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Hierarchically Structured Lipid Systems

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Without Abstract

Synonyms

Lipid phase equilibria; Lipid self-organization; Lipid superstructures; Multiscale structural ordering of lipids

Definition

Lipids, being amphiphilic, spontaneously organize into a broad range of nanoscale self-assemblies in an aqueous environment, which often turn out to be a part of multiscale architectures found in cellular membranes as well as drug carriers and consumer products used in daily life.

Lipid Self-Assembly and Simple Biomembranes

The cell membrane primarily constitutes of lipid molecules. Lipids have an anisotropic structure, generally composed of hydrophilic or polar head group and a long alkyl tail that is hydrophobic (lipophilic) or nonpolar in nature. Hydrophilic (and sometimes electrostatic) forces at the head group favor interactions with water molecules whereas hydrophobic (and van der Waals) forces, prevalent in the chain region, try to minimize them. This principle essentially allows lipids to self-assemble into various shapes and structures in aqueous milieu similar to cellular environments (Seddon and Templer 1995) .

The cell membrane commonly adopts quite simple – spherical shape formed of a closed lipid bilayer, which itself has a 'locally planar' structure (given the size of the cell ~1-100 μm compared to the bilayer thickness ~3-4 nm). Planar bilayer structure is a consequence of an assimilation of diverse molecules in such a way that an average molecular shape is rendered cylindrical (**Fig. 1d** Left), while the composition of both (outer and inner) membrane leaflets is not necessarily the same (van Meer and de Kroon *2011*, Furse and Shearman *2018*). Biological membranes are typically formed of lipids

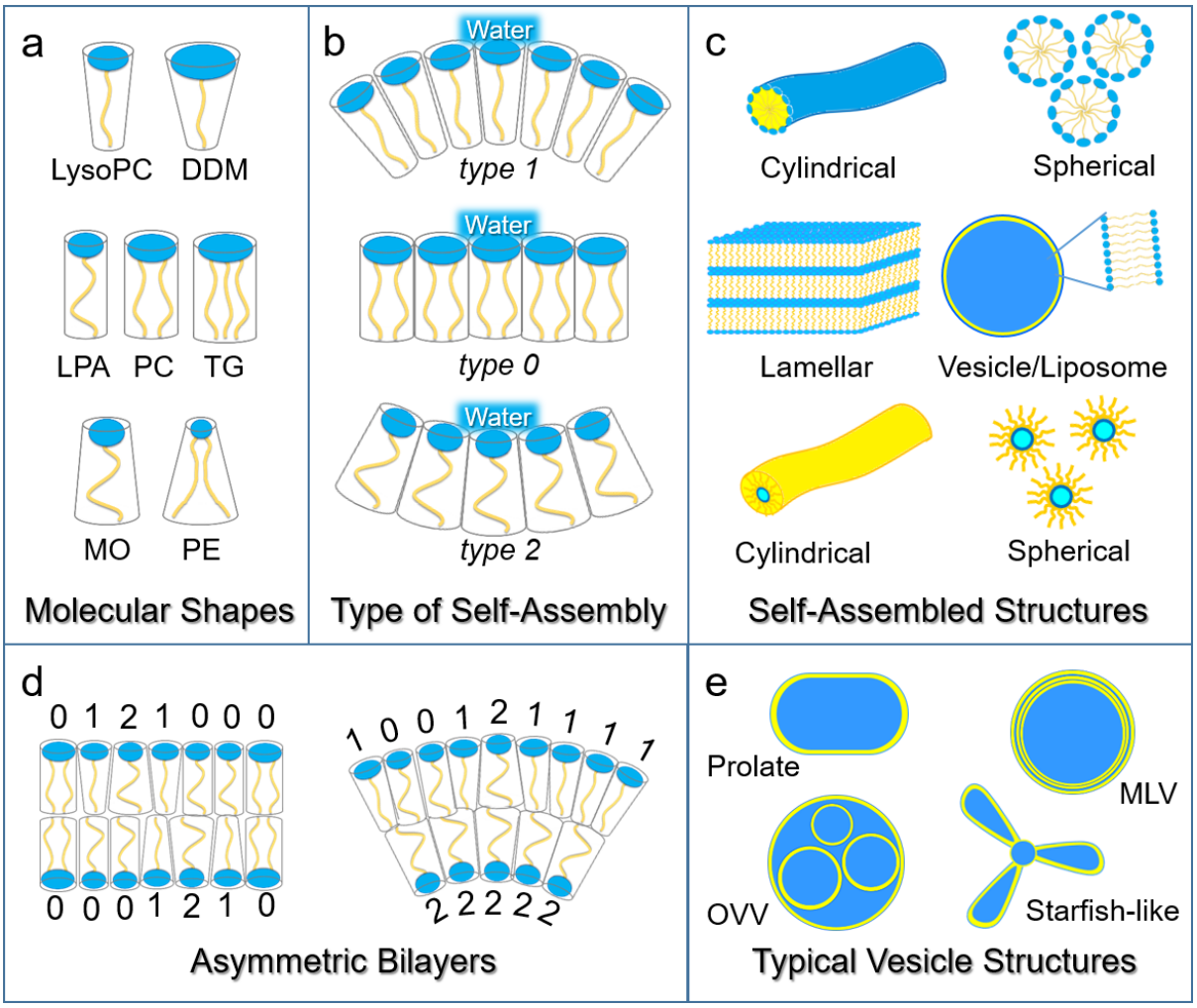
from three main classes (van Meer and de Kroon *2011*) – phosphoglycerides (~65 mol%), sphingolipids (~10 mol%) and steroids (~25 mol%). However, there are ~43,000 biologically relevant unique lipid structures known today; based on their chemical structure, these lipids are grouped into eight different categories (**Table 1**). Chemical structures of these lipids, primarily, govern their average molecular shapes in corresponding self-assemblies. Lipids with small head group and/or '*cis*' unsaturation/s in alkyl chain/s attain inverse conical shapes. The *cis* unsaturation induces a 'kink' in the chain region thereby elevating the chain splay; more the unsaturations, more is the chain splay (Kulkarni *2019*). Consequently, the cross-sectional area at the chain end increases as compared to the interfacial area at the head group. It has direct influence on the molecular packing, thus increasing the membrane fluidity (van Meer, Voelker et al. *2008*). Important membrane lipids, phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) exhibit inverse conical shapes (**Fig. 1a**); all of which contain at least one unsaturation in their chains (van Meer, Voelker et al. 2008, Furse and Shearman 2018). Phosphatidylcholine (PC) is another important membrane lipid but it adopts the cylindrical shape (**Fig. 1a**). In this case, the interfacial area created by the bulky head group, composed of three methyl moieties, closely balances the cross-sectional area generated by two long alkyl chains. Molecules with one or three alkyl chains may also adopt cylindrical shapes as exemplified by, a phospholipid derivative - lysophosphatidic acid (LPA) and common food constituent- triglycerides (TGs) (**Fig. 1a**). A few lipids like, for example, lysophosphatidylcholine (lysoPC) acquire conical shape owing to the large head group size (**Fig. 1a**) (Kulkarni *2019*). The detergent molecules like dodecyl-β-D-maltoside (DDM) and octylglucoside (OG) also adopt conical shapes (Kulkarni *2019*).

