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Review

Dynamics of raft molecules in the cell and artificial membranes: approaches by pulse EPR spin labeling and single molecule optical microscopy

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Abstract

Lipid rafts in the plasma membrane, domains rich in cholesterol and sphingolipids, have been implicated in a number of important membrane functions. Detergent insolubility has been used to define membrane "rafts" biochemically. However, such an approach does not directly contribute to the understanding of the size and the lifetime of rafts, dynamics of the raft-constituent molecules, and the function of rafts in the membrane in situ. To address these issues, we have developed pulse EPR spin labeling and single molecule tracking optical techniques for studies of rafts in both artificial and cell membranes. In this review, we summarize our results and perspectives obtained by using these methods. We emphasize the importance of clearly distinguishing small/unstable rafts (lifetime shorter than a millisecond) in unstimulated cells and stabilized rafts induced by liganded and oligomerized (GPI-anchored) receptor molecules (core receptor rafts, lifetime over a few minutes). We propose that these stabilized rafts further induce temporal, greater rafts (signaling rafts, lifetime on the order of a second) for signaling by coalescing other small/unstable rafts, including those in the inner leaflet of the membrane, each containing perhaps one molecule of the downstream effector molecules. At variance with the general view, we emphasize the importance of cholesterol segregation from the liquid-crystalline unsaturated bulk-phase membrane for formation of the rafts, rather than the affinity of cholesterol and saturated alkyl chains. In the binary mixture of cholesterol and an unsaturated phospholipid, cholesterol is segregated out from the bulk unsaturated liquid-crystalline phase, forming cholesterol-enriched domains or clustered cholesterol domains, probably due to the lateral nonconformability between the rigid planar transfused ring structure of cholesterol and the rigid bend of the unsaturated alkyl chain at C9–C10. However, such cholesterol-rich domains are small, perhaps consisting of only several cholesterol molecules, and are short-lived, on the order of 1–100 ns. We speculate that these cholesterol-enriched domains may be stabilized by the presence of saturated alkyl chains of sphingomyelin or glycosphingolipids, and also by clustered raft proteins. In the influenza viral membrane, one of the simplest forms of a biological membrane, the lifetime of a protein and cholesterol-rich domain was evaluated to be on the order of 100 μs, again showing the short lifetime of rafts in an unstimulated state. Finally, we propose a thermal Lego model for rafts as the basic building blocks for signaling pathways in the plasma membrane.

Keywords: Raft molecule; Artificial membrane; Single molecule optical microscopy

1. Introduction

Many important functions of cellular membranes are closely associated with various specialized domains in the membrane. These membrane domains include stable micrometer-sized domains such as adherens junctions and focal adhesions, and 100-nm domains like clathrin-coated pits (whose lifetime may be less than a minute) and synapses, and could include very small molecular complexes like receptor clusters [1–6]. In particular, raft domains, which are present in the plasma and Golgi membranes, have recently been drawing extensive attention, because these domains may play important roles as a platform form signal transduction and protein sorting in these membranes [7–19]. Therefore, understanding the molecular mechanisms by

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which these domains are formed, maintained, and disintegrated has become one of the central issues in membrane biophysics and cell biology today [4–16]. Rafts might arise through lipid–lipid interactions that form liquid-ordered-phase-like domains, and might be stabilized or coalesced by GPI-anchored or transmembrane proteins cross-linked by lectins, ligands, or antibodies. Caveolae may be thought of as rafts stabilized by caveolin assembly [20–22]. It should be noted that many membrane domains are very dynamic structures and may be forming and dispersing (or becoming internalized) continually, and/or their constituent molecules may frequently enter/exit from the domain [5]. For example, even clathrin-coated pits, which appear to be large and stable in electron micrographs, take several tens of seconds to form and become internalized [23]. These dynamic processes associated with rafts are the major subjects of the present review.

Detergent insolubility is mainly used to define raft domains biochemically in the membrane [7,24–28]. In fact, a hallmark of a raft-constituent molecule is that it is recovered in the low-density fraction after cold Triton extraction and sucrose density gradient centrifugation. This low-density fraction is often called the detergent-resistant membrane (DRM) fraction. However, such biochemical approaches provide limited information on the rafts and the mechanism by which they function in the membranes of live cells [22,29–34]: one would want to know the morphology, lifetime, molecular organization, and dynamics of the raft-constituent molecules and the raft itself in the membrane.

