Preliminary evaluation of the capacity of Surface-Active Phospholipids to provide semipermeability in a saline filtration environment

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Running title: Semipermeability of Surface-Active phospholipids

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Summary

Background. Semipermeability to fluid transport is one of the principal attributes of a tissue like articular cartilage. Consequently, this characteristic can be exploited in attempts to understand the functional roles of the biological layer of Surface Active Phospholipids (SAPL) which form on its surfaces. A previous study, relevant to peritoneal SAPL was carried out in which hypertonic glucose solution was dialysed against physiological saline through SAPL membrane and concluded that SAPL possessed semipermeability. Our analysis extends this previous study by dialysing hypertonic and hypotonic saline solutions against physiological saline via SAPL membranes which is more relevant to the articular joint environment..

Material and Methods. Membranes were produced from either synthetic or bovine cartilage SAPL and used to carry out tests involving the dialysis of hypotonic and hypertonic sodium chloride solutions against physiological saline, using an Ussing chamber to hold both the membranes and dialysis fluids.

Results: The dialysis produced osmotic pressures which are commensurate with our experimental constraints, but strongly indicated that it is indeed possible to generated osmotic pressures using SAPL membranes, indicating the semipermeability of this lipid structure..

Conclusion: It is widely accepted that the collagen-proteoglycan membrane provides the semipermeability of articular despite the low levels of osmotic pressure recorded in our experiments, our results demonstrate that SAPL aggregation can constitute a semipermeable layer with a strong capability to contribute to the semipermeablity of the collagen-proteoglycan system especially on the surface of the tissue. Consequently its deficiency, as seen in osteoarthritis could lead/contribute to cartilage dysfunction.

Keywords: Surface active phospholipids; Cartilage; Osmosis pressure; Semipermeability; Hypotonic saline solution, osteoarthritis.

Background

Articular cartilage is covered by a thin layer of Surface-active phospholipids (SAPL) of microscopic thickness which is believed to contribute to lubrication[1] and load processing[2]. SAPL is known more commonly as "surfactant" in the lung, where it is produced by alveolar Type II cells in the form of lamellar bodies, which are secreted onto the alveolar surface[3].

Studies indicate that surface active phospholipids are also synthesized and secreted in other parts of the body[4-5], where its adsorption onto the surfaces of tissues at these sites has been demonstrated using techniques such as electron microscopy, epifluorescence microscopy and autoradiography[3, 6-8]. SAPL imparts highly desirable physical and physiological properties, including: reduction of surface tension[9], boundary lubrication[3], release (anti-stick) [10] and physical barrier formation[11].

The probability that SAPL, might contribute to the semipermeability of soft tissue membranes was proposed in one recent study[12] in an attempt to explain the cause of ultrafiltration (UF) failure in peritoneal dialysis (PD) patients. It was suggested that a deficiency of SAPL adsorbed to the surface of peritoneum could cause the partial or total loss of the semipermeable membrane on which hypertonic glucose solution relies in order to generate ultrafiltration. In a previous study[13], commercially available clinical dialysis fluids (hypertonic glucose solution) was dialysed against saline through a membrane made with either synthetic or human peritoneal SAPL. The results indicated that SAPL is capable of imparting membranous semipermeability. In this study the emphasis is on extending this previous work to the dialysis of hypotonic and hypertonic solutions against physiological saline through SAPL membranes.

It has been established that the surface of osteoarthritic human hip and knee articular cartilage is significantly deficient in SAPL relative to the normal healthy tissue[14]. Based on this finding, a clinical trial was performed in which a single injection of SAPL was delivered into one knee of 10 patients with bilateral moderate-to-severe osteoarthritis[15]. It was reported that a significant reduction in pain sensation occurred in these patients over the period of post-injection monitoring. In our opinion this finding underlies the importance of conducting

further studies on SAPL which will enable further insight into its characteristics and potential for the management of joint cartilage degeneration and disease.

In this present study we hypothesis that the ability of these phospholipids to adsorb to the surface of articular cartilage and thus contribute to the semipermeability of its proteoglycan-collagen membrane may be the reason for the effectiveness of the SAPL injection. We will test this hypothesis by evaluating the semipermeability of SAPL under conditions that are similar to those found in articular joints. To this end both hypotonic and hypertonic sodium chloride solutions will be dialysed against physiological saline (0.15M saline solution) using membranes constructed from commercially available SAPL.