Hierarchically Structured Lipid Systems, Table 1.
Number of structures per lipid category. Data obtained from LIPID MAPS Structure Database (LMSD) https://www.lipidmaps.org/resources/databases/ on 15/07/2019.

No.	Lipid Category	Curated	Computationally-generated	All
1.	Fatty acyls	7468	1792	9260
2.	Glycerolipids	228	7379	7607
3.	Glycerophospholipids	1606	8312	9918
4.	Sphingolipids	1242	3176	4418
5.	Sterol lipids	2826		2826
6.	Prenol lipids	1258		1258
7.	Saccharolipids	22	1294	1316
8.	Polyketides	6810		6810
	TOTAL	**21460**	**21953**	**43413**

Typically, normal (*type 1*), planar (*type 0*) and reverse or inverse (*type 2*) phases are formed (by pure lipids in water) (**Fig. 1b**) when they acquire average *conical*, *cylindrical* and *inverse conical* shapes, respectively (Seddon and Templer 1995). The general convention is: the phase is *normal* when the interfacial curvature is (positive) towards chain region, *planar* when it is zero and *inverse* when it is (negative) towards the aqueous region (Seddon and Templer 1995). Molecular shapes play important roles in administering the membrane curvatures in cellular organelles. For instance, locally planar membrane is not only assembled with cylindrical shaped molecules, but it can be also constructed from an appropriate mixture of conical and inverse conical molecules (**Fig. 1d** Left). The existence of such molecules, resulting asymmetry of the membrane and congruent fluidity, offer the membrane its stability and perhaps, the capability of reorganizing when there is a demand for curvature induction (**Fig. 1d** Right). The coupling of biomembrane composition and membrane curvature are in good

agreement with this principle (van Meer, Voelker et al. *2008*, van Meer and de Kroon *2011*). For instance, inverse cone shaped PE, PS and PI are abundant in the inner-cytosolic membrane leaflet whereas the cylinder shaped PC is accommodated in both leaflets, but a bit excess in the outer leaflet of the membrane (van Meer, Voelker et al. *2008*, van Meer and de Kroon *2011*, Furse and Shearman *2018*). In this perspective, it is perhaps needless to mention that the average curvature of the inner leaflet is more than that of the outer leaflet of the plasma membrane.



