Two sides of the coin. Part 2. Colloid and surface science meets real biointerfaces

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Abstract

Part 1 revisited developments in lipid and surfactant self assembly over the past 40 years [1]. New concepts emerged. Here we explore how these developments can be used to make sense of and bring order to a range of complex biological phenomena. Together Part 1, this contribution is a fundamental revision of intuition at the boundaries of Colloid and Science and Biological interfaces from a perspective of nearly 50 years.

We offer new insights on a unified treatment of self assembly of lipids, surfactants and proteins in the light of developments presented in Part 1. These were in the enabling disciplines in molecular forces; hydration, oil and electrolyte specificity; and in the role of non Euclidean geometries—across the whole gammut of physical, colloid and surface chemistry, biophysics and membrane biology and medicine.

It is where the early founders of the cell theory of biology and the physiologists expected advances to occur as D'Arcy Thompson predicted us 100 years ago.

Keywords

anaesthesia, membrane(s), cubosome(s), surfactant(s), lipid raft(s), self-assembly, lipid(s), curvature, packing, hydration, specificity.

PART 2 - COLLOIDS AND SURFACE SCIENCE MEET REAL BIOINTERFACES

1. Introduction

It is exactly 100 years since the publication of D'Arcy Thompson's book *On Growth and Form* [2]. We learn from him that Kant said of the chemistry of his day and age that it was a science, but not Science – "eine Wissenschaft, aber nicht Wissenschaft" - for that the criterion of a true science lay in its relation to mathematics.

On the other hand Auguste Comte said that if chemistry ever were to rely on mathematics it would cause a rapid degeneration of that science. Whence these two philosophers derived their authority is unclear. Neither ever did an experiment. Kant spent his whole life walking around Königsberg which must have been awful for him, and Comte was a social scientist. The quotations may be out of context. But the opposing views serve to focus the mind on a venerable issue posed by Thompson: How to bridge the divide between the physical and biological sciences?

Again, from Thompson, Emile Du Bois-Reymond said 100 years after Kant that chemistry would only reach the rank of science when – in modern language – it derived its laws from statistical mechanics, using molecular forces.

In Part 1 we outlined new concepts in lipid and surfactant self assembly over the past 40 years [1]. Here we apply these to make sense of some biological phenomena.

2. A Miscellany of Interactions of Surfactant, Polycation and Lipid Membranes

The complexity of real biological membranes - rather than membrane mimetic lipid models - is obvious. However some inferences on mechanisms of action can still be drawn from the latter.

Some provide useful hints for drug applications. Some examples:

2.1. Bacteriocidal action of Cationic Surfactants

Cationic surfactants as bacteriocides have wide usage, from hospitals to households - toothpaste, washing detergents, skin care, eye lubricants and for a time even spermicides! Their action can be understood in terms of changes in membrane interfacial curvature induced by their adsorption. At the cmc (in physiological saline) the single chained surfactant ($p \sim 1/3$) adsorbs into the double chained lipid membrane ($p \sim 1$). The mixed surfactant then has $p_{\text{effective}} < 1$. The membrane is destroyed [3].

The argument is not sufficient. If that were the whole story then an anionic or non-ionic surfactant of the same (hydrophobic) chain length could also do the job just as well. They do not. The example in Part 1 on sulphate DDAB microemulsions shows explicitly that the hydration of sulphate ion and that of the didodecyl dimethylammonium terminal group are incompatible [1,4].

Since the terminal groups of a phosphatidylcholine lipid is much the same as DDAB, a common surfactant like SDS can not adsorb. Similarly for nonionic surfactants. The head group hydration is just not compatible.

This difference in specific hydration seems at the root of the superactivity of sulphate in the common enzyme horseradish peroxidase [5]. The toxicity of specific ions like La(III) and Co(III) and their much different toxicity can be traced to similar lipid head group hydration interactions [6].

2.2. Immunosuppression of Cationic Surfactants

At concentrations far below the cmc, cationic surfactants behave as potent

immunosuppressants. This discovery in 1981 was very surprising. Since the surfactants are so widely used in first world countries the implications are as enormous and ignored. They are more effective as immunosuppressants than the once used drugs of choice for organ transplants. The mechanism has been identified and confirmed in *in vitro* mixed lymphocyte culture experiments, *in vivo* mouse thyroid rejection studies and in human models.

Here is how they work: Below the cmc the surfactant does not disrupt the membranes of T-cells that trigger the immune response. Uptake and its chain length dependence follows a Langmuir isotherm. That is, the process is physical chemistry.

After adsorption the cationic surfactant flips over to the inner side of the membrane which contains anionic phosphatidylserine lipids. The cationic surfactant neutralises its negative charge. Consequently the membrane bound calcium drops. Calcium is crucial to structure of the major histocompatibility transmembrane protein complex responsible for recognition of antigens. The immune response is switched off [7,8].

2.3. Local Anaesthesia

Another example that illustrates the interplay between lipids and an actual medical response is the action of local anaesthetics.

The most typical is lidocaine and similar molecules. They have a large bulky cationic head group and a relatively short hydrophobic portion. When injected in excess in a localised region, the law of mass action forces the hydrophobic tail to anchor in the the lipid region of a nerve cell.

This changes the membrane surface charge and its potential. So the propagation of an electrical signal is switched off. After a time the lidocaine leaks out.

This picture is oversimplified. But basically that is it. The hydration of the bulky head group has to be compatible with that of the membrane phospholipids.

Similar effects are achievable with cationic surfactants, with some venoms, in snakes, jellyfish, toads, and octopuses that use (polycationic) polymeric poisons [3].

2.4. General Anaesthesia and Alkane Poisoning

To anticipate the more detailed intricate account of section 4 on this problem we refer to a potted history of earlier ideas on the topic [9]. From the perspective of a physical chemist, or an intelligent physiologist the literature ideas are crude.

Conceptually simpler ideas of how these phenomena occur emerge in the light of recent developments in self assembly. The key to it is this:

As discussed in Part 1, just as there are many phases of lipids possible in three dimensions, so too there are in two dimensions (mesh phases). This is so also for the two dimensional world of biomembranes.

