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Review

"Rafts": A nickname for putative transient nanodomains

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ABSTRACT

The membrane raft hypothesis, proposed in 1997 by Simons and Ikonen, has played a paradoxical role in the history of biomembrane research. While it has generated a large amount of investigations, thus helping to increase our understanding of membranes, the object that gives name to the hypothesis, i.e. the raft itself, has been and still is an object of controversy, in which its very reality is often questioned. In this contribution I review the history of the hypothesis and its reception by membrane biologists, and summarize some of the valuable physico-chemical results that have been obtained while testing the raft hypothesis. To save a useful concept from its many misuses I propose that the expression "(transient) nanodomains" be employed instead of "rafts".

1. Introduction

The membrane raft hypothesis, proposed by Simons and Ikonen in 1997 (Simons and Ikonen, 1997) has been enormously fruitful, originating thousands of published papers on the lateral structure of cell membranes. It is unfortunate that the hypothesis has not always been correctly understood in its proper terms, giving rise to misinterpretations and even laboratory artifacts, notwithstanding the important conceptual and experimental advances fueled by that proposal.

In this contribution I will review the historical path of the raft hypothesis and of its developments, and will also summarize some of the physico-chemical foundations of membrane lateral inhomogeneity.

2. Historical developments

The original authors proposed "the dynamic clustering of sphingolipids and cholesterol to form rafts that move within the fluid bilayer" (Simons and Ikonen, 1997). They also suggested that "the function of these microdomains is to serve as rafts for the transport of selected membranes or as relay stations in intracellular signaling". The origin of this idea was apparently the observation (Simons and van Meer, 1988) that glycosphingolipid clusters were formed within the exoplasmic leaflet of the Golgi membrane and that these microdomains would be sorting centres for proteins destined for delivery to the apical plasma membrane in epithelial cells. An important aspect, already mentioned in Simons and Ikonen (1997), that has remained rather constantly associated to the subsequent raft descriptions, is that these microdomains consist primarily of sphingolipid-cholesterol clusters.

The reception granted by the scientific community to the raft hypothesis was enthusiastic. The reasons for such an unusual success are difficult to ascertain with precision, and belong to the sociology of science rather than to any other branch of knowledge. At the risk of committing major mistakes or gross misapprehensions, I will mention three possible causes of success, namely that the hypothesis provided food for thought at a moment when Membrane Biophysics was not at a peak of creativity, that it appeared to provide a link between two traditionally diverging areas, i.e. Cell Biology and Biophysics/Physical Chemistry, and that the hypothesis was presented with great vigor by a very distinguished scientist, its main proponent Kai Simons. What happened in the following years was that a host of scientists embarked in this suddenly fashionable area, with the outcome of a cascade of reports in which every conceivable membrane protein was associated to rafts, so that, if true, the plasma membrane would in fact have been a giant raft, punctuated here and there by small "islands" of non-raft character.

The 1998–2005 era of raft enthusiasm was moderated with the publication of a series of sobering reports both from the points of view of Cell Biology and of Biophysics, of which some selected examples can be seen in Edidin (2003), Hess et al. (2005), Pike (2006), Veatch and Keller (2005), Heerklotz (2002), Sot et al. (2002). As a result of the Keystone Symposium on Lipid Rafts and Cell Function (March 23–28, 2006, Steamboat Springs, CO), Pike published a meeting report (Pike, 2006) that presented in a balanced and accurate way the state of the art at the time. Pike not being one of the original proponents of the hypothesis, her opinion carries an additional weight. The main point that, according to Pike, was agreed on in Steamboat Springs was the raft definition: "Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that