Hierarchically Structured Lipid Systems, Fig. 1

a) Illustrations of molecular shapes adopted by various amphiphilic molecules including lipids (LysoPC: lysophosphatidylcholine; DDM: dodecyl-β-D-maltoside; LPA: lysophosphatidic acid; PC: phosphatidylcholine (dioleoyl phosphatidylcholine); TG: triglyceride, MO: monoolein (a mono-glyceride) and PE: phosphatidylethanolamine). b) Types of self-assemblies formed by conical (*type 1*), cylindrical (*type 0*) and inverse conical (*type 2*) molecules. c) Schematic diagrams of simple forms of self-assemblies formed by pure *type 1, type 0* and type *2* lipids; hydrophobic chains (yellow) are shielded from aqueous regions (light blue). d) Mixture of lipids exhibiting different molecular shapes tend to form asymmetric bilayers (similar to biological membranes) (Furse and Shearman *2018*). The numbers 0, 1 and 2 indicate corresponding lipids of cylindrical, conical and inverse conical shapes. e) Other than the spherical geometry, the vesicles/liposomes also attain prolate and starfish-like morphologies; depicted with simple drawings. Moreover, they also form multi-lamellar (MLV) and oligo-vesicular vesicles (OVV) containing multiple bilayers.

Among the simple geometrical shapes, spherical isotropic morphologies like uni-lamellar (with single bilayer) vesicles, multi-lamellar (multiple bilayers) vesicles (MLV) and oligo-vesicular (vesicles inside vesicles) vesicles (OVV) as well as some anisotropic structures including prolate and starfish-like shapes are seen in cells (**Fig. 1e**). Some of these single or multi-compartment assemblies are employed to carry functional molecules across the cell, and for the delivery of active ingredients in some biotechnological applications. Anisotropic shapes are commonly seen during the structural transformations, driven by homeostasis, and/or as demanded by cellular processes/functions.

Structural Hierarchy in Liquid Crystalline Phases

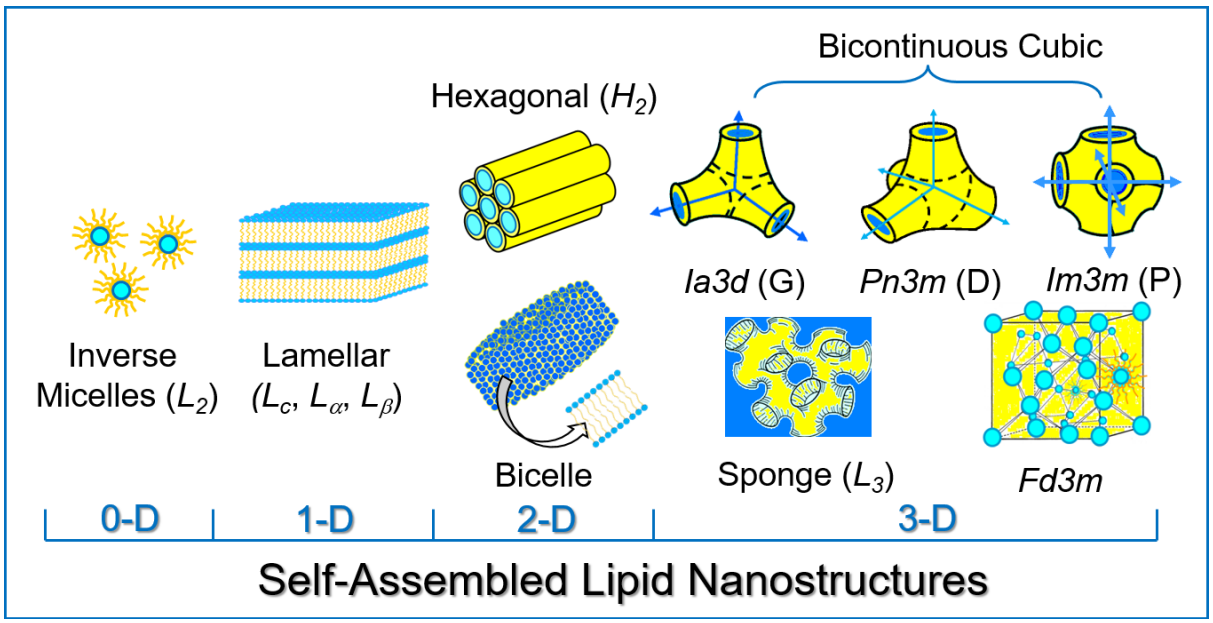
Locally planar, cylindrical and spherical shapes are simple geometries (**Fig. 1c**), but self-assemblies with an intermediate curvatures like cuboid, ellipsoid, disc and torus shapes are also formed under various conditions (**Fig. 2a**)(Hyde, Andersson et al. 1997). Both, the type and the magnitude of lipid molecular shape affect the curvature of the self-assembly (Kulkarni *2019*). The lipid molecular chain splay increases with increasing temperature (pressure usually has an opposite effect), which makes the molecule more inverse conical, deviating from the cylindrical shape (Kulkarni, Wachter et al. 2011). This, in turn leads to the formation of highly curved phases. The formation of phases is also governed by other important physicochemical factors including: lipid chain length, degree of unsaturation, head group area, type and composition of solvent, composition of lipid mixture, addition of other additives (like buffers, salts, other amphiphilic molecules), charge and pH (Seddon and Templer 1995, Kulkarni, Wachter et al. 2011). Israelachvili's dimensionless packing parameter or shape factor (Israelachvili, Mitchell et al. 1976) qualitatively describes average molecular shapes in corresponding self-assemblies of amphiphilic molecules. It is calculated as a ratio between lipid molecular volume and a product of an interfacial area and lipid length (usually hydrophobic chain length) (Israelachvili et al. *1976*). Recent efforts, however, have formulated a method to calculate a crucial parameter the 'chain splay' (Kulkarni, *2019*), which is an important step towards the quantitative estimation of shapes of molecules that constitute self-assemblies as well as biomembrane structures.