Material and Methods

Materials

The synthetic SAPL used was dipalmitoyl phosphatidylcholine (DPPC), purchased from Lipoid GmbH (Ludwigshafen, Germany). Bovine cartilage SAPL was extracted from the articular surface of fresh bovine joints according to the protocol adopted in the previous study[14]. All chemical reagents (sodium chloride, chloroform, methanol, and acetone) were at least analytical reagent grade and were purchased from Ajax Chemicals (Auburn, New South Wales, Australia) or BDH Laboratory Supplies (Poole, U.K.). Saline (0.15M sodium chloride solution) was purchased from Baxter Healthcare (Old Toongabbie, New South Wales, Australia). Hypotonic sodium chloride solutions (0.0375M and 0.075M) were made by proportionally diluting saline with distilled water. Hypertonic sodium chloride solutions (0.3M and 0.6M) were made by dissolving sodium chloride powder in distilled water. The difference in sodium chloride concentrations between these sodium chloride test solutions and saline provided the concentration gradient for generating osmotic pressure.

Sample preparation

Bovine cartilage SAPL was extracted from the articular surface of ten bovine cartilage samples by a standard lipid extraction procedure[16]. The lipid solvent was chloroform:methanol (2:1), known as Folch reagent. During the collection procedure, soft facial tissues soaked with Folch solvent were used to wipe SAPL off from the articular surface. The contact time between solvent and articular surface at each of these selected areas

was all less than 10 second as our pre-test showed that this time frame did not cause any histological change or damage to cartilage tissues. After decanting the lipid fraction, neutral lipids were removed by precipitation of the SAPL in cold acetone according to the procedure described in a published study[17]. SAPL collected from ten cartilage samples was pooled together. Final removal of solvents was effected by evaporation under Nitrogen. The SAPL residue was weighed and re-dissolved in chloroform to give a solution of known concentration. Based on the experience obtained from our previous study[13] 54 µl of reconstituted SAPL solution was deposited on each side of a disc of Nylon filter paper (0.2µm, pore diameter) white nylon (Millipore Corporation, Bedford, MA, U.S.A.) that was used as the carrier to produce the SAPL semipermeable membrane. The solvent was removed by evaporation, and the weight deposited per unit area was recorded as the effective "thickness". Therefore in order to achieve effective membrane thickness of 2.36mg or 3.78mg, concentrations of 21.85 and 35.0 mg/ml reconstituted SAPL chloroform solutions were used respectively such that the SAPL residue covered an exposed area of 0.95 cm^2. Accordingly, synthetic DPPC was also prepared in chloroform to the same concentrations.

Measurement of osmosis

Osmotic pressure was generated by clamping a SAPL membrane prepared as mentioned above between the two compartments of an Ussing chamber (Jim's Instrument Manufacturing, Iowa City, IA, U.S.A.). The left compartment was always filled with saline and the right with the sodium chloride test solutions of different concentrations of solute. Osmotic pressure was measured as the difference in hydrostatic pressure of the compartments needed to stop further water transmission across the membrane. The total capacity of each compartment was approximately 0.7ml, and the contact area between the two compartments was 0.44 cm^2. For the various experiments, 2.36mg SAPL (DPPC or bovine cartilage surface SAPL) and 3.78mg SAPL (DPPC) were used. Two vertical tubes with inner diameters of 1.2mm were connected to the side of each compartment to measure osmotic pressure. Figure 1 illustrates the device.

At the beginning of each experiment, the fluid heights indicating pressure were set to be equal on both sides of the membrane. The whole device was maintained at 37^oC in a water bath, and the fluid heights indicating osmotic pressure difference (ΔP) were measured and recorded until no further movement of fluid was seen. At the end of each experiment, ΔP was recorded

as the difference in heights between the two fluid columns. Since both hypotonic and hypertonic sodium chloride solutions were used as test solutions that were dialysed against saline, the fluid height of the test solutions could be either lower or higher than that of saline at the end of dialysis. Therefore, a negative ΔP was generated if the fluid height of the test solution was lower than that of saline. Accordingly, when the fluid height of the test solution was higher than that of saline, the ΔP generated was expressed as a positive value. The mean and standard error of the mean (SEM) were calculated for each group of data points and the one-way ANOVA test was used for statistical analysis.