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compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions." The only major aspect not present in the 1997 paper is the (debated) agreement on size. Other important accepted features were: (i) the insistence on "membrane rafts" over "lipid rafts", (ii) opening the possibility of rafts in membranes other than the plasma membrane, (iii) dismissing the liquid-ordered state (Ipsen et al., 1987) as a characteristic of rafts (in fact, accepting the possibility of rafts with low cholesterol concentrations), and (iv) rejecting detergent resistance as a mark of identity for rafts (Pike, 2006). A further definition of rafts was offered by Simons and Sampaio in 2011 (Simons and Sampaio, 2011), as follows: "Presently, membrane rafts are defined as dynamic nanoscale sterol, sphingolipid-enriched, ordered assemblies of specific proteins, in which the metastable resting state can be activated to coalesce by specific lipid–lipid, protein–lipid, and protein–protein interactions". Here the rafts are included among the nano-objects. In parallel with the above refinements of the raft concept, it became clear that proteins in general partitioned in the fluid-disordered (L_α) rather than in the fluid-ordered (L_o) domains [see e.g. Yang et al., 2010], thus making the raft-related proteins a minority group in membranes.

Let us now review briefly a series of raft-related aspects that were mentioned, at least implicitly, in the original paper, and have been later dealt with from either similar or different perspectives.

2.1. Caveolae

Caveolae had been described by Yamada as cave-like depressions in the mouse gall bladder epithelium, in 1955 (Yamada, 1955). Much later, in 1993, Simons et al. found similarities between caveolae and glycolipid microdomains in the trans-Golgi network, and suggested that caveolar plasma membrane domains were related to the processes of protein and lipid sorting at the TGN (Dupree et al., 1993). In their 1997 seminal paper (Simons and Ikonen, 1997) Simons and Ikonen proposed that, since caveolae were rich in clustered GPI-anchored proteins and glycosphingolipids, proteins and lipids associated with sphingolipid – cholesterol rafts might become trapped in caveolae, thus establishing a link between rafts and caveolae that would become stronger along the years. Pike, in 2003 (Pike, 2003) proposed that caveolae should be considered as a subset of rafts, characterized by the presence of the membrane protein caveolin, and they have remained so to this day. One important property of caveolae, not shared with the other rafts, is that they are clearly visible and their limits recognizable under the electron microscope, the tool that led to their discovery.

2.2. Raft lifetimes

According to Simons and Ikonen (1997), rafts would be formed by lipid clusters from/into which "individual lipids may move in and out" explaining "why sphingolipid–cholesterol clustering is difficult to detect spectroscopically", thus underlining the transient nature of these structures. Attempts to measure raft lifetimes have taken place all along raft history, in some sense lifetime being an essential property that would give or detract credibility to/from these structures. Unfortunately, no consensus appears to have been reached, apart from the "dynamic" (Simons, 2016) or even "highly dynamic" (Pike, 2006) character of these assemblies. Veatch et al. (2008) have noted the possibility of compositional fluctuations in the plasma membrane at physiological temperatures, that would imply the rapid formation and disappearance of nm-sized inhomogeneities.

2.3. Cholesterol removal from rafts

Cyclodextrins were known to stimulate cholesterol efflux from cell membranes (Kilsdonk et al., 1995). Very soon after the raft hypothesis was proposed, they were used with the purpose of removing cholesterol from the raft microdomains (Keller and Simons, 1998). Then a variety of effects of cyclodextrins were interpreted as the result of raft cholesterol depletion, and as proofs of the existence of rafts. However, this would only be true if cholesterol in cell plasma membranes were only, or predominantly found in rafts, and/or if cyclodextrins showed a particular affinity for cholesterol in rafts. These points have not been demonstrated, and in fact the use of cyclodextrins is a frequent source of artifacts in cell membrane studies, either raft-related or otherwise.

2.4. Detergents

Simons and Ikonen (1997) incorporate into their proposal previous suggestions (Brown and Rose, 1992; Morrow et al., 1995) according to which sphingolipid-cholesterol clusters would be insoluble in Triton X-100 at 4 °C. However, they add: "One problem with Triton X-100 extraction is that the original subcellular locations of DIGs [detergent-insoluble, glycolipid-enriched complexes] are unknown. […] Only by isolating specific organelles before detergent extraction can the origin of DIGs be determined; neither can it be assumed that two proteins are in contact with each other on the basis of their partioning into DIGs". If only these wise words had been taken into account by the raft followers, many an artifact would have been spared to the scientific community.