Par excellence is this so for lipid-protein-cholesterol mixtures that form real membranes [10]. Transitions from one form to another occurs with extravagant ease. That is, not too much energy is required to trigger such transitions. Such lipid transitions (section 4) are integral to the transmission of the nerve impulse.

That such lipid-protein-cholesterol phase transitions *must* occur follows if admit cubic and mesh phases (noneuclidean geometries) into the pantheon of allowed phases [11]. This is new. Literature discussion of mechanisms of how general anaesthesia operate ignore these structures. So the literature ignores the source of the mechanism it is trying to explain. It is therefore incomprehensible [12,13].

The modern view envisages anaesthesia as a process where the active molecule accumulates in the hydrophobic region of the neuronal membrane, directly interacts

with the membrane lipids, and alters the fluidity, the curvature and phase structure of the bilayer. Eventually this change affects the conformation and functional performances of ion channels, receptors and more in general of transmembrane proteins [13-18]. In 1988, Larsson first introduced this hypothesis of an anaesthetic induced change of the membrane phase state. The hypothesis implied that the transmission of the nervous impulse involved not just ion channels. It was rather driven by reversible lipid phase changes that propagated, coupled to, and triggered opened and closed ion channels. So an inhalation anaesthetic- induced lipid phase change would be too extreme to allow the functioning of ion channels [3,19].

A moment of reflection gives that hypothesis support. There was no other means by which channels opened and closed except by Hodgkin–Huxley equations that simply characterise the process, but say nothing of mechanism. We later deal with later presently popular refinements proposed to control the effects of anaesthetics.

The origins of anaesthetic action may well be manyfold, but such a conclusion ignores the lipid phase changes that physics demands must be integral to the process [20].

We can go further. For in any case lipid rearrangement *i.e.* a change in the curvature (surfactant parameter, p) of the bilayer must be induced by the uptake of a hydrophobic anaesthetic. This does not conflict with a modification of the hydration of the lipid head groups. Indeed it is a necessary consequence [9].

For example, lidocaine hydrochloride changes the phase transitions of lipid dispersions, by inducing a lowering in the transition or pre-transition temperature in DPPC or DHPC bilayers [21]. This effect depends essentially on the hydrophobicity of the anaesthetic. Adsorbed into the lipid bilayer it induces the corresponding change [9,22].

One outstanding issue is that xenon is a most effective anaesthetic, widely used in the Netherlands [23]. Pauling suggested that its potency was related to the formation of clathrates [24]. Why, he did not say. Possibly since xenon has a high polarisability (its static polarisability is larger than 4 $Å^3$ [25]) and therefore can interact via dispersion self free energy to partition into the lipid hydrocarbon chains. That could probably be established by looking up its solubility in oils. Recently, it was shown that xenon can modify the domain structure in "lipid raft" membranes [26].

If opening and closing of channels, propagation of the nervous impulse are coupled to lipid phase changes, it is of no consequence whether membrane phase state is perturbed by adsorption of an anaesthetic molecule at the hydrophilic or hydrophobic side, of the interface. Both will change curvature. So the debate is empty. Pauling was probably right for xenon as patients have little side effects.

When the anaesthetic is at least partly polar, its activity has been supposed to be at the water-membrane interface, and its specific action mediated by the release of hydration water molecules that are strongly bound to the polar head groups [27].

This is obvious. Membrane curvature and lipid phase state changes if hydration changes. That effect has been confirmed by spectroscopic experiments on halothane (F_3C-CBrClH), a typical inhalatory partially polar anaesthetic (the dipole moment is about 2 D [28]), in lamellar phases of DMPC [29,30].

A most dramatic simple illustration of lipid phase changes induced by anaesthetics is illustrated by isoflurane (F_3C-CHCl-O-CHF_2). After exposure to the gas, the isoflurane molecule is taken up in the hydrophobic region of the membrane and swells the hydrophobic core. This means the required lipid transition for conduction of the impulse is no longer possible. Fluorocarbons and hydrocarbons are structurally incompatible [31,32]. So after a time the fluorocarbon is released and the membrane

goes back to its normal resting state.

This can be seen with a simple experiment (K. Larsson and B. W. Ninham, unpublished results): Bubble isoflurane gas through a suspension of phosphatidylcholine multilayer vesicles. (They appear as a white emulsion and are easily made by sonication). After a few minutes the suspension transits to a clear viscous cubic phase which proves the point. If left for 15 minutes the isoflurane which resides in the hydrophobic membrane core comes out. The system returns to its original state.

What can be drawn from that experiment is inevitable.

The change in lipid bilayer curvature and phase state induced by adsorption of the anaesthetic can not be disputed. If a corresponding change does *not* occur in real biological membranes one has to ask why. It is clearly the key factor underlying the phenomenon.

The structure of nerve membranes is much more complex than an assumed lipid bilayer, for example a multilayer for which there is some evidence [33]. But the same conclusion on coupling of lipids to channels remains.

The same accounts for the invidious effects of octane and other hydrocarbons on the brain (petrol sniffing). Hydrocarbons are miscible with lipid chains and impossible to remove. The “high” achieved by sniffing analogous to many other recreational drugs induces a permanent state of idiocy or sub optimal brain performance [34].

Again, the perturbation of the interfacial hydration layer in the biological membrane may result in conformational changes of the membrane proteins.

3. More Structural Changes Inside Lipid Bilayers

Some other biological and physiological functions and their linkage to physical chemistry will now be discussed. For these functions it will again be clear that molecular reorganisations in the membrane hydrophobic regions that dictate structures and phase transition mechanisms together determine function. One essential factor here is the relation between the rigid cholesterol skeleton to the liquid-like disordered hydrocarbon chains of the bilayer. Another trigger of structural changes lies with the bilayer spanning peptide chains of membrane proteins. These are usually in an α-helical conformation with hydrophobic side chains. Very minor conformational changes of the hydrocarbon chain geometry can trigger transitions into periodically curved (mesh phase) bilayers.

3.1. Cubic Biomembranes

A major inhibition to progress in bridging the physical–biological sciences divide has been a dispute for over 40 years on the complex structures of lipid phases. The French School under Luzzatti vigorously advocated a set of structures that was impossible.