Experimental procedure

The experimental study was divided into three sections:

Section I (n=7): Measurement of osmotic pressure produced by dialysing saline against hypotonic sodium chloride test solution (0.075M) using a DPPC “membrane” of “thickness” 2.36mg.

Section II (n=5): Measurement of osmotic pressure produced by dialysing saline against hypotonic sodium chloride test solution (0.075M) using extracted bovine cartilage surface SAPL for the “membrane” at a “thickness” of 2.36mg.

Section III (n=8): Measurement of osmotic pressure produced by dialysing saline against sodium chloride test solutions of varying solute concentrations (0.0.0375M, 0.075M, 0.3M and 0.6M) using DPPC as the “membrane” at a “thickness” of 3.78mg.

Results

At the end of dialysis, hypotonic sodium chloride test solutions (0.0375 M and 0.075M) generated negative osmotic pressure and hypertonic sodium chloride test solutions (0.3 M and 0.6 M) generated positive osmotic pressure according to the definition described above. Specific results included the following features:

1) In each of the five runs using pooled bovine cartilage SAPL, 0.45% hypotonic sodium chloride test solution generated an osmotic pressure difference (ΔP) averaging –2.68 ± 0.9cm H_2O as shown in Figure 2.

2) For the same membrane thickness, synthetic SAPL (DPPC) was equally effective (ΔP= -2.63 ± 0.7cm H_2O) as bovine cartilage SAPL.

3) As shown in Figure 2, thicker membranes of DPPC (3.78mg thickness) produced a ΔP similar to that produced by thinner membranes (2.36mg thickness).

4) For the same membrane thickness (2.36mg thickness) and composition (DPPC), the osmotic pressure was almost unchanged at two hypotonic solute concentrations (0.0.0375M and 0.075M) and increased with two hypertonic solute concentrations (0.3M and 0.6M), as shown in Figure 3.

Discussion

It is well known that proteoglycans, being negatively charged and fixed within a collagen matrix, are the main components of a network that regulates osmotic pressure in cartilage. The reported osmotic pressure for articular cartilage is in the range of 0.02 to 0.2 MPa[18], which is several magnitudes higher than the value of $3x10^{-4}$ MPa measured in this present study. However, it is arguable that the real osmotic pressure from SAPL could be much higher as the inherent limitation is the capacity of our membrane system to accurately mimic the physiological condition in the joint could be have contributed to the low values measured as discussed below.

Firstly, our study used nylon filter paper as the carrier for SAPL; however, while this is convenient as a preliminary medium for carrying the SAPL layer, the chemical bond between the filter paper and SAPL is weak. Pre-tests showed that this membrane system was only stable for 24 hours. If the system was used for longer than 24 hours, SAPL would detach and migrate away from the surface of its nylon filter paper career resulting in destruction of the membrane system; as this process of disintegration of the membrane system progressed an associated decay of the osmotic pressure difference (ΔP) was observed.

Secondly, this tendency for the membrane to disintegrate could have limited the possible total that was diffused through the membrane to the chamber in which the pressure head was measured, i.e. if the pressure difference could have been higher if the flow of water through the membrane could have continued for a longer time. In other words, a higher osmotic pressure might have been achieved if it was possible to allow a much longer dialysis. An optimisation of this membrane system may be necessary before the true picture of osmotic pressure generated by SAPL can be established.

Though the osmotic pressures measured in this study are considerably smaller that those reported previously for articular cartilage, this work further demonstrates the capability of SAPL to impart semi-permeability onto surfaces to which it is adsorbed.

However, our study demonstrates that the membranes we have made from synthetic DPPC or endogenous SAPL (bovine cartilage) possess excellent semipermeability, expressed as ΔP,

the difference in head of water. These results are consistent with the findings from previous study[13], where it was proposed that SAPL adsorbed to the peritoneum imparts the semipermeability necessary to be part of a system that maintains ultrafiltration in peritoneal dialysis patients.

Although this study could not accurately measure the osmotic pressure generated by SAPL, our results still have important implications. Firstly, we were able to demonstrate that a level of osmotic pressure (ΔP) can be generated when dialysing hypotonic sodium chloride solutions against saline through a membrane of SAPL extracted from articular cartilage. This result indicates that SAPL could be an essential component to the membrane structure that is responsible for the semipermeability of the articular cartilage; and that the osmotic process those not only depend on the semipermeability provided by the proteoglycan-collagen membrane alone.