To address these issues, we and our colleagues have developed and applied pulse EPR spin labeling methods and single molecule optical techniques [35–40]. These methods are sensitive to the entry and exit of the probe molecules from the rafts. The aim of the present review is to briefly summarize our results on cholesterol-enriched domains and rafts obtained during the past 15 years. For broader reviews of this field, the readers are encouraged to read the other excellent reviews cited here and also those collected in this special issue of Reviews of Biomembranes. The main points we would like to emphasize here are the following. (1) In the steady state without an extracellular stimulus, the rafts may be very small in size, perhaps consisting of only several molecules, and their lifetimes are quite short, perhaps on the order of a millisecond or less [41,42]. This may be coupled to the short residency times (about the same time spans) of the raft-constituent molecules in the raft. (2) When the cells are stimulated, activated GPI-anchored receptors, such as CD59, are clustered, and the cluster induces a stabilized raft in and around it (core receptor raft, lifetime over a minute), and the core receptor raft diffuses as a small entity [41,42]. (3) As the core rafts diffuse around in the cell membrane, they induce greater but transient rafts (transient confinement zones (TCZs), lifetimes <1 s), perhaps by coalescing small rafts, each of which may contain one downstream effector molecule (signaling rafts) [41,42]. (4) In the binary mixture of cholesterol and an unsaturated phospholipid, such as L-α-dioleoylphosphatidylcholine (DOPC), cholesterol is segregated out from the bulk DOPC phase, forming cholesterol-enriched domains or clustered cholesterol domains, due to the structural conflict between the rigid ring skeleton of cholesterol and the rigid bend of the unsaturated chain at C9–C10. However, such a domain is small and likely to consist of only several cholesterol molecules, and is short-lived, on the order of 1–100 ns [43]. The following two points are our speculations. (5) These cholesterol-enriched domains may be stabilized by the presence of the saturated alkyl chains of sphingomyelin or glycosphingolipids, and also by the clustered raft proteins. In the influenza viral membrane, one of the simplest forms of a biological membrane, the lifetime of a protein-rich, cholesterol-rich domain was found to be on the order of 100 μs [44], again showing the small/unstable nature of rafts in unstimulated cells. (6) At variance with the general view (e.g., Ref. [18]), the main thrust for the formation of rafts may not be the affinity of cholesterol and saturated alkyl chains, but rather the segregation of cholesterol out of the liquid-crystalline unsaturated bulk-phase membrane. Saturated alkyl chains may be associated with both the cholesterol-enriched domains and the disordered bulk domain, optimizing both structures (and possibly the interface between the two domains [45]) for the minimum (possibly local and temporal minimum) free energy of the whole membrane.

2. Cholesterol is segregated out in unsaturated phospholipid membranes due to the lateral nonconformability between the rigid steroid ring of cholesterol and the rigid bend of the *cis* double bond at C9–C10

Employing both conventional and pulse EPR spectroscopies, Subczynski, Kusumi, and their colleagues observed the behavior of both fatty acid probes (*n*-doxylstearic acid spin labels, *n*-SASLs; 14-eicosanoic acid spin label, 14-EASL) and spin labeled cholesterol analogues (cholestane spin label, CSL, and androstane spin label, ASL) in both saturated and unsaturated phosphatidylcholine (PC) membranes containing various amounts of cholesterol [46–51]. The chemical structures of these spin labels and their approximate locations in the lipid bilayer are shown in Fig. 1. We assume that, although *n*-SASLs and 14-EASL partition into both the bulk and cholesterol-enriched domains, the presence of the bulky doxyl group (see the ring structure attached to *n*-SASLs in Fig. 1) on various places on the alkyl chain causes the fatty acid spin probes to partition into the bulk domain more than the cholesterol-enriched domain. This tendency may decrease as the position of the bulky doxyl moiety moves down along the alkyl chain toward the methyl terminal (toward the middle of the bilayer; recall that these fatty acids are saturated chains and could partition into raft domains). As shown later, in the