Lipids self-assemble into a broad range of thermodynamically stable zero, one-, two- and/or three-dimensional architectures (Hyde, Andersson et al. 1997).Their phase behavior mediates solids and liquids, and they exhibit only long-range order hence they are called liquid crystalline (LC) phases. Their structural length scales lay in the range of ~2–40 nm; therefore they are also referred as nanostructures. The most simple lyotropic (formed by solvent) system is with two components, i.e., a pure lipid plus water, whose behavior is usually studied at different temperatures, and is represented on a temperature–composition phase diagram (Kulkarni, Wachter et al. 2011).

The lamellar phase is quite common in many lipid systems and is an indispensable structural constituent of biological membranes. This phase consists of one-dimensional stacking of planar lipid bilayers, whose behavior may differ in the degree of chain fluidity, head group ordering, tilting, inter-digitation of monolayers, etc. These structural distinctions reflect into an interesting set of polymorphism in lamellar phases which also has a vital significance in biomembrane functioning. Most common polymorphs are crystalline (L_C), fluid (L_α) and gel (L_β) lamellar phases (**Fig. 2**); however, other phases (tilted gel $L_{\beta'}$, interdigitated gel $L_{\beta I}$ and rippled gel $P_{\beta'}$) are also possible under various conditions (Seddon and Templer *1995*). These polymorphs are usually distinguished by wide-angle X-ray scattering studies. The fluid (L_α) lamellar phase is sometimes referred, as liquid-ordered (*Ld*) phase, whereas liquid-ordered (*Lo*) phase is estimated of having similar characteristics as that of the lamellar gel (L_β) phase (Kaiser, Lingwood et al. *2009*). This categorization is generally used by biophysical researchers to describe the phase separation in membranes where *Lo* phase is composed of rather tight packing of cholesterol and saturated (sphingo)lipids and the *Ld* phase is predominantly

formed of unsaturated glycerophospholipid in the disordered state (Kaiser, Lingwood et al. *2009*). These features bear implications in the discussion of lipid rafts and constitutions therein.

The inverse hexagonal H_2 phase (**Fig. 2**), comprising of long water channels enclosed by lipidic cylinders, is also common among many lipid systems (Seddon and Templer 1995). A few lipid systems, e.g. monoglycerides (Kulkarni, Wachter et al. 2011) or a mixture of selected phospholipids form bicontinuous cubic phases (Barriga, Tyler et al. 2015), which are quite elegant phases. Most common bicontinuous cubic phases are indicated with their space groups *Ia3d*, *Pn3m*, and *Im3m*, whose architecture is based on mathematical minimal surfaces G, D, and P, respectively (**Fig. 2**) (Hyde, Andersson et al. 1997). These intricate cubic phase assemblies exhibit finely designed and ordered topology where an individual phase consists of a continuous lipid bilayer enclosing two interwoven but separate aqueous channels. The numbers, dimensions, and angles at which the aqueous channels meet at single points in the unit cell of the cubic phases differ among the phases (**Fig. 2**). Other LC nanostructures (**Fig. 2**) such as micellar cubic *Fd3m* phase, bicontinuous sponge phase (disordered) and disk-shaped bicelles are displayed when additional chemical or biological component/s is/are added to the binary lipid–water system (Kulkarni, Wachter et al. *2011*). These 3-D phases (except micellar phases) can already be understood as hierarchical ordering of lipids, as each of them consists of a self-assembled lipid bilayer, which folds further in a highly organized manner (except sponge phase where it is disordered) to produce three-dimensional architecture. In case of micellar phases, e.g. *Fd3m*, discrete micelles arrange into a cubic lattice to form hierarchically organized structures.