The definitive proof of the existence of cubic bilayer phases, a then completely new concept in structure, emerged from x-ray studies on the monolein-water system [35]. These structures are periodically curved bilayers with zero average curvature. (They are termed cubic phases because their symmetry is the same as that of ordinary cubic crystals) [3,11]. They are quite unlike asymmetrical lipid vesicle (or multibilayer) liposomes. Here the outer normal curvature of a bilayer is positive. The inner curvature is negative (see Section 2.3 of Part 1) [1]. In cubic phases the inner lipid frustration depicted in Figure 2 of Part 1 is relieved for cubic phases by having lipids arranged so that curvature in one direction cancels that in the orthogonal direction everywhere. The net curvature is everywhere zero, *i.e.*, the same as that of a planar

bilayer. The gaussian (product of normal curvatures) varies continuously over the surface. The number of such bicontinuous (non Euclidean) geometric shapes possible is very large [11,36]. They are now known to occur in solid state physics as surfaces along which electrons transit. The most well known structures occur with zeolites. They are hard to recognise with lipids because their symmetry allows misidentification without exceptional care, *e.g.* as hexagonal phases. Similar, myriad such structures occur in Nature in symmetric and asymmetric regular two dimensional arrays, *e.g.* in the bark of trees and drying of clay surfaces [36,37].

Their distinguishing special characteristic is that they can transform from one form to another with extravagant ease by cooperative rearrangements.

These surfaces, the P (primitive), D (diamond) and G (gyroid) are illustrated in Figure 1.

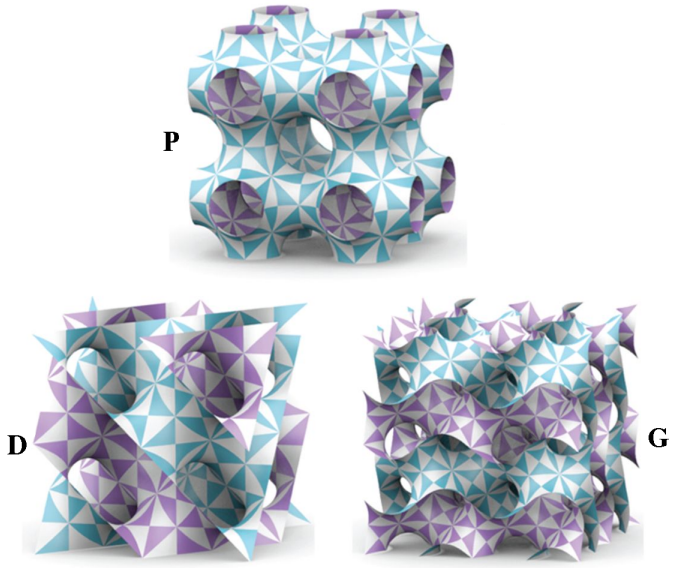

Fig. 1. Schwarz' P (primitive), D (diamond) and G (gyroid) surfaces. The blue and purple colours show the two distinct channels of the surfaces. Reproduced from Ref. 38 with permission of the International Union of Crystallography, Copyright 2013.

The aqueous monolein system exhibits two cubic phases.

There are three periodic minimal surface cubic phase structures. They are the gyroid (G), the diamond (D) and the primitive (P) phase. They correspond to FCC, BCC and

simple cubic packing of spheres. If we like they can be envisaged as the dual of these sphere packings with the bilayer wrapping around the space between the spheres. The gyroid structure is formed above the swelling limit of the lamellar liquid-crystalline phase, and at increased water content, the diamond type of phase is formed as shown in Figure 1.

The third type of cubic phase, described by the primitive minimal surface, has been observed in a range of lipid systems incorporating proteins, *e.g*. the monoolein-water system with cytochrome c at high water content [39] and lysozyme in monoolein/distearoyl phosphatidylglycerol mixed systems [40]. The incorporation of a protein into the aqueous channels of a bicontinuous cubic phase induces both changes in the head group region and a higher conformational order in the acyl chain region [41]. The same cubic phases occur in the ternary phase diagrams of cationic surfactant-water-oil microemulsions systems discussed above [42]. The most common structures with oil-water-surfactant mixtures are ubiquitous, disordered and bicontinous; if we like disordered cubic phases of constant, not zero, average curvature. An important work relevant to our discussion of lung surfactants below is reported in Ref. 43. The transition in microstructure from disordered (or ordered) monolayers of surfactants dividing oil and water to bilayers involves complex topological rearrangements unravelled in that paper.

Many other papers dealing with the matters of this section are collected together in Ref. 44. See also Refs. 11 and 36.

3.2. Cubosomes

Monoolein, a single chained lipid used as a model system to study phase behavior, is widely exploited for drug delivery. A phase transition from a closed multilamella state

to an open bicontinuous cubosome can take place with change in temperature or physico-chemical conditions in a patient. A drug can be incorporated and delivered in the lamella liposomes. Then the transition to an open bicontinuous state (a cubosome) is ideal for *in situ* delivery [37]. This technique was developed by Kåre Larsson. Colloidal particles of the (bicontinuous) cubic phase, termed *cubosomes* [38], can easily be made for optimal delivery. The cubosome consists of several unit cells with a closed structure formed by a single curved lipid bilayer. The inside space is separated by the bilayer to form two separate systems of water channels.

Archaebacteria are found under extreme conditions, like the base of underwater volcanic mountains, where they grow at high heat, large salinity and at very low pH. Their membrane consists of chains with methyl branches instead of double bonds. There are ether-linked polar head groups at each side of the two chains without any end group opening in the middle (bolaform surfactants) [45]. Luzzati with coworkers have shown that these membrane lipids form cubic phases under physiological conditions [46].

An ultrastructure that resembles a cubosome was described early on by Gunning from electron microscopy analysis. It is formed by the thylakoid membranes. They represent a vegetative state of thylakoid membrane stacks termed prolamellar bodies in plants. They form, for example, during winter without photosynthesis. The structure was shown to be consistent with the diamond type of cubosome [47]. Later Yuru Deng discovered a similar vegetative state in mitochondria of amoebae. It was found to be formed reversibly at starvation [48].