Pike (2006), reporting on the consensus view of a notable assembly of biophysicists and cell biologists, declared that detergent resistance should not be included among the defining characteristics of rafts. To no avail. To this day, "cold extraction with Triton X-100" remains a standard procedure for raft isolation. The physico-chemical reasons why this is an essentially flawed methodology are discussed in Section 3.

2.5. Seeing the rafts

The difficulties in observing the proposed small, transient structures were made explicit in the original publication (Simons and Ikonen, 1997). From then on, a dozen or so different experimental approaches have been tried for the detection of non-caveolae rafts. According to Pike (2006) these include electronic microscopy, heterofluorescence and homofluorescence resonance energy transfer, fluorescence quenching, fluorescence lifetime imaging microscopy, fluorescence recovery after photobleaching, fluorescence correlation spectroscopy, image correlation spectroscopy, and single particle tracking. A review by van Zanten and Mayor (2015) discusses the relevance of these and other techniques in the study of membrane organization. Fig. 1 is taken from that paper. Interestingly, the lower, left angle region, where rafts should belong considering their estimated size and lifetimes, is virtually empty, i.e. no available technique could be applied to their study. However, the super-resolution microscopy techniques that are becoming available to us today and the use of membrane, lipid and protein dyes allow us to reach a spatial resolution of the order of 10–15 nm, so that these techniques could be applied to raft studies. If the FRET techniques could be reach the microsecond regime, something that appears to be feasible, the yellow region in Fig. 1 should be extended to occupy the regions ofthe nanodomains and the lower, left angle of the figure would be populated. In a recent, well-documented review, Eggeling et al. (Sezgin et al., 2017) conclude that "direct detection of these elusive domains in cell membranes, while remaining challenging, may be within reach" . An important move in the correct direction is the work by Yan et al. (2018) who have developed a method of spectrally resolved and functional super-resolution microscopy that allows the observation of low-polarity nanodomains, perhaps below 10 nm in diameter, in the plasma membrane of live mammalian cells.

2.6. Lipid rafts, and raft membrane models

The raft hypothesis was proposed for cell membranes, thus the nanodomains involved should be referred to as membrane rafts. However, perhaps because the presence of sphingolipids and cholesterol was from

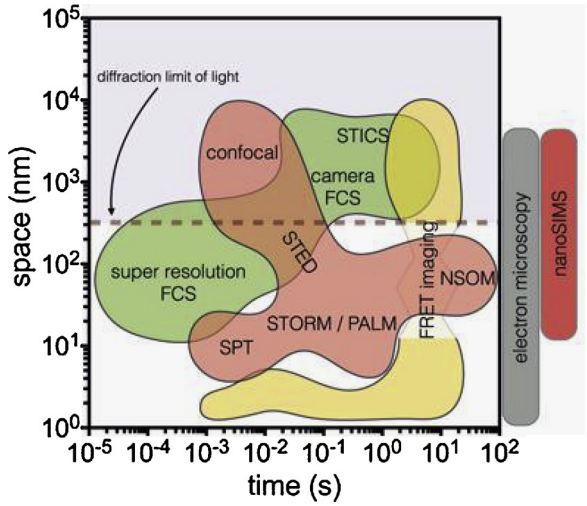

Fig. 1. Space and time resolution of current methods shedding light on the spatio-temporal fluctuations of functional chemistry that underlie cellular behavior.
Representation of the landscape that current methods occupy in the space and time axes. Abbreviations: FCS, fluorescence correlation spectroscopy; FRET, Forster resonance energy transfer; SOM, near-field scanning optical microscopy; PALM, photoactivatable localization microscopy; SPT, single particle tracking; STED, stimulated emission depletion; STICS, spatio-temporal image correlation spectroscopy; STORM, stochastic optical reconstruction microscopy. Taken from Keller and Simons (1998). Note that recent technical developments might allow the occupation of the small-size, short-time angle in the figure (see main text, *Seeing the rafts* section).

the very beginning considered as definitory for rafts, the expression "lipid rafts" has been in common use to this day as a synonym of membrane rafts. This is against the advice of the Steamboat Stream Symposium conclusions (Pike, 2006). Moreover, model membranes consisting essentially of defined lipid mixtures were, from the early years, applied to the study of membrane rafts/caveolae (Veatch and Keller, 2003a; Villar et al., 1999), even if they usually consisted of μm-sized domains in equilibrium, thus clearly different from the putative biological counterparts.