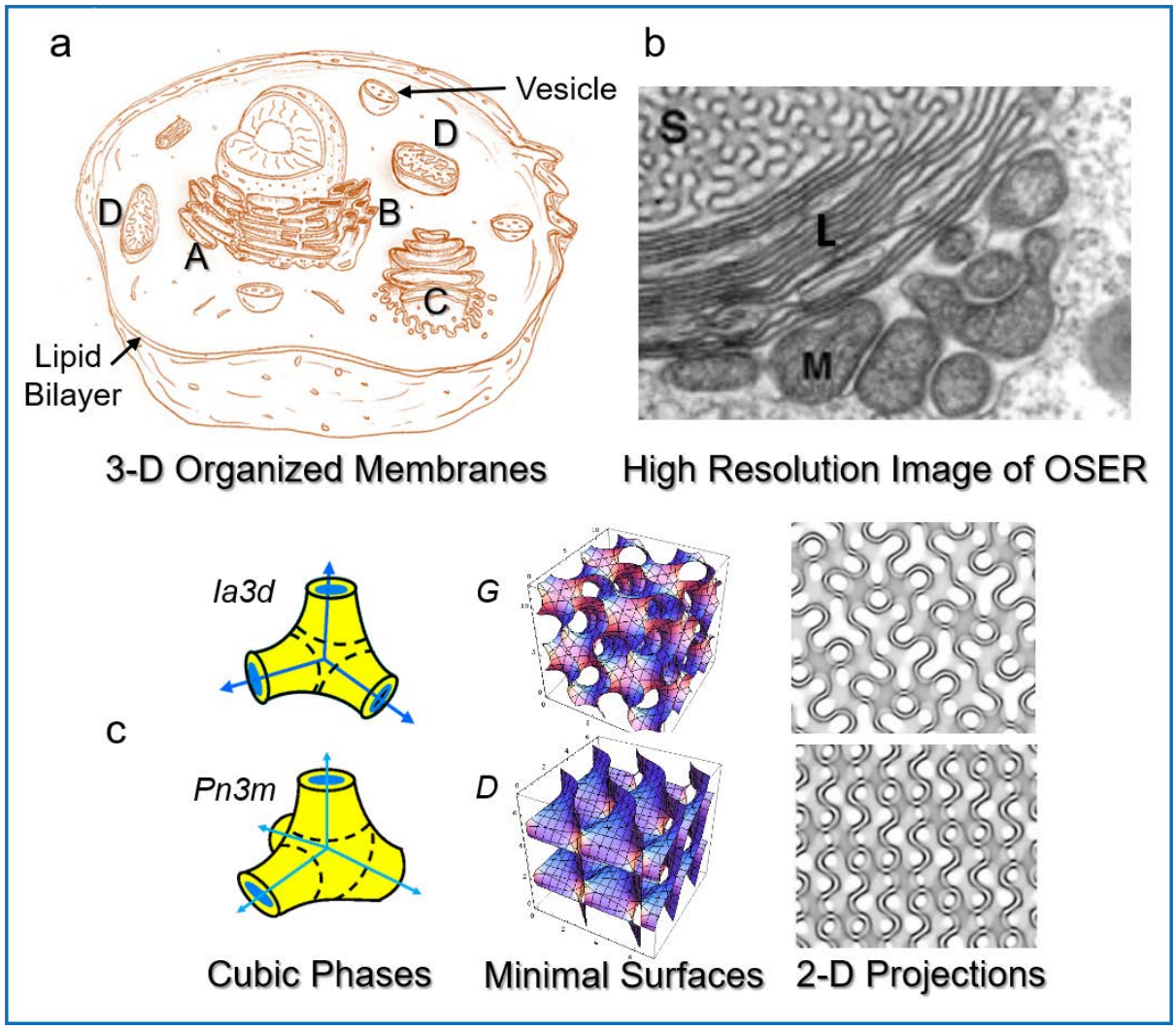
Hierarchically Structured Lipid Systems, Fig. 2
Schematics of commonly observed 0, 1, 2 and 3 dimensional (D) self-assemblies of lipids in water. Arrows in bicontinuous cubic phases designate aqueous channels. Bicelles contain lipid bilayer/s assembled in a disc shape (covered by lipid/detergent molecules pointing their head groups outwards). Figure modified from .

Mimicry of Biomembrane Structures

Plasma membrane and intracellular liposomes show simple membrane geometries but ER (endoplasmic reticulum) membranes, Golgi membranes, inner nuclear membrane, mitochondrial inner

membrane and chloroplast thylakoid membranes are far more complex arrangements. Moreover, these structures undergo continuous reorganization under physiological conditions. These dynamic and convoluted structures can be better termed as ‘kinetically stabilized’ and/or ‘metastable’ rather than purely ‘self-assembled’ and ‘thermodynamically stable equilibrium’ systems.