Evidence for cubic membranes as possible functional states of biomembranes were reported in several ultrastructural descriptions in the literature [49]:

1. The dendritic organ grandular cells.

2. The double membranes in some Gram-negative bacteria.
3. The Gram positive *Streptomyces hygroscopicus*.
4. The inner and outer membranes of secreted milk fat globules.

A large number of cytomembranes exhibiting cubic membranes have been identified [11,50]. Most involved diseased conditions.

Present opinion is that it is unlikely that a symmetric bilayer can exhibit an active single-bilayer structure (cubosome) in biological systems. Membrane asymmetry is supposed to be critical for a functional membrane. The asymmetry is maintained by a phospholipid transferase. This maintains the serine group of the phosphatidylserine head group exposed towards the inside. When serine occurs at the outside, it indicates that the transferase is not working, and this is a marker for apoptosis (programmed cell death) [50,51]. (This "accepted" explanation of membrane asymmetry, maintained by an enzyme, begs the question of how it works). If in such a situation symmetric membranes occur, however, it may be that they provide the least biological disturbances if they are condensed into the different cubic membrane assemblies identified so far [11,50]. The opinion contrasts with the existence of *Archaebacteria* and extremophiles.

A different point of view emerges from the considerations of Section 2.3 Part 1 on vesicles.

There we saw that with either single walled or multilamellar liposomes the curved membrane is necessarily asymmetric. Below a certain radius, typically 100 nm for most lipids, the curved bilayer is forbidden by geometry. The interior can however easily collapse into a cubic phase. The whole structure, a state of supraself-assembly, a bicontinuous cubic organelle surrounded by a protective layer of bilayers is a

necessary consequence of local packing conditions of lipid chains. If such structures *do not* form one has to ask why they do not.

The channel sizes of simple – one component - lipid cubic phases are typically around 3 nm. Those water channels for most cubic membrane assemblies observed in living systems are much larger, around 100 nm. As for cubic phases it is very challenging to obtain lipid based cubosomes with a channel diameter larger than 10 nm. This can be achieved by adding cholesterol and charged lipids [52]. With the large channel objects this indicates that they might be formed by a mechanism like double diffusion, Gibbs–Marangoni hydrodynamic processes, but in biological systems there are also protein components that promote the formation of larger channel sizes.

A less complex explanation for the formation of large channel aggregates might be seen if we consider the microemulsions of Part 1. There we saw that bicontinous structures of widely varying size form as a result of global packing constraints. But water structure (hydration) features as a hidden parameter.

3.3. The Lung Surface

A prime example of bilayer structural transformation induced by molecular rearrangements *inside* the bilayer is the mammalian lung surface. The lung surfactant is a lipid-protein complex with about 90% lipid and 10% protein. This bounds the surface of the *alveoli*. It forms an organised coherent phase. The structure is remarkably similar to the minimal surface membrane assemblies above. The *physical* properties of this state of self organisation has direct implications for lung function and pathological conditions involving the alveolar surface.

The lipid fraction of the lung "surfactant" is dominated by dipalmitoyl-phophatidylcholine (DPPC) with phosphatidylglycerol (PG) as the second most

abundant component (DPPC:PG ratio 9:1). In addition it contains about 10% cholesterol [53]. There are two hydrophilic proteins in the surfactant. These are termed SP-A and SP-D. Then there are two extremely hydrophobic proteins; SP-B and SP-C [53]. The proteins are essential to lung function. Numerous ultrastructural studies had indicated some organisation in the subphase. A common observation was that of a periodic arrangement of planar layers. These were assumed to be lipid bilayers. Accompanying that apparent arrangement were bilayers along two perpendicular directions. This texture was labeled tubular myelin. It had been assumed to form a depot for a monolayer at the air/water interface. The addition of different combinations of the constituent proteins SP-A, SP-B and SP-C to extracted lung phospholipids have been studied. They showed that a combination of SP-A and SP-B is needed in order to form tubular myelin.

The conditions that the lung surface has to satisfy are stringent. It has to rearrange and expand vastly, in the process expelling water and CO_2. It then takes up oxygen at essentially zero work, and folds back. It repeats this several times a minute and does so for the decades long life of an individual.

For 50 years the accepted model of the lung surface was a lipid monolayer that sits above an aqueous subphase. The monolayer interfacial tension of such a model would be far too large by orders of magnitude. Despite this, most research groups in the field played with monolayer models from surface chemistry. The irrelevance of such studies did help in acceptance of the actual surface *phase* model of the lung surface.

Here is the story: The cubosomes of cell biology (Section 3.2) have membranes that are complex mixtures of lipids and proteins. The dimensions of the water channels, typically 500-1000 Å, are much larger than water channels (about 30 Å) formed by membrane mimetic lipids. The latter, lipid-water mixtures alone form bulk

equilibrium phases. These occur simply as a result of the requirement that local curvature (molecular packing) and global packing constraints have to be satisfied. Cubic membrane assemblies probably form as a result of double diffusion gradients set up by steady state biochemical processes. They are evidently involved in cell fusion and biochemical traffic, where bicontinuity confers clear, essential advantages. Such structures are obviously ideal candidates to do the lung surfactant job. In 1999 it was reported that the lung "surface", the "air/water interface" of the alveoli, is formed by a surface phase organized into a periodic minimal surface structure formed by the surfactant lipid bilayer–protein mixture (see Figure 2) [54,55].

This triggered off studies embodied in a 2008 review by Pérez-Gil [56]. He concluded that there are different surface phases that form the alveolar surface. See also Refs. 57 and 58 for later work.

The alveolar surface phase first reported by Larsson *et al*. [59] (see Figure 2) was revealed by cryo-TEM studies of freshly opened alveolar surfaces of rabbit lungs, by transfer of the surface layer to the grid and immediately freezing the structure (without ice crystal formation) [59].