3. Biophysical aspects

3.1. Phases and phase separation

This section is abridged from Goñi (2014), Goñi et al. (2008) where the reader can find more details. A phase is defined as a region of space throughout which all physical properties of a material are uniform. "Phase" is synonym of "state of matter". E.g. water can exist in the solid, liquid or vapour phases, or states. Phases are thermodynamic concepts, i.e. ideal entities to which real objects resemble more or less. The condition of uniformity included in the definition must be understood macroscopically, at least at the micrometer scale in the context of membrane lipids.

Along the last century a number of phases were identified with properties intermediate between liquid and solid. They are collectively known as mesophases. A well known example is the liquid-crystalline phase, in which cell membranes appear mostly to exist, that is characterized by exhibiting a liquid-like fluidity, with its molecules being oriented in a crystal-like way (Goñi, 2014).

Pure membrane lipids dispersed in water adopt usually one of the lamellar phases (L). These consist of two lipid layers whose non-polar moieties are in contact and away from water. This is the disposition spontaneously adopted by most phospholipids and glycolipids, but not by cholesterol, unless in mixtures with other lipids. Several lamellar phases are known, that are relevant in the study of rafts. Most saturated membrane lipids can give rise, at room temperature, to a *gel* (or solid) L_β phase, while fully hydrated unsaturated lipids give rise to a *fluid* (or liquid-crystalline) lamellar phase L_α. In the L_α, but not in the L_β phase, the lipids exhibit unhindered translational and rotational diffusion (alkyl chain disorder). In addition a liquid ordered L_o lamellar phase has been described (Ipsen et al., 1987) that is formed in the presence of certain phospholipids and cholesterol. In this phase the lipid molecules have free lateral diffusion, i.e. they are fluid, but rotation around the alkyl chain C—C bonds is restricted (fatty acyl chains are ordered). This ordering effect of cholesterol, while keeping the lipids in the fluid phase, had been observed earlier by Rice et al. (1979). Ahmed et al. (1997) suggested that, rafts being microdomains enriched in sphingolipids and cholesterol, they could exist in the L_o state.

Not all membrane lipids are miscible (Goñi et al., 2008; de Almeida et al., 2003; Chiang et al., 2005). As early as in 1970 Chapman et al. observed, using differential scanning calorimetry, that certain saturated phosphatidylcholine species were not miscible, so that, under certain conditions, gel and fluid phases could coexist in a bilayer. Sankaram and Thompson later found that the presence of cholesterol could give rise to fluid-phase immiscibility, i.e. coexistence of separated $L_d + L_o$ phases. A large body of evidence has since confirmed these observations (see e.g. Fig. 2). Triangular phase diagrams of mixtures of phospholipids and cholesterol, constructed using a variety of techniques suggest the presence of multiple coexisting phases, e.g. $L_d + L_o$, $L_d + L_o + L_\beta$, $L_o + L_\beta$, at a given temperature, see a detailed review in Goñi et al. (2008). Importantly, the study of these mixed lipid systems in equilibrium using a combination of fluorescence recovery after photobleaching (FRET) and confocal microscopy has led to the conclusion that very small L_o domains can exist, less than 20 nm in diameter, when the proportion of L_o phase is small (de Almeida et al., 2003). Thus the possibility of nanodomains in the L_o phase in cell membranes is not against the physico-chemical principles.

It should be stressed that nanodomains in the cell membrane environment will occur influenced not only by the phase properties of lipids, but also by lipid-protein and protein-protein interactions. In this context the work by Goswami et al. (2008), Raghupathy et al. (2015) in which they show ordered membrane nanoassemblies (< 100 nm) containing GPI-anchored proteins and immobilized through the interaction of one of the lipid components with cortical actin constitutes an excellent example of nanodomains stabilized by lipid-protein interactions. In the words of Sezgin et al. (2015), commenting on Raghupathy et al. (2015): "the sources of cell membrane heterogeneity [may be] themselves heterogeneous".