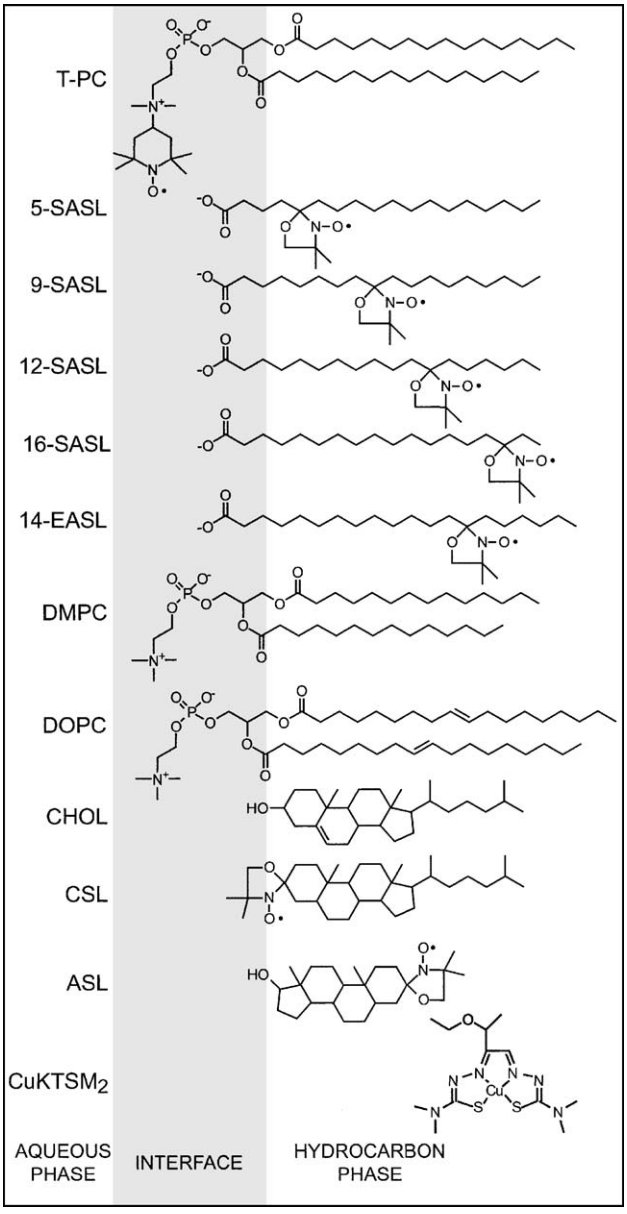

Fig. 1. Chemical structures of lipids (DMPC, DOPC, cholesterol = CHOL), phospholipid-type spin probes (T-PC and *n*-SASL), cholesterol-type spin probes (CSL and ASL), and $CuKTSM_2$ described in this review. Approximate locations across the membrane are also illustrated. However, since alkyl chains tend to have many *gauche* conformations, the chain length projected to the membrane normal would be shorter than that depicted here, and the tetracyclic ring structure of cholesterol would reach the C9–C10 *cis* double bond in DOPC in the liquid-crystalline phase.

case of 14-EASL, which has the doxyl group in the inner part of the membrane (on C14), each 14-EASL molecule stays in the bulk domain two-thirds of the time in the influenza viral membrane (and one-third of the time in the cholesterol-rich, protein-rich raft domains), or at any moment, two-thirds of the 14-EASL in the membrane is localized in the bulk region (or one-third of the 14-EASL is localized in the raft domain [44]).

As such, it is important to realize that very few molecules are exclusively localized in either the raft or the bulk domains. Even in the cases of typical raft-preferring molecules like Lyn, approximately half of the molecules reside in the bulk domain (unpublished observation, Iino and Kusumi). The partitioning behavior depends on the cell type, perhaps reflecting the amount of the raft domain in the membrane as well as the aggregation state of the molecule, which may be controlled by extra- and intracellular signals. Therefore, the difference between raft and non-raft molecules is quantitative, and not either/or. However, if one is considering the number density (concentration) of molecules in the raft domain, then it could be considerably higher than that in the bulk domain. Even if half of a molecular species is localized in the raft, if the raft occupies only 10% of the membrane area, then the molecule is 10 times more concentrated in the raft domain. Much confusion in the raft research field appears to stem from the mix-up of the concepts of concentration (the number density) and the absolute amount (the total number) of a molecule of interest located in the raft domain.

The following are two key observations made in conventional EPR studies. Conventional EPR of the nitroxide spin probes (N–O, a free radical) is sensitive to reorientational diffusion (thermally driven random rotational motion) in the time scale (rotational correlation time) between 0.01 and 10 ns.

(1.1). In *cis*-unsaturated membranes (DOPC and egg-yolk phosphatidylcholine (EYPC)) the cholesterol effect on the reorientation diffusion of the nitroxide group attached to cholesterol-analogue spin probes (CSL and ASL) is much greater than that on *n*-SASLs.

