-monolayer. However, the peptides did change the near-
collapse-behavior of the monolayer. Both the shift in the collapse area (~47mN/m, a shift of ~3 mN/m) and pressure (~23 Å², a shift of ~4 Å²) appeared.

The shift in the collapse area with the peptide addition may imply molecules take up some space of palmitic acid molecules, and may require further study.

The comparison between the PA+WT and PA+Oxmut suggested that at low concentration of peptide WT was more effective than Oxmut in fluidizing the monolayer. The PA+WT gave a higher collapse pressure than the PA+Oxmut monolayer (50mN/m > 48mN/m). Moreover the former gave a higher liftoff area. The FM images showed that the morphology change due to the peptide addition. Adding the peptide does not change significantly the FM image at the liftoff. However, when the le-lc phases coexist, the addition of peptide made the dark lc domains much smaller (compare fig 8A and 8B). It hinted the fluidizing properties of the peptides. However, the distinction of the two peptides was not apparent by just viewing the FM image. (fig. 8B and 8C)

Fig 8 The FM images of le-lc coexistence region for different monolayer: A)PA monolayer B)SP-B-WT11-25 +PA monolayer C)SP-B 11-25Oxmut +PA monolayer

At high concentration, the two species of peptide segments behave differently from the case of low peptide concentration. The WT (23.2% by weight)+PA monolayer had a liftoff area similar to the pure PA film. The isotherm(fig 8) is
smoother than that of PA film along the decreasing area. The shift of collapse area also appears (~21 Å², a shift of ~2–3 Å²) and the the collapse pressure increases too (~47 mN/m, an increase of 5mN/m from the pure PA case)

The Oxmut of the same molar percentage (~22.4% by weight) also showed the similar changes. The liftoff area increases to ~52 Å², and the curve again run smoothly and rising more uniformly before the kink. Such behavior is explained by the increased fluidity of the monolayer. The collapse occurs at the same area as the WT+PA do. However, the collapse pressure (~49Nm/m) is slightly higher (a difference of ~2 mN/m) than the WT+PA.

The FM images in these two cases are similar to their respective low-concentration counterpart. The images do not have significant difference, both in the structure and the density of features.

**SP-B 1-25 WT and Oxmut and PA**

Fig. 9 The isotherm of the Oxmut+PA and WT+PA at 28°C at water subphase. The concentration of peptides are 22.4% and 23.2% respectively. The black curve represents the pure PA film, The blue curve represent WT11-25+PA, and the red curve represents Oxmut11-25+PA

Experiments with the longer peptide segments served as 1. Confirmation of the comparison between the WT/ PA and Oxmut/ PA ; 2.Comparison with experiments using 11-25th residues peptide segment.

1. PA+WT 1-25 and its oxidation resistant mutant at water 16°C
Both WT+PA and OxFmut+PA have a concentration of 20% in weight of peptides. The slight difference of the molar density of peptide is not taken into account due to the small difference in molecular weight. The isotherms turned out to be very similar(fig 10). Both the WT+PA and OxFmut+PA gave a liftoff area of 37mN/m, which is much larger than the pure PA film. In fact, the shape of the curve is very similar. Moreover, these two films collapse at the same area (~20 Å³).

Along most parts of the isotherms, they are similar. The plateau region of the isotherm corresponds to the coexistence of lc-tc phases. From the FM image, the nucleation grows as well as merges. At the end of the plateau, the lc nucleation nearly takes up the whole monolayer. The collapse occurs then upon further compression. The collapse pressure of the WT+PA layer is 49mN/m, which is lower than OxFmut+PA layer (collapse pressure ~54mN/m) by 5mN/m. The result indicates OxFmut is working more effectively against collapse than the wildtype. It is in good agreement to the result of the previous experiments with SP-B 11-25 segment and its mutant at high concentration.

Another interesting feature is the difference in liftoff area from the pure PA film was not obvious (~1 Å³), in the contrary to the isotherm of the 11-25th segment. It suggested missing the first ten residues might have an effect in changing the positioning of the peptide on the layer.