3.2. Early physico-chemical studies

As mentioned above, the publication of the raft hypothesis elicited a renewed interest in the biophysical studies of lipid bilayer properties. Apart from the increased attention paid to the L_o phases, a number of interesting observations appeared in the years immediately following the original publication, and clarified important aspects of Membrane Biophysics. Studies were devoted to the reconstitution of rafts/caveolae, with defined phospholipids, cholesterol, and sometimes GPI, or GPI-bound proteins (Villar et al., 1999; Milhiet et al., 2002; Morandat et al., 2002). Marsh and et al. applied ERS spectroscopy to examine the molecular interactions of sphingomyelin and phosphatidylcholine, between them and with cholesterol (Veiga et al., 2000, 2001), and observed that cholesterol stabilized the coexistence of gel-phase and L_o domains in membranes containing sphingolipids. These authors later found, in mixtures of sphingomyelin and cholesterol studied as a function of temperature, the coexistence first of gel (L_β) and L_o phases and then of L_o and fluid (L_α) phases, with progressively increasing temperature (Collado et al., 2005). These phase coexistences are detected over a limited range of cholesterol contents. A series of studies were published on the partition of different lipids, phospholipids, sphingolipids or sterols, in "raft-like" lipid mixtures, with the result that

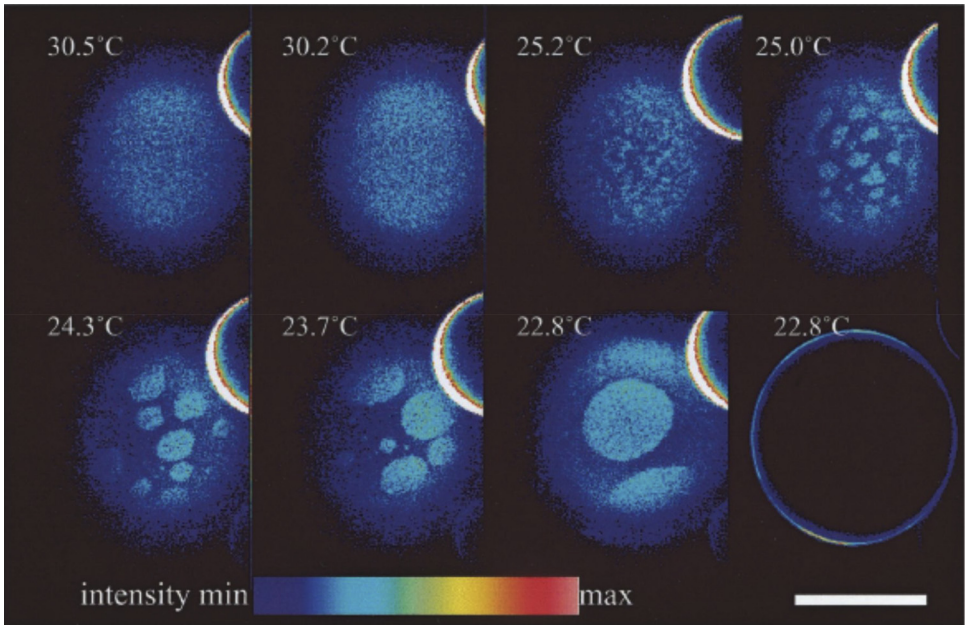

Fig. 2. An early example of membrane domain visualization in giant unilamellar vesicles (GUV) using fluorescence confocal microscopy. Two-photon excitation fluorescence intensity images (false color representation) of LAURDAN-labeled GUV as a function of temperature. Vesicles formed from DOPC/cholesterol/sphingomyelin (1:1:1) with 1 mol % GM1 ganglioside added. The lower right panel shows an equatorial section demonstrating the absence of internal vesicles. Bar, 50 μm. Domains of the order of 10 μm were seen in polar sections of the "raft lipid" GUV by scanning two-photon fluorescence microscopy as the vesicles were cooled through 25 °C. Domains were not seen in GUVs formed from non-raft lipids. Taken from Dietrich et al. (2001).

polar head groups, alkyl chains and sterol structure were all very important in promoting raft formation (Wang and Silvius, 2000; Xu and London, 2000; Miao et al., 2002; Shaikh et al., 2002; Armstrong et al., 2002). Xu and London (2000) found that those sterols that promoted tight packing of saturated lipids promoted domain formation. Miao et al. (2002) found that cholesterol was more efficient than its metabolic precursor lanosterol in forming L_o phases.