(1.2). In saturated PC membranes, the rates of reorientational diffusion of both groups of spin labels (cholesterol- and fatty acid-analogue probes) are greatly reduced by the presence of cholesterol, and the extent of this reduction is much greater than that in *cis*-unsaturated PC membranes.

These results indicate that *cis*-unsaturated PC is less miscible with cholesterol at physiological temperatures than saturated PC, i.e., cholesterol and cholesterol-type spin labels are segregated out from the bulk domain of unsaturated PC, but not as much in saturated PC, while the major effect of cholesterol on saturated PC is to mix with the PC at certain ratios and to enhance the *trans* configuration of the saturated hydrocarbon chain. This leads to great reductions of the reorientational diffusion of both cholesterol- and fatty acid-analogue probes, consistent with the observations made by Shin and Freed [52,53], Shin et al. [54], and Vist and Davis [55].

In order to explain these results, it was proposed that the key feature of the interactions between *cis*-unsaturated PC and cholesterol is the considerable nonconformability (conflict) between the molecular shapes of these molecules in the membrane (Fig. 2a). The cholesterol backbone is the rigid planar transfused tetracyclic ring structure of a sterol (Fig. 1), which reaches up to the *cis* double bond at C9–C10 of the

extended alkyl chains and to a somewhat deeper level in the hydrophobic region of the membrane in the liquid-crystalline phase [56,57] (in Fig. 1, the extended alkyl chain in PCs is shown, and therefore, the tetracyclic ring structure of cholesterol seems to barely reach the C9–C10 double bond, but in the liquid-crystalline state, the rigid ring structure of cholesterol reaches the double bond easily). The rigid skeleton of cholesterol and the rigid bend structure of the *cis* double bond in DOPC (and in EYPC, which mainly contains palmitoyl and stearoyl chains on the C1 position of the glycerol backbone, and oleoyl and linoleyl chains on the C2 position of the glycerol) are not likely to conform to each other when they are in direct contact in the membrane (Fig. 2a). Although the effect of this sharp rigid bend could be somewhat reduced by the simultaneous occurrence of kinks in the oleoyl chain [56,57], the *cis*-double bond would certainly create serious problems in the packing of cholesterol and unsaturated chains in the membrane.

We propose that, due to such nonconformability, the cholesterol molecules tend to be excluded from the unsaturated bulk domains and segregated out, as shown in Fig. 2c, whereas they tend to mix to a certain degree with saturated alkyl chains (Fig. 2b). In biological membranes where unsaturated phospholipids and cholesterol are the dominant lipid species, it is likely that cholesterol-rich and unsaturated PC-rich domains coexist.