It has been demonstrated that the dominant component of SAPL is phosphatidylcholine (PC) which is capable of forming a closely-packed network serving as an effective barrier or membrane[12]. As illustrated in Figure 4a and b, PC contains a polar moiety made of a phosphate ion which is negatively charged, and a terminal quaternary ammonium (QA) ion which is strongly positively charged, rendering the whole PC electrically neutral. It also contains a non-polar moiety, made of two fatty acid chains. The PC molecules have the same cross-sectional areas in both polar and non-polar moieties, which enable them to pack together closely and form an effective membrane or barrier. These PC molecules can be strongly bound to a negatively charged solid surface through their positively charged QA ions, which leaves the negatively charged phosphate ions free to interact with any mobile cations (eg Ca^{++} or Na^{+}) that are commonly found in any biological fluids. Under the experimental conditions in the current study, the mobile cations such as Na^{+} are freely available. Therefore, the interspersion of Na^{+} and phosphate ions can pull the PC molecules together into a close matrix to impart strong cohesion to the adsorbed monolayer (Figure 4c). We believe that this very well structured matrix or network forms an effective semipermeable membrane which allows only the transport of water but not small ions like Na^{+} and Cl^{-} through the SAPL membrane.

It is our opinion from this present study that in cartilage, SAPL could strongly bind to negatively charged proteoglycans and become an important part of the whole semipermeability system in regulating water transport. The unique location of SAPL makes it even more important in maintaining the normal functions of cartilage. Also a SAPL lining on the articular surface may offer a possible front line defence to any physical or chemical attack, such that its deficiency would compromise or weaken the overall function of cartilage. A SAPL lining that covers the intracellular gaps may also be a necessity for the whole semipermeability system to be functional because proteoglycans may not be sufficient.

Conclusions

Our present study further supports the concept of semipermeability imparted by both synthetic and endogenous SAPL. This finding may be important as a SAPL lining on the articular surface could offer in addition to its role as boundary lubrication, the role of regulating osmotic pressure as an important part of the whole semipermeability system. Further studies are needed to explore the implications of these findings to those clinical issues associated with a deficiency of SAPL.