An important breakthrough was the development of giant unilamellar vesicles (GUV) in which domain separation could be conveniently monitored by fluorescence confocal microscopy (Veatch and Keller, 2005; Dietrich et al., 2001; Veatch and Keller, 2003b). Similar lateral inhomogeneities were observed in supported planar lipid bilayers (Samsonov and Mihalyov, 2001). Note however that in these systems stable, μm-sized domains are observed and used as models for the transient nanodomains, an often forgotten limitation of these studies. The same size/lifetime limitation applies to the reports in which atomic force microscopy was applied to the observation of lateral phase separation in planar bilayers (Rinia et al., 2001; Yuan and Johnston, 2001; Yuan et al., 2002). This and other related problems are aptly discussed in de la Serna et al. (2016).

Although many more details have been elucidated in subsequent studies, the data described in this section are enough to substantiate the main physico-chemical contributions to the raft hypothesis, namely that mixtures of certain sphingolipids (or saturated glycerophospholipids) with unsaturated phospholipids and cholesterol give rise to macroscopic lateral phase separation of sphingolipid-cholesterol domains. Thus similar phenomena of lateral inhomogeneities occurring in the cell membranes would not be in violation of thermodynamic rules.

3.3. Detergent resistant membranes

The early identification of detergent resistant membrane fractions with rafts became soon, in spite of the caveats expressed by Simons and Ikonen (1997) (see above), a source of possible artifacts. This was shown at a rather early stage by Sot et al. (2002), who concluded from their studies that "detergent resistant membranes obtained after detergent treatment may be the result of detergent-induced bilayer partial solubilization and reassembly, instead of corresponding to structures existing in the native membrane" (Fig. 3). Shortly afterwards, Heerklotz (2002) demonstrated, mainly on the basis of calorimetric measurements, in which an equimolar mixture of unsaturated phosphatidylcholine, sphingomyelin and cholesterol (presumably an ideal "raft mixture") was treated with increasing Triton X-100 contents, that addition of the detergent promoted the formation of L_o domains. Therefore, detergent-resistant membranes "should not be expected to resemble functional rafts regarding their abundance, size, composition, or even existence". Lingwood and Simons (2010) emphasized the limitations of the detergent method for raft isolation in their 2007 review.

Other flawed assumptions have pervaded the raft preparation methodology, *e.g.* that at low temperature the detergent would be less effective, when it is usually the opposite, or that sphingolipids would be detergent-insoluble, when they are, if anything, easier to solubilize than glycerophospholipids. These and other related matters were reviewed by Lichtenberg, Goni and Heerklotz in a paper whose title proclaimed that "Detergent-resistant membranes should not be identified with membrane rafts" (Lichtenberg et al., 2005). It is unfortunate that, against all the physico-chemical evidence, presumed rafts are still being isolated "by the method of cold Triton treatment", perhaps the single main source of artifacts in the whole membrane field.

4. The inconsistencies, and a proposal

Criticism of several aspects of the raft hypothesis, and of the experimental work developed thereof, appeared rather soon (Munro, 2003). Pike, in 2006 (Pike, 2006), referred to rafts as "an entity that is difficult to visualize, has an ill-defined molecular composition, and whose very existence has been questioned". As we have seen in the above paragraphs, the situation has not changed much along the years. There are still important inconsistencies to be clarified, among them:

- Membrane raft models currently in use often consist of bilayers containing μm-sized domains in equilibrium, while the biological structure they are purported to represent is a transient (i.e. short-lived) nm-sized object.
- There is no agreement about the size and lifetime of rafts in cell membranes. In the words of Enrico Gratton (personal communication) "what was generically called rafts is in reality a much richer collection of nanostructures that we are deciphering as new super-resolution and fast imaging techniques are becoming available".
- Cold extraction with Triton X-100 remains a standard procedure for raft isolation, despite the abundant evidence that precludes its use for being a source of artifacts.