A range of model systems have been developed to mimic structural and functional aspects of plasma membranes and simple subcellular membranes (Chan and Boxer *2007*). Freestanding (free-spanning) bilayers (also called black lipid membranes-BLM), Self-assembled monolayers (SAM), Langmuir-Blodgett films, supported lipid bilayers (SLB) and vesicles from pure or mixed lipid systems have been more popular models, but hybrid membranes including tethered lipid bilayer membranes (t-BLM), tethered vesicles, vesicles ruptured on supported bilayers, membrane nanodiscs and bicelles are also used at varying levels of applicability (Chan and Boxer *2007*). These models, however, are not capable of mimicking complex biomembrane structures. Some efforts in this direction have demonstrated the mimicry of compartmentalized architectures using multi-lamellar vesicles (MLV), multi-vesicular vesicles (MVV) - also called oligo-vesicular vesicles (OVV), polymersomes and multi-compartment vesicles (Rideau, Dimova et al. 2018). These ensembles were successful as function-specific cell mimics (Rideau, Dimova et al. 2018).



Hierarchically Structured Lipid Systems, Fig. 3
a) Rough endoplasmic reticulum – ER [A], smooth ER [B], Golgi apparatus [C] and mitochondria [D] are important cellular organelles formed by complex 3-dimensional

arrangement of lipid bilayers. The cell membrane and vesicles are also formed of lipid bilayers. b) Biogenesis of cubic biomembranes in cell lines of COS-7. 'M' and 'L' represent mitochondrial and lamellar biomembranes whereas 'S' represent sinusoidal membrane organization of organized smooth ER (OSER). c) Bicontinuous cubic phases, *Ia3d* and *Pn3m*, have similar architecture to that of the folded arrangement of ER; as demonstrated by comparing electron micrograph of OSER (as shown in b)) with 2-dimensional projections of *G* and *D* type minimal surfaces, which form the basis of *Ia3d* and *Pn3m* lipid phases, respectively. (Figure modified from (Kulkarni, Ces et al. 2013).

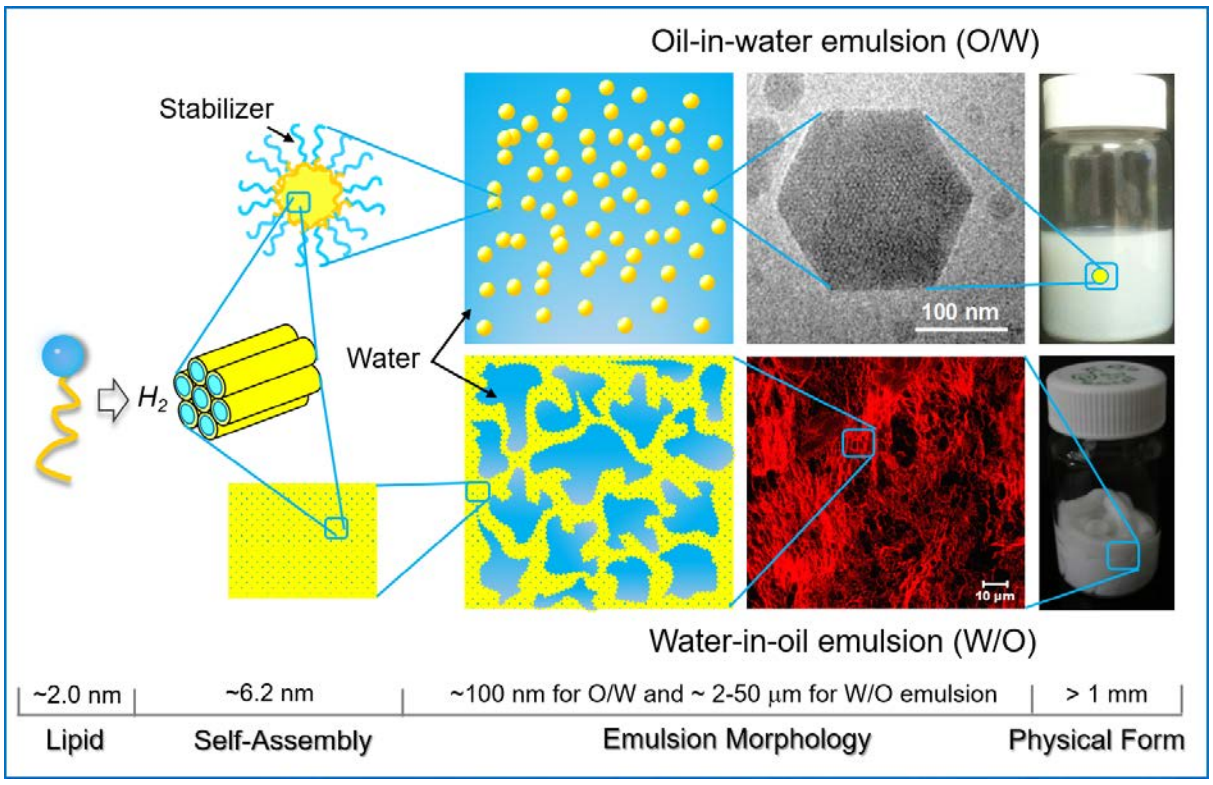
Intracellular membranes are much more complex than mere compartmentalized structures (**Fig. 3a**). For instance, the ER consists of stacked membrane sheets and tubules, efficiently packed in the restricted space of a cell whose form and distribution varies with physiological state and phenotype of cells. The structure of Golgi apparatus is like a stack of pancakes formed of membrane bound sacs also called cisternae; whereas the mitochondrion (plural- mitochondria) is formed of a double membrane; a lipid bilayer along with proteins constitutes the outer membrane while the inner folded membrane contains lamellae and tubular structures (**Fig. 3a**). It is hypothesized that the majority of above structural aspects are already displayed by lipid phases, discussed earlier (**Fig. 2**) (Landh 1995). Explicit evidences have confirmed that two-dimensional projections of gyroid and double diamond minimal surfaces closely resemble the structural patterns of intracellular biomembranes of various living organism (**Fig. 3b, c**) (Almsherqi, Margadant et al. 2010). These topologies are capable of generating very large surface areas as employed in the ER to accommodate large number of functionally important molecules. Not only folded and continuous sheets but the tubular configuration of smooth ER also mimics aqueous channels within cubic phases or perhaps from the hexagonal phase (**Fig. 2**) (Landh 1995).