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Figure 1

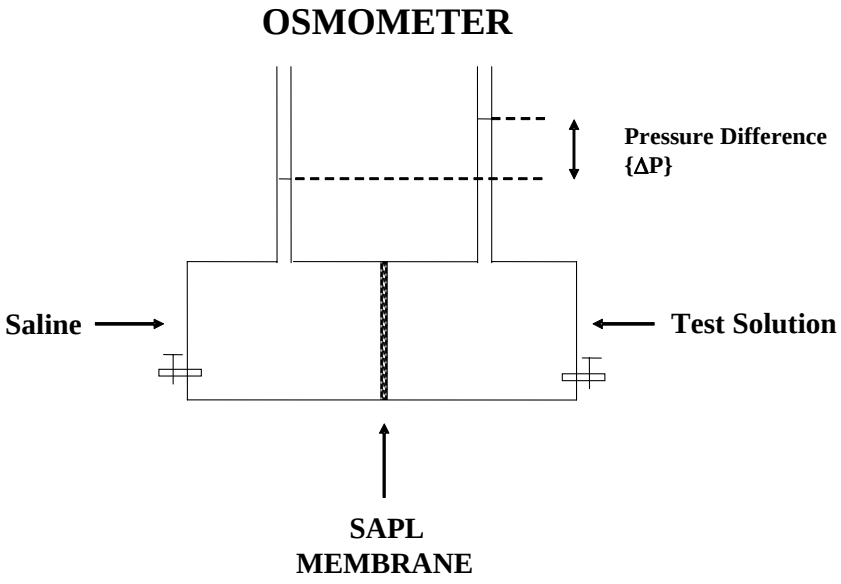

OSMOMETER
Pressure Difference
{ΔP}
Saline
Test Solution
SAPL
MEMBRANE

Figure 2

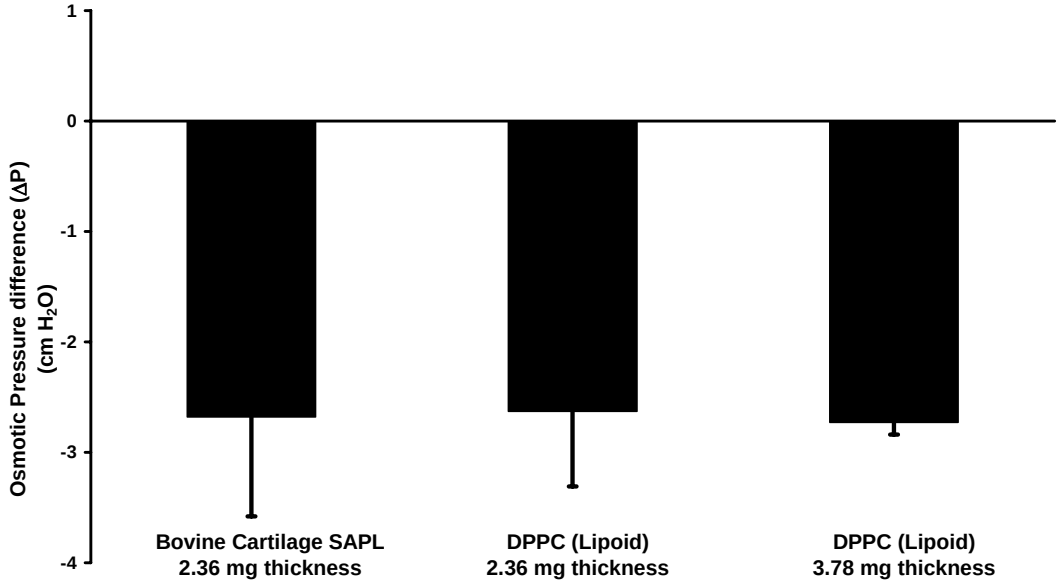

1
0
-1
-2
-3
-4
Osmotic Pressure difference (ΔP)
(cm H2O)
Bovine Cartilage SAPL
2.36 mg thickness
DPPC (Lipoid)
2.36 mg thickness
DPPC (Lipoid)
3.78 mg thickness

Figure 3

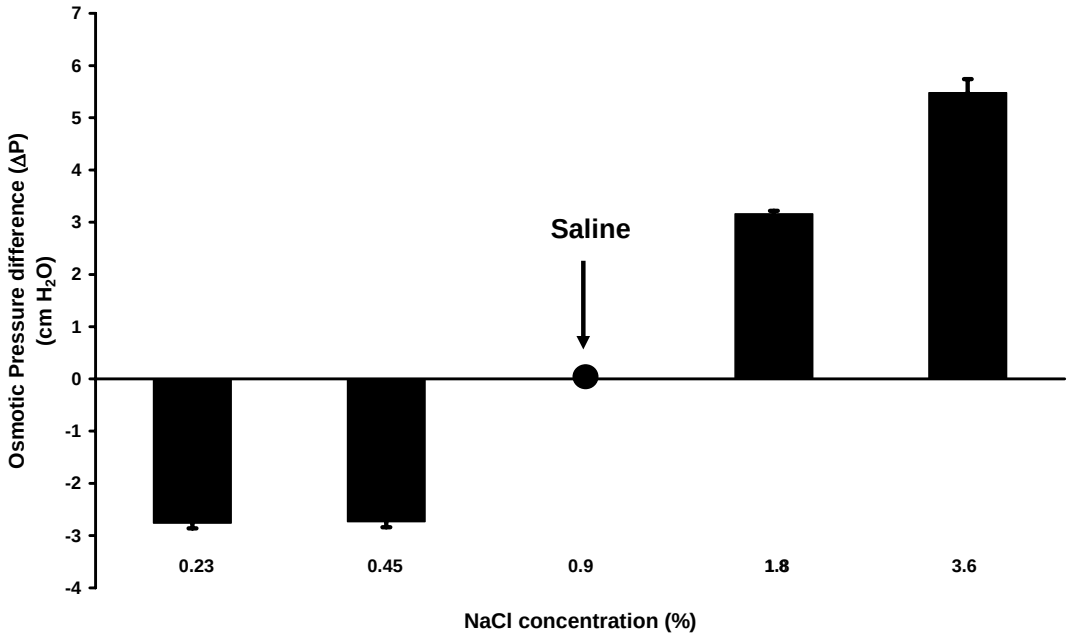

Osmotic Pressure difference (ΔP)
(cm H2O)
7
6
5
4
3
2
1
0
-1
-2
-3
-4
Saline
0.23
0.45
0.9
1.8
3.6
NaCl concentration (%)