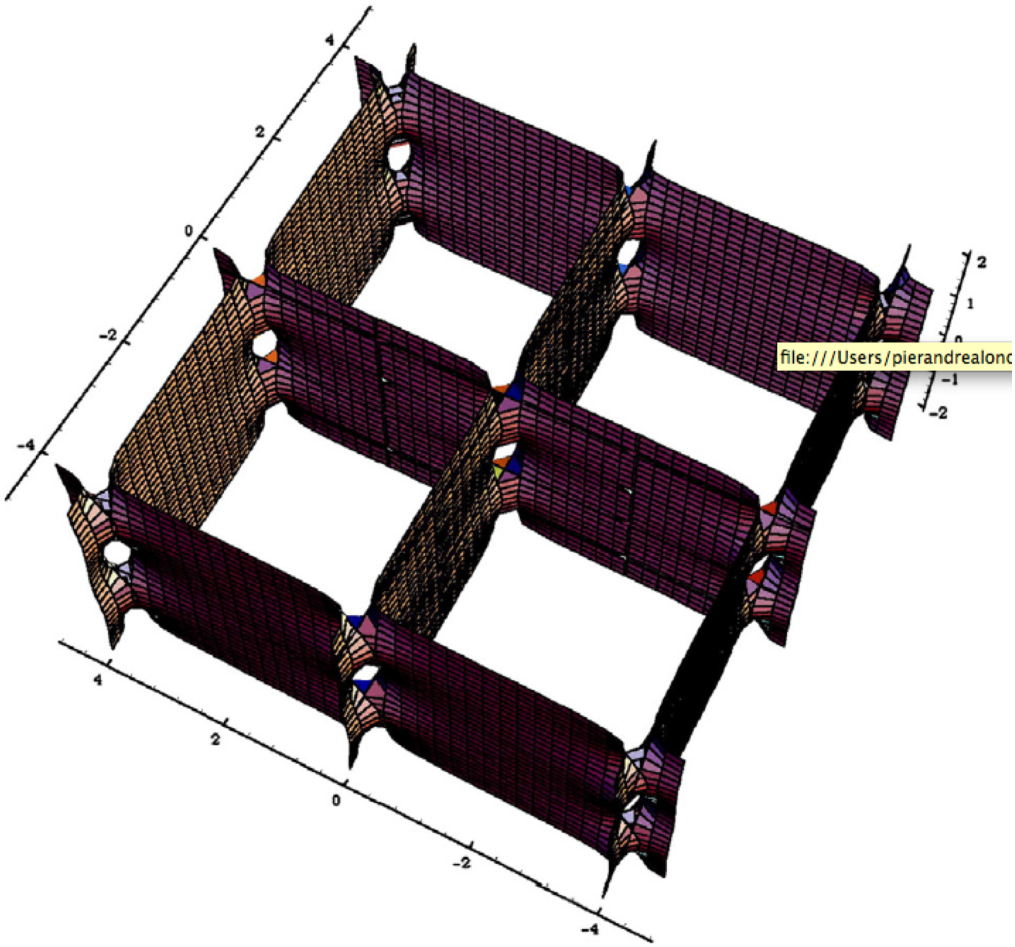

Fig. 2. The first reported complex bicontinuous structure formed by lung surfactants. Reprinted from Ref. 60 with permission from Elsevier. Copyright 2014.

The dimensions were in agreement with a well-known ultrastructure in the alveolar region termed tubular myelin (TM). It had been assumed to consist of two intersecting stacks of bilayers forming a tubular network. This was regarded as a storage silo of surfactant bilayer and the water-soluble protein SP-A. The observations from cryo-TEM showed how a uniform and coherent phase could form by a single bilayer without self-intersections based on a known periodic minimal surface lipid bilayer termed CLP (crossed layers of parallel) minimal surface. The structure is shown in Figure 3.

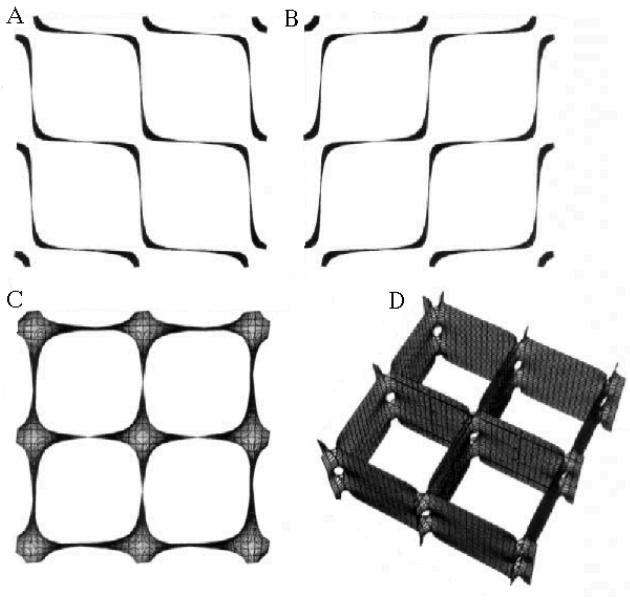

Fig. 3. The bilayer of TM/CLP is seen in two adjacent cross-sections (A and B), these cross-sections overlapping (C), and finally in a calculated structure (D) of the corresponding minimal surface (using Mathematica and adopting the nodal surface approximation). Reproduced from Ref. 59 with permission of Springer. Copyright 2002.

The driving force behind the transition from the multilamellar surface phase and CLP/TM is a tendency of the water soluble octadecameric protein SP-A to associate (perhaps electrostatically) with the SP-B complex. Two SP-A octadecamers are associated at their collagenous ends and such dimers span the diagonals of the tubuli.

The possibilities of other minimal surface conformations have been analysed and only one other alternative appeared possible: a tetragonally deformed cubic P-surface [60]. However, mainly due to its mechanical properties, it seemed a less likely candidate. The three-dimensional character of the TM/CLP structure and its ability to be viscoelastically deformed provides a mechanism behind reduction of the work required for breathing. The physiological aqueous lipid-protein lining of the alveoli forms the lung "surfactant" region that controls the transport of O_2 in from, and CO_2 out to the air. Based on model studies it is tempting to assume the presence of microbubbles of carbon dioxide on expulsion as such bubbles are inhibited from

fusing at physiological salt concentration [61]. The inhomogenous nature of the boundary region may itself be the source and sink of the rearrangements in structure that allow diffusion. Calcium ion is known to interact with the phosphatidylcholine head groups. This can change curvature and unzip the folded bilayer pegged together by the proteins so exposing hydrophobic monolayers for oxygen uptake. The mechanism by which such a topologically difficult process can occur has been elucidated in the parallel case of tetradecane-water emulsions (see Part 1) by Hyde *et al*. [43].