![Graph showing the isotherm of OxFmut+PA and WT+PA at 16°C at water subphase. The concentration of peptides are both 20%. The black curve represents the pure PA film, The blue curve represent WT1-25+PA, and the red curve represents OxFmut1-25+PA.](image-url)
The FM image is qualitatively similar to that of the 11-25 peptides +PA monolayer at the same temperature. The 1-25\textsuperscript{th} segment +PA monolayers have a higher density of nucleation at the same area per molecule than the 11-25th segment+PA. Since quantitative analysis was not taken out, further description was not available.

2. PA+WT 1-25 and its oxidation resistant mutant at water 28°C
The isotherms with adding either type of peptide do not make a very big difference in the shape of the isotherm. This time we have a even closer resemblances of the two curves. The only difference is the collapse pressure. The WT+PA monolayer has a collapse pressure of 38mN/m, which is lower than that of the Oxmut+PA monolayer (It has a collapse pressure ~ 44mN/m). The FM images have yielded no new special features. Both WT+PA and Oxmut+PA have a smaller nucleation than the pure PA film at the same area per molecule.

So this set of experiments have revealed that: 1. in general, the Oxmut is likely to be more effective to resist collapse; 2. The change in collapse area per PA molecules indicated that the positioning of the peptide at the interface might have changed.

3. PA+WT 1-25 and its oxidation resistant mutant at buffered subphase at 16°C
There are two reason for using buffered subphase instead of water:
1. To simulate the physiological situation better; 2. DTT can be added to the subphase to remove both inter- and intra-peptide sulphur bonding. The pure PA film will behave similarly to the pure water case. At collapse, there is no drastic fall of pressure corresponding to a catastrophic loss of material. Instead, from the FM images, we saw small fracture structure. It remained attached to the monolayer. It turned out such structure appeared whether the peptide is added. (fig 12)

The addition of peptides does not make much obvious difference in the morphology in this case. Isotherms (fig 13) with the WT+PA added, using subphase either with or without DTT, shows an increase in the liftoff area (~41-44mN/m). But the collapse pressure does not have an increase at all, and it is the same either with or without the DTT. It may be accounted by the invalidity of DTT in reducing the sulfur in the peptides.

It is interesting that, as OXmut+PA shows similar behavior to WT+PA in most regions, it gives a much higher collapse pressure.
2. PA+WT 1-25 and its oxidation resistant mutant at buffered subphase at 28°C

The phase behavior of pure PA film is like the pure water case. It follows a g-le-lc-sc sequence. The major difference is, at the collapse there are fractures visible under FM. (fig 14)

In general, the monolayer is fluidized by the peptide and isotherms of the peptide+PA monolayer were having a smoother curve(fig 15). In addition, collapse structure was observed no matter if the peptide is added.

The DTT addition was not affecting the behavior of the peptide. The isotherms of WT+PA, whether the DTT is added in the subphase, are nearly identical.

As the 16°C case, The Oxmut+PA showed different behavior from the WT+PA. The curve shape is different, most notably the greater fluctuations beyond collapse.

In short, it needs further confirmation to the difference of function in Oxmut and WT peptide.

Fig. 14. The formation of fracture structure (circled) in the PA films at 28°C at the buffer subphase. The structures’ size is much larger than the case of 16C.

Fig. 15 The isotherm of the Oxmut+PA and WT+PA at 28C at buffer subphase. The concentration of peptides is both 20%. The black curve represents the pure PA film, The deep blue curve represent WT1-25+PA using buffer without DTT, The Cyan curve represent WT1-25+PA using buffer with DTT and the red curve represents Oxmut 1-25+PA
**WT and G-Flex and PA**

There are 3 proline residues in natural SP-B 1-25th segment. As the proline is known to restrict the change of conformation in many situations, we would like to investigate if these residues have any impact on SP-B functioning. To study the importance of proline, the proline in SP-B 1-25\textsuperscript{th} segment is replaced with Glycine and we call the mutated segment G-Flex. 20\% by weight of G-Flex is added to PA to form the G-flex+PA mixture. The isotherms are taken with water subphase, at both 16 \textdegree C and 28 \textdegree C.