Figure 4c

4a

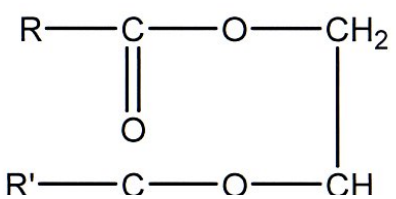
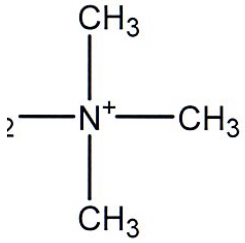
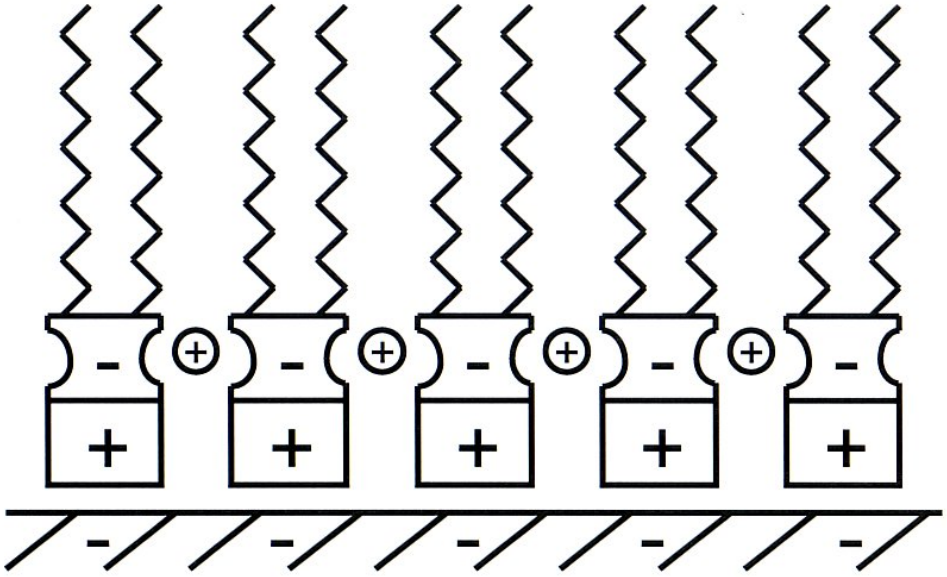


Figure 4 a, b



4c

⊕ **Mobile cation**



Close packed fatty Acid chains

Solid surface (negatively charged)

ɔiety with a
te ion and a
ammonium ion

Figure Legends:

Figure 1 Illustration of the osmometer in which the "membrane" is clamped between the two compartments of an Ussing chamber in which the test solution is "dialysed" against saline. The osmotic pressure difference (ΔP) is measured as the difference in hydrostatic pressure of the two compartments needed to balance ΔP.

Figure 2 Mean osmotic pressure difference (ΔP) generated by three "membranes" of bovine cartilage surface-active phospholipid (SAPL) using 0.075M hypotonic sodium chloride test solution against saline, reaching statistical significance ($P < 10^{-8}$). Also depicted are ΔP values for DPPC (a synthetic SAPL from Lipoid Germany) at the same membrane thickness (2.36mg) and sodium chloride gradient. It is further shown that a thicker (3.78mg) membrane generated a similar ΔP for the same (0.075M) sodium chloride driving force.

Figure 3 Osmotic pressure difference (ΔP) generated after "dialysing" four sodium chloride test solutions against saline through a DPPC membrane of 3.78mg thickness. Hypotonic sodium chloride test solutions (0.0375M and 0.075M) generated negative ΔP while hypertonic sodium chloride test solutions (0.3M and 0.6M) generated positive ΔP. Also depicted is the increase in osmotic pressure with sodium chloride driving force.

Figure 4 The chemical structure and activity characteristics of surface active phospholipids (SAPL); (a) chemical structure, (b) schematic representation of the SAPL molecule, (c). Typical surface film network architecture of SAPL.