3.4. Lipid Rafts and Caveolae

A lipid molecular packing transition is induced in phospholipid bilayers by cholesterol. In a neutron diffraction study of DMPC-cholesterol aqueous phases, Mortenssen *et al*. in 1988 reported that it induces a unique kind of phase transition within the bilayer. The result is ordered strips that are cholesterol-rich. These alternate with cholesterol-poor less ordered strips. These are called L(o) and L(d) respectively [62]. Such microsegregation is a general phenomenon of statistical mechanics for solids and liquid crystals. It is a property of alloys and occurs for any two component system [63].

Another topographic alternative is the formation of islands of L(o)-conformation of the bilayer within an L(d) environment. These so-called “lipid rafts” are regions where membrane embedded proteins are localized. Such regions might also be curved into so-called *caveolae* (little caves) due to special proteins present.

The phase separation of cholesterol-rich regions L(o) for DPPC, one of the major lipids in lung surfactants, starts at about 8% mol/mol cholesterol in the bilayer, and the two-phase region ends at about 20% mol/mol cholesterol [64]. This segregation

and X-ray diffraction effects at physiological temperatures have been demonstrated in lung surfactant bilayer systems [55,65].

The phenomenon will reappear in connection with the nerve signal conduction in Section 5.

3.5. Fats, Oils, Phospholipids and Digestion

Phase transformations in lipids are central to gastrointestinal digestion. The processes involve absorption from emulsions - via micellar solutions - to lipoproteins in the circulation. Oil and fat components work with these lipid phase transformations and bile salts via global packing changes to produce a remarkably efficient machinery. Bile salts are very different kinds of surfactants and different from lipids in possessing very rigid structures. The field is so vast and technical that we leave this story to Refs. 66-70.

4. Signaling Systems within the Bilayer

4.1. Axon Pulse Propagation, Synaptic Chemical Signal Transfer

Axon Pulse Propagation

The classical (1952) theory of electrophysiology for nerve signal conduction is due to Hodgkin and Huxley [71]. It “explained” the local positive exterior electric charge of the axon membrane and its progression by the opening of the sodium channels. And it explained the relaxation to negative resting surface charge outside the membrane by sodium and potassium ion gradients. At least it provided a mathematical characterisation of the process. This became core dogma.

Astoundingly, no mechanism was advanced until 2005 [72]. The “soliton wave theory” couples phase transitions known in lipid bilayers to opening and closing of ion

channels [73]. It also takes account of cooperative membrane protein localization into lipid rafts within the membrane [74]. The coupling of lipid phase transitions to ion channel association and transport is a necessary consequence of membrane constitution to condensed matter physicists.

We will return to axon membrane signal conduction and its coupling to lipid phase transitions in the further discussion below of anaesthetic effects.

Inconvenient Truths

However satisfactory such a state of affairs appears, there is a problem. The "theory" rests on a foundation provided by Hodgkin-Huxley. That is an application of classical double layer theory of colloid science. No ion specificity or mechanism for ion partitioning exists. We saw in Part 1 that the attempts by Ling to explain cationic distribution asymmetry via Donnan equilibrium – essentially the same theoretical foundation - were dismissed. Ion pumps were then postulated and became part of the theology. When it is repaired the necessity for ion pumps is not obvious [3]. The inconsistency remains. The Hodgkin–Huxley foundation relies still on classical theory which we know is wrong! It can only be resolved once theories take account of Hofmeister effects [75].

4.2. Chemical Signaling at the Nerve Synapses. Vesicles and Supraaggregation

From one axon to another the signal can be transferred *via* a variety of transmittor molecules. The different transmittor molecules affect different functions of the brain; *e.g.*, for the brain to muscle, the transmittor substance is acetylcholine.

When the spike in an axon reaches the synapse, the influx of calcium ions induces a mass-cooperative fusion of transmittor molecule-loaded "vesicles" at the presynaptic

membrane. The postsynaptic membrane will then induce a new action potential. If a muscle cell is targeted the actin-myosin contraction cycles are started or continued.

The chemical signal transport over the synapses takes place under stringent time and signal strength control. The mechanisms are not known. But we can make a guess.

Recall our earlier discussions of cubic membranes. And of supraaggregation with vesicles, the interior which contains bicontinuous cubic phases. The mass-fusion of vesicles may draw on such conformational changes. Calcium ions can induce fusion between these vesicles and simultaneously a fusion with the presynaptic membrane.

Knowing what we expect on supraaggregation of lipids, from Section 2.3 of Part 1, the interior of vesicles is likely to contain cubic microphases with excess calcium for efficient delivery across the synapse. Such a possibility is expected on grounds discussed in earlier sections. It would also explain another related mystery.

Electrical transmission of signals in the brain can be tracked over many synapses and large distances. This apparently leads to calcium depletion. Yet restoration to resting potential conditions is rapid. It seems reasonable to assume that lipid phase transitions associated with the conduction process can "bleb off" vesicles containing calcium *along* the axon as well as at the synaptic junction. These would act as reservoirs to "recharge" the axon after the signal has passed.

In any event, whatever the complexities, several observations relevant to our thesis on the role of hydrocarbon lipids emerge.

It seems that:

1) Microphase separation into rafts that bring proteins into association occurs. It is a consequence of the defects induced by cholesterol. The phase separation is an unavoidable expectation from statistical mechanics.

2) The curvature due to the cholesterol-lipid mixture in a raft is necessary to allow the proteins to pack within the membrane.

3) The whole process is underlaid by lipid bilayer phase transitions.

4) Cubic microphases due to supraaggregation are necessary to effect trans-synaptic transfer. The open bicontinuous structures allow rapid delivery.

All four concepts that follow as in Part 1, from local curvature and global packing constraints alone. These have been missing in interpretations of experiment which have mass-fusion of vesicles to "phase-inversion conformational changes".