Although the structural resemblance of lipid phases with intracellular morphologies has been validated, there are some major bottlenecks in their mimicry. First and foremost problem is the matching of their size and dimensions: the unit cell dimensions of most common lipid phases lay in ~6 nm to ~14 nm range but intracellular biomembranes are about an order of magnitude larger than these length scales. Second issue is the dynamics; the intracellular membranes are highly dynamic which undergo continuous reorganization whereas the lipid phases are under the state of thermodynamic equilibrium. Third difficulty arises due to compositional variation: lipid phases are usually prepared from one or just a few components, on the contrary, the intracellular membranes are composed of a large number of structurally and chemically diverse molecules. Nonetheless, recent efforts have been promising towards resolving some of these issues (Kulkarni, Ces et al. 2013, Barriga, Tyler et al. 2015).

Hierarchical Lipid Systems for Biotechnological Applications

Lyotropic LC phases are known for their applications in various biotechnological fields, ranging from drug delivery to membrane protein crystallization (Kulkarni, Wachter et al. 2011). However, direct use of bulk LC phases in certain applications is hampered, for instance, due to their high viscosity and variable domain consistency. To overcome these concerns, bulk phases are usually transformed or kinetically stabilized into other forms and/or hierarchical structures using high-energy input and/or external stabilizer. Depending on the method of preparation and the composition of components, LC nanostructures reorganize into oil-in-water (O/W) or water-in-oil (W/O) emulsions (**Fig. 4 top**). The hydrophobic phase (lipid or lipid+oil) itself displays a wide range of nanostructures (as shown in **Fig.**

2). When the bulk phase is dispersed in water in the form of particles, it is generically called 'isasomes' (Yaghmur, de Campo et al. 2005), i.e. internally self-assembled *somes* (particles). More specifically, the particles with hexagonal and cubic internal structures are termed, 'hexosomes' and 'cubosomes,' respectively. The cubosomes were the first among such emulsions prepared about three decades ago (Larsson 1989). Isasomes are also known with some other names including nanostructured lipid particles, lipid nanoparticles, lipid phase nanoparticles and liquid crystalline nanostructured particles. The reverse type of emulsion, where a hydrophobic phase (with an LC nanostructure) forms a continuous wall-like architecture enclosing water droplets (up to ~95% by wt), is called a W/O nanostructured emulsion, and more importantly, it does not require any external stabilizer (**Fig. 4 bottom**) (Kulkarni, Mezzenga et al. 2010).



Hierarchically Structured Lipid Systems, Fig. 4

Lipids form various self-assemblies (shown in **Fig. 2**), which can be further (kinetically) stabilized into oil-in-water and water-in-oil nanostructured emulsions. Multiple length scales in these systems depict their hierarchical structural levels (the length scales are approximate). O/W emulsions have particulate form with the particle size in the range of 100-400 nm (TEM image)(Yaghmur, de Campo et al. 2005). As the particles are kinetically stabilized into large volume of water, they generally have fluid consistency (top vial). On the other hand, W/O emulsions are viscous and have creamy or paste-like texture (bottom vial). The water droplets are anticipated to have anisotropic shapes and their sizes range widely from 2-50 micron as shown by the confocal microscopy image (lipid stained in red)(Kulkarni, Mezzenga et al. 2010). In both of the above systems, the lipid self-assembly itself can have aqueous nano-channels of ~2-5 nm diameter (dots in W/O emulsion schematics).