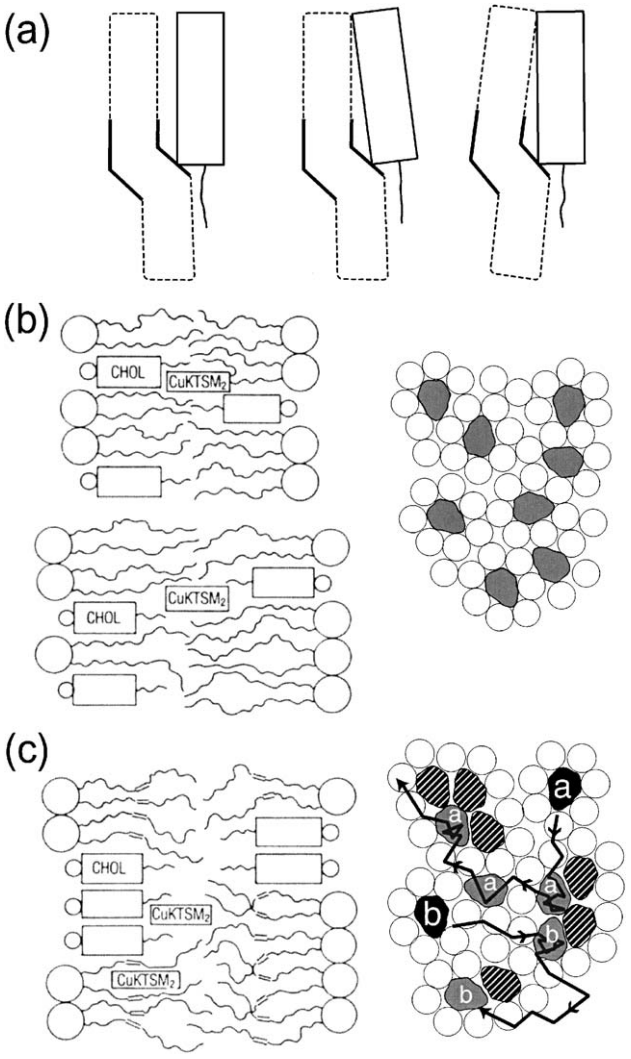

Fig. 2. Schematic drawings showing the interaction of cholesterol with *cis*-unsaturated and saturated PC molecules in the membrane. (a) A static view of the nonconformability between the rigid structure of cholesterol (rectangles with short tails) and the rigid bend at the C9–C10 *cis* double bond in an unsaturated DOPC hydrocarbon chain (bent rods). Due to this nonconformability, placing cholesterol and unsaturated alkyl chains side-by-side in the membrane creates packing problems. This figure also shows that due to the mismatch in the lengths between the cholesterol and the alkyl chains, the assembly of cholesterol could induce vacant pockets (packing defects) in the central part of the membrane. (b and c) Snapshot drawings of the side view (left) and the top view (right) of DMPC-cholesterol (b, upper), DSPC-cholesterol (b, lower), and DOPC-cholesterol (c) membranes with $CuKTSM_2$. In the top view, the open circles represent phospholipid hydrocarbon chains, and the solid structures indicate cholesterol molecules (after Martin and Yeagle [100]). The top view of c shows that cholesterol molecules, like those designated as "a" and "b", frequently and repeatedly become incorporated in cholesterol clusters and dissociated. To simplify the presentation, the movement of the cholesterol with hatching is not shown. This figure is designed to convey this message, and is not intended to show the two-dimensional arrangement of phospholipid and cholesterol. See Refs. [104,105] for further information.

3. Tiny cholesterol-rich domains may be continuously forming and dispersing with a lifetime on the order of 1–100 ns in artificial unsaturated PC-cholesterol bilayers

The information gained by conventional EPR spectroscopy is based on the reorientational diffusion of the nitroxide moiety placed on lipid probes in the membrane, and is limited to the events that take place in time scales shorter than 10 ns. In order to gain more insights into the size and the lifetime of the cholesterol-rich domain, pulse EPR experiments were performed, in which the spin–lattice relaxation times (T_1's) of the nitroxide spin probes were measured. Since the T_1 of spin labels (1–10 μs) is much longer than the reorientational correlation time of the nitroxide group on lipid probes (0.01–1 ns), membrane dynamics in longer time–space scales can be observed in pulse EPR experiments. In particular, dual-probe pulse EPR experiments turned out to be useful. In these investigations, small paramagnetic molecules, molecular oxygen (O_2, MW = 32) [50,51,58] or a square-planar copper complex, 3-ethoxy-2-oxobutyraldehyde bis(N^4,N^4-dimethylthiosemicarbazonato)copper(II), called $CuKTSM_2$ (MW = 394, see Fig. 1 for its chemical structure) [43,59], were introduced in the membrane, and the rate of bimolecular collision between O_2 or $CuKTSM_2$ with the nitroxide moiety of a lipid-type spin label placed in the membrane was evaluated from the T_1's of the spin labels. Since the T_1's of O_2 and $CuKTSM_2$ are much shorter than those of the nitroxide spin probes, the collision of O_2 or $CuKTSM_2$ with the nitroxide induces instantaneous relaxation of the nitroxide spins.

The oxygen transport parameter (oxygen transport rate) was introduced as a convenient quantitative measure of the rate of the collision between the spin probe and molecular oxygen by Kusumi et al. [58] as:

$$\text{Oxygen transport parameter } W(x) = T_1^{-1}(\text{Air},\ x) - T_1^{-1}(N_2,\ x), \quad (1)$$

where the T_1's are the spin–lattice relaxation times of the nitroxides in samples equilibrated with atmospheric air and nitrogen, respectively. The collision rate is also proportional to the local oxygen diffusion–concentration product (thus, it is called the "transport" parameter, and has nothing to do with active transport across the membrane) at a "depth" x in the membrane that is in equilibrium with atmospheric air. Kusumi et al. [58] concluded that the oxygen transport parameter is a useful monitor of membrane fluidity that reports on translational diffusion of small molecules.

By analogy to the oxygen transport parameter, the $CuKTSM_2$ transport parameter was defined as:

$$\text{CuKTSM}_2 \text{ transport parameter } (x) = T_1^{-1}(2 \text{ mol\% CuKTSM}_2, x) - T_1^{-1}(\text{No CuKTSM}_2, x). \quad