The mutation does not change the behavior of the peptide+PA film significantly. At both 16 \textdegree C and 28 \textdegree C, The isotherms of the G-Flex+PA films are very similar to the WT+PA film at high area region (fig 16 & 17). At low area region, the G-Flex+PA can stand a higher pressure. At 16 \textdegree C, the collapse pressure of G-Flex is 56mN/m. It is 8mN/m higher than the WT 1-25\textsuperscript{th} segment+PA.

However, the FM image looked similar. Before the liftoff, the monolayer is at fluid-gas coexistence, as the WT+PA is. But the gas phase domain was much smaller in size compared to the WT+PA monolayer. The contrast between the fluid and gas phase gradually vanishes along compression. When the monolayer arrives at the pressure equivalent to the kink in pure PA film, the monolayer become homogenous.

Fig. 16 The isotherm of the Oxmut+PA and WT+PA at 16C at water subphase. The concentration of peptides is both 20\%. The black curve represents the pure PA film, The deep blue curve represent WT1-25+PA and the green curve represents G-Flex 1-25+PA.
At 28°C, the similarity of the isotherms are also obvious, as the shape is nearly the same, and liftoff and collapse of G-Flex+PA appear at the same area as the WT+PA. The collapse area of G-Flex+PA is then 43mN/m, higher than WT+PA. However, there was an unexpected fluctuation of the pressure at the point of collapse. It may need further verification.

The FM image illustrated that the morphologies of G-Flex+PA is similar to the case of WT+PA, but there are smaller lc domains. (fig. 18)

![Fig 17 The isotherm of the Oxmut+PA and WT+PA at 28°C at water subphase. The concentration of peptides is both 20%. The black curve represents the pure PA film, The deep blue curve represent WT1-25+PA and the green curve represents G-Flex 1-25+PA](image)

![Fig 18(a.b) The comparison of G-flex+PA and WT+PA at similar area (~34Å²). The G-flex+PA (left,a ) have shown much smaller region of the lc phase than the WT+PA(right,b ) did.](image)

Discussion

From the experiments, we find:

1. Both SP-B 1-25 and 11-25 Oxmut are fluidizing the monolayer better than the natural sequenced peptides. However, using DTT subphase had failed to prove the sulfur bonding’s role in the peptide. Therefore, it may require
further study if the sulfur bonds in those residues is the reason for
difference in behavior of the systems.

2. The Proline in the peptide segment may not be helping the fluidization. It
was unexpected at the first sight. But as the series of the experiment is
using excess amount of Fluorescent dye, verification is needed on the
validity of the result.

3. The comparison of the area at which collapse occurs between segment of
SP-B 11-25 and SP-B 1-25 suggest SP-B 11-25 may take a different
positioning from the SP-B 1-25. Other methods investigating such
possibility, like X ray reflectivity, are needed. It may help us in
understanding the exact role of insertion sequence.

4. Further experimentation can be done on systems with more similar
composition as the Lung Surfactant. For example, the DPPC:POPG binary
mixture can be used as the model system.

Conclusion
Employing the Fluorescence microscopy and the isotherm measurement, we
discovered the SP-B peptide 11-25 th segment function is less effective in
fluidizing and in increasing the collapse pressure of the PA monolayer.
Moreover, we discover that the area per molecule at which collapse occurs is
larger for SP-B 11-25 segment.
Comparison between both SP1-25th and 11-25th natural sequenced peptide with
the corresponding Oxmls revealed that removing sulfur containing residues
increases the fluidization further. This holds for different temperature and
different subphase used. However, the role of the sulfur containing residues in
the peptide is not well proved in the series of the experiments, and need further investigation.
The experiments using the proline-removed mutant indicate the proline removal help the fluidization of peptide and increasing the collapse pressure.

Acknowledgment
I would like to thank everyone I knew in U. of Chicago, of course Prof. Ka Yee Lee and my project supervisor Dr. Josh Kurutz. I would also thank Ajaykumar Gopal, Yuji Ishitsuka, and Henry Lam and other Lee group members for their great help and I had great time living with them. Finally, I would thank CUHK Physics Department for giving me such a great opportunity to learn.