Recent research advances have extended the boundaries of internal nanostructures (of isasomes) beyond the cubic (*Pn*3*m*, *Im*3*m* or *Fd*3*m*) and hexagonal (H_2) phases so as to include the microemulsion (L_2), lamellar, and even sponge (L_3) phases; the resulting isasomes are called emulsified microemulsions (*EME*), lamellarsomes, and spongisomes (name given here), respectively .

Sometimes, the vesicles and liposomes were seen to coexist and/or enclose the isasomes. Note that the lamellarsomes and liposomes are not one and the same, as the interior of liposomes is usually filled with water whereas lamellar phase is enclosed by the lamellarsomes. Being similar in size as liposomes, the isasomes are comprised of LC phases thereby providing considerably higher volume fraction of hydrophobic environment. The latter is already equipped with well-defined nanostructural units facilitating loading of poorly water-soluble and amphiphilic functional molecules. The same dispersion also allows loading of highly water-soluble molecules in the continuous aqueous medium. Increased diffusive path length provided by the internal nanostructures of isasomes thus demonstrate an enhanced capacity for sustained release of the loaded components, which can be further improved by encapsulating the isasomes within hydrogel films (Kulkarni, Moinuddin et al. 2015). In this manner, the isasomes provide certain advantages over vesicle/liposomal structures and conventional (*unstructured* oil phase) emulsions; the latter are most common for detergent-based amphiphiles. Although the nanoscale structure is similar to the bulk LC phases, isasomes offer some advantages over them; for instance, isasomes are easy to handle (because of rather low viscosity materials) using conventional industrial designs customized for liquid handling. The overall cost is also dropped significantly when a dispersed form (requires only 5-10% lipid) instead of pure LC phase (requires about 50% lipid) is utilized. Hierarchical lipid structures therefore find several applications in fields of pharmaceuticals, foods, cosmetics, agriculture and bioadhesives given the possibility of their customization from food grade or biocompatible components (Kulkarni and Glatter 2012).

There are several other types of hierarchically ordered forms of lipids being developed and investigated by researchers for various applications, including poly-high internal phase emulsions (poly-HIPE); bicontinuous interfacially jammed emulsion gels (bijels); polymerized LC phases; lipid nanoparticles (LNP), i.e., liposomes loaded with functional molecules; lipid nanocapsules; etc. (Kulkarni and Glatter 2012). Solid lipid nanoparticles (SLN) developed in the early 1990s, and their successive variants, nanostructured lipid carriers (NLC), prepared by the high-pressure homogenization technique, are also under investigation, particularly for drug delivery applications. Hierarchical systems (proteoliposomes, proteocubosomes, or lipid bilayer nanodiscs - proteins with bicelles) fabricated by loading of lipid structures with proteins and other biomolecules are also exploited for aforementioned applications. A few authors have also employed isasomes with dissimilar nanostructures, charges, or functional molecules to intermix or transform into fascinating layer-by-layer assemblies (Kulkarni and Glatter 2012). A majority of these systems have a great potential in biotechnological applications. Nonetheless, structured emulsions, formed primarily from simple monoglycerides, have emerged to be quite successful lipid-based materials that are utilized for various commercial applications . There are enormous opportunities to further develop, explore, and utilize new and novel types of lipid-based hierarchical structures.

Summary

In presence of water, lipids spontaneously self-assemble into a broad range of micelles and bilayers to thermodynamically stable LC phases of various sizes, shapes, and properties. These, as well as hierarchically organized lipid self-assemblies, exist not only in the form of simple biomembranes and subcellular complex structures but they are also nurtured in valuable configurations such as structured emulsions. Morphological behavior of these lipid systems extends in multiple length scales (nano, micro and millimeters). The research in understanding the roles of lipid systems in fostering life and the development of innovative applications from them is quite an open area for the years to come.

Cross-References

Membrane Lipids
Chemical Diversity of Lipids
Electron Microscopy of Membrane Lipids
Functional Roles of Lipids in Membranes
Lipid Bilayer Asymmetry
Lipid Mesophases for Crystallizing Membrane Proteins
Lipid Organization, Aggregation, and Self-assembly
Lipidomics
Membrane Fluidity
Phase Transitions and Phase Behavior of Lipids
Pressure Effects on Lipid Membranes
Supported Lipid Bilayers
Thermodynamics of Lipid Interactions

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