This is a very fashionable field of research. Witness another of the ubiquitous Nobel Prizes in Physiology or Medicine (2013) on the machinery regulating vesicle traffic. It involves so-called SNARE proteins that play a role in the vesicle fusion process. However the role of the lipids in this process or for that matter how authority for direction of the process is assigned to the proteins is sufficiently arcane for us to avoid it.

4.3. The G-protein System illustrated by the Physiology of the Olfactory System and of the Pheromone Activity

4.3.1. The G-protein System

A membrane-localized protein called a G-protein with a G-protein-coupled-receptor (GPCR) forms a complex signaling system in eukaryotic cells. An understanding of the physiology is new. (More Nobelers! R. Leifkowitz and B. Kobilka, Nobel Prize in Chemistry 2012) [76]. A signal starts with a ligand bonding to the G-protein coupled receptor (GPCR). This consists of seven α-helical peptides that span the membrane bilayer. The receptor is located at the outside of the membrane. This binding of a ligand to a GPCR induces a conformational change of the GPCR helical membrane

complex. This is sensed and influences a G-protein, which can be located at a short distance from the receptor. The G-protein is heterotrimeric, with an α-region, a β-region and a γ-region. All three are reversibly associated components. The binding of the receptor induces dissociation of the α-unit with one anchor to the inside of the bilayer from the β-unit and γ-unit, which also has a similar anchor into the bilayer layer inside.

This binding also induces a transition at the α-protein of guanisine triphosphate (GTP) into the diphosphate (GDP), which in turn *via* membrane-bound enzymes results in the release of second messengers (for example diglycerides) to the target cells. In this way one ligand binding to a GPRC can result in a signal cascade involving hundreds of target cells!

Adrenalin, which induces "fight-or-fly" physiological reactions, is one example [77]. Other examples of signaling addressing the GPCRs are histamine, seretonin and dopamine. Almost half of the number of drugs known at present function *via* this G-protein system.

4.3.2. Physiology of the Olfactory System and of Pheromone Activity

We consider the olfactory system specifically to illustrate this signaling system.

With mammals, the receptors for smell and pheromone recognition are located together in the upper region of the nasal cavity. In primates there is a special region for pheromone receptors located near to the receptors for the sense of smell; the vomeronasal glands. In other mammals the pheromone receptors are located within the olfactory receptor region. The olfactory receptor system in humans covers about 4 cm^2. We can differentiate between about 30,000 – 40,000 different smell identities by the olfactory cells and their GPCRs located in the mucous surface [78]. These cells

are in fact neurons ending in the region of the cortex allocated to smell. For pheromones the signals end in the limbic system of the brain.

The significance of pheromones for humans is open, but their role in other mammalians is well known. They are specialized, for sexual signals. In mice they have been shown to inhibit inbreeding. The receptors for pheromones in insects are located at the surface of their antennae. They are extremely sensitive. Electrophysiological measurements show that a single pheremone molecule can induce a signal.

This is reminiscent of light detection where a single photon is enough to trigger reception via rods and cones [79]. *A sensitivity so universal must involve cooperative transitions of the lipid matrix*. Even some single celled eucaryotes have eye-like structures called "oecelloids" [80].

The influence of salts in the Hofmeister series on the prototypical G-protein visual receptor rhodopsin and subsequent work reinforces this universality [79].

4.3.3. A Problem with Pheromones

The triggering of a lipid phase transition like that described for conduction of the nerve impulse seems to provide a generic cooperative mechanism. This is so from vision to brain function and pheromones and anaesthesia.

One still insuperable difficulty remains: It is well known that insects can sense sex pheromones at incomprehensibly low concentrations. The signals can be triggered by direct adsorption of a receptor molecule to the receptor site. And the consequent biochemistry and phase change triggered off is well explored.

The difficulties are: How is the species dependent pheromone molecule information

transferred to the initial receptor protein on the antenna? All pheromones are more or less simple molecules and van der Waals (visible frequencies) adsorption energies will be virtually identical. All differences lie in the infra red region.

It is possible for the pheromone to be emitted in a metastable excited state. Then long range information exchange is possible via photon transfer between a metastable state to excite the receptor protein conformation change [11].

In a recent study we investigated the effect of the pheromone "olean" (1,7-dioxaspiro[5.5]undecane, one of the sex pheromones of the olive fly) by bilayers of dioleoyl-phosphatidylcholine (DOPC). The results indicated that the uptake of the active molecule induces two phase transitions in the the phospholipid dispersion: one from lamellar L_{α} to inverse hexagonal H_{II} and then to inverse cubic I_{II} phase. Moreover the phase changes reduce the water reorientational motions and lower the freezing temperature of water [9]. This is another example of a phase transition – induced by hydrophobic adsorption and curvature change - being involved in a complex biological mechanism.

4.4. The Prostaglandins

Two different kinds of lipid have important physiological effects, *prostaglandins* and *estolides*. They are complex fatty acids with hydroxyl groups attached to the acyl chains. Their importance is unquestioned. They must have structural effects on the hydrophobic interior of membranes. What those effects are is unexplored.

The first group are *prostaglandins*. There are two kinds, called PGE_2 and $PGF_{2\alpha}$. They derive from arachidonic acid and contain 20 carbon atoms and a cyclopentane ring in the middle region. The detailed chemical structure is classified into groups A to H,

according to the type of ring structure, and the number of double bonds. The first crystal and molecular structure determination that revealed the architecture of prostaglandins were determined by Sixten Abrahamsson in the 1960s [81].

They have hormone-like effects. But their applications in medicine are still limited as they act in very diverse ways. Some expand the bronchial channels of the lung. Others contract them. Some lower the blood pressure. Others increase it.

The other group are the *estolides*. Recently it was reported that these molecules exhibit a drastic effect on appetite and therefore on obesity [82]. The molecules are linked hydroxy-fatty acids, in which the hydroxyl group in one chain is ester-linked to a fatty acid carboxyl group in another fatty acid. If this also is a hydroxy-fatty acid, molecules with three or four chains may be formed. Such large complexes have been shown to exist in oat cereal lipids [83]. Oats are unique in this respect due to their content of a hydroxy-fatty acid termed avena-acid. Eat your porridge.