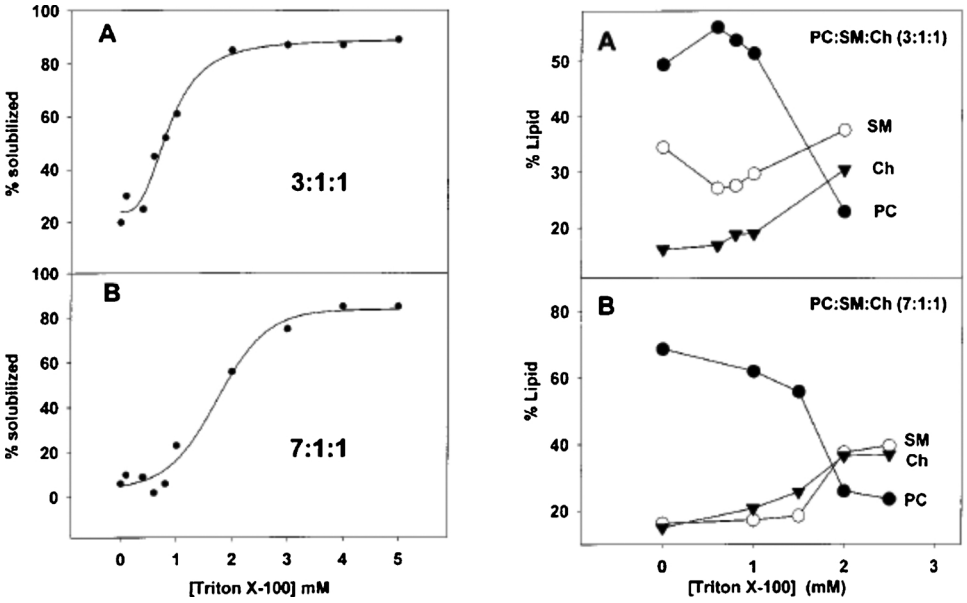

Fig. 3. Generation of raft-like detergent-resistant membranes as a result of Triton X-100 partial solubilization of lipid bilayers. *Left-hand side.* Pure lipid vesicle solubilization by Triton X-100 assessed by centrifugation. The percent lipid phosphorus recovered in the pellet after centrifuging the detergent-treated membranes is taken as the fraction of non-solubilized membrane. (A) PC/SM/Chol, 3:1:1 mol ratio. (B) PC/SM/Chol, 7:1:1 mol ratio. The pellets were resuspended and the lipids extracted in chloroform/methanol (2:1). The organic extracts were applied to thin-layer chromatography plates so that the three lipids, PC, SM, and Chol, could be separated and quantitated. The results of pellet analysis are shown in the *right-hand side* panels. For both lipid compositions, the pellet became depleted in PC, thus relatively enriched in SM and Chol as solubilization progressed. For example, at 2 mM Triton X-100, the non-solubilized fraction of the original 7:1:1 sample contained PC/SM/Chol at an approximate molar ratio of 0.7:1:1 and the pellets from the 3:1:1 sample contained PC/SM/Chol at ca. 0.8:1:1.2, which is in both cases relatively close to the 1:1:1 ratio that some authors have considered as paradigmatic of detergent-resistant membranes. All experiments were performed at 4 °C. Taken from Sot et al. (2002).

- Attempts to define structural elements like rafts by alluding to their functional properties should be avoided.

While the raft hypothesis deserves a place of honor in the history of biomembrane studies, mainly for the amount of interesting research it has generated, the unfortunate truth is that the name “raft” has ended being associated to too many artifacts and inconsistencies, with basic doubts that remain unsolved two decades after its proposal. I would suggest, following an increasing number of authors, that for the sake of precision we speak of *(transient) nanodomains* when we want to refer to the putative, heterogeneous cell membrane structures, 10–200 nm in size, often known as *rafts*.

Conflict of interest

None.

Acknowledgments

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