4.5. A comment on air dissolved in oils

In surface and colloid science a mineral oil is often considered as similar to a triglyceride oil. But one important aspect of the behaviour of an "oil" phase is its content of dissolved air. A mineral oil can solvate almost 10% of its volume of air, whereas only something in the range between 30 and 40 mg/kg has been reported for olive oil and palm oil [84]. Due to the high solubility in mineral oil it should be of interest to know the difference in air solubility between pure paraffins of different chain lengths. Furthermore, we recall that - due to the very low intermolecular forces - perfluorinated oils dissolve an incredible amount of gases [31,32,85].

The mapping work by Fontell, Ekwall and Mandell of the system decanol, sodium caprylate, water is also interesting to consider in this respect. The decanol-corner-

located L_2 phase is often regarded as an oil corner. What are the effects there of dissolved air? And in this connection the remarkable works on enhanced oil solubility in degassed water [86], on the variation of the cloud point of dioctanoyl-phosphatidylcholine upon removal of dissolved gases from its water dispersions [87], and on the kinetics of formation of pseudopolyrotaxanes from cyclodextrins [88].

Long range "hydrophobic" interactions disappear on removal of dissolved gas, effects of which are ignored in classical physical chemistry [75,89]. The implications are large and have hardly been recognised.

5 Cubic Bilayer Vesicles, Exosomes, and Cubosomes.

New liquid-crystalline phases of lipids emerged in the 1960s [90]. The structures involved bicontinuous cubic phases. This opened up new perspectives. The real structures, vigorously misinterpreted by the Luzzatti School for 40 years held up developments. Similarly Fontell had often observed cubic phases 20 years earlier, and forbidden to publish them by Ekwall [K. Larsson and B.W. Ninham, private communication]!

Liquid-like disorder for hydrocarbon chains in these phases is too simplistic for biology. Cholesterol has a key role. It induces local order. At high concentration it causes inhomogeneity due to microphase segregation with consequences as above. Membrane-embedded proteins are furthermore localized at the inner side of the bilayer, a consequence of lipid membrane asymmetry due to packing constraints (Part 1). The same packing constraints led to the universality of periodic minimal surface conformations in amphiphile-water systems [3,11,75].

We have explored some physiological consequences of this wider class of lipid geometries.

Further conceptual developments, the consequences of which have not been known and not explored at all, are embodied in Section 2.3 of Part 1. The necessary asymmetry in disposition of lipids between the outer, and the inner surface of any vesicle or curved membrane (see Figures 2 and 3 in Ref. 1) in even a single component vesicle was proven quite generally in 1981 by one of us and D.J. Mitchell and forgotten [91]. This realisation invalidates a great deal of theoretical work on vesicles that assume the hydrocarbon chain region of bilayer is symmetric. The hydrocarbon chains of lipids on the inside of a curved bilayer necessarily have reverse curvature (splayed configuration). The lipids on the outside have normal curvature. That has consequences for transmembrane protein disposition and packing across membranes, that favour the inner layer.

It follows from this that prostaglandins and estolides with their small head groups will pack on the inner side of vesicles and interact strongly with transmembrane proteins to affect their activity. How they do so is obviously specific depending on chain length, flexibility and polarity. That observation may be sufficient to explain the variable activities they induce (Section 4.6) A similar geometric physical packing effect will affect the disposition of lipids like phosphatidyl serine that has not yet been studied.

A major conceptual development is embodied in the phenomenon of supraaggregation as an equilibrium and necessary state of aggregation. This was explored in Part 1 Sec. 2. with implications too wide to include here.

With those remarks noted, we continue.

5.1. Exosomes

An isolated vesicle that contains a cubic lipid bilayer will be surrounded and protected

by a few asymmetric bilayers in a high state of stress. See discussion of supraaggregation (see Part 1). The vesicle will then be very fusogenic, and ideally poised for the intensive exchange of materials from a cell and its outside. Endocytosis and exocytosis are activities of living cells. It is trite to say that are governed by and respond to strict concentration levels, external and internal physico-chemical conditions that are the source and sink that drives their self assembly. The intensive research going on on exosomes and their function ignores lipid self assembly. To explore such arcana, see Refs. 92-94.

5.2. Cubosomes

Cubosomes in cells were discovered by one of us (KL). It is a word that now labels colloid dispersions of cubic bicontinuous phases in water. They are very much in vogue, *e.g.* for thermo-responsive and targeting formulations in drug delivery [95].

6. Conclusions. A Muddled Progress

Our conclusions to the analysis of Part 1 holds equally for this Part 2.

Over the past 40 years lipids, previously almost invisible background to the proteins and biopolymers, have emerged as central players. The states of self assembly accessible and their supporting roles were non existent. What is satisfying is that these new states emerge from a combination of indisputable local packing constraints due to opposing interfacial forces, and global packing constraints only. These renormalised variables emerge from detailed statistical mechanics and are parameter free. The necessary asymmetry of curved membranes and vesicles, supraaggregation, cubic phases bring a certain unity and simplicity that cuts through some very complicated biological problems.

But progress is muddled. On the other side of the coin the complementary face of physical chemistry has been evolving fast on molecular forces, Hofmeister effects and hydration, the role of dissolved gas, on "hydrophobic" interactions, pH, buffers, and more [1,3,75,89,96].

The consequence is that the fundamentals and intuition deriving from them are subject to major revision [75].

Yet biological theories *e.g.* Hodgkin Huxley equations and "ion pumps" had inevitably built on the older classical and invalid theories of physical chemistry, - the double layer and electrochemistry, membrane potentials, pH buffers, ion binding and transport, zeta potentials - the list is finite but long. Much progress in biology relied on measurements that depend, for their interpretation on a flawed theory of physical chemistry. The observation means that just as the intuition in physical chemistry has to undergo revision, so too will some of the biological intuition that we presently hold indisputable.

There is more. Hot air bubble columns have shown that beyond the still inexplicable behavior of bubble- bubble interactions, the reactivity of gas bubbles is amazingly and unexpectably catalytic [97]. They kill cells and viruses with extreme efficiency.

We believe we have made some progress in the spirit of D'Arcy Thompson's plea.

There is more to do and the road for young scientists is more open now than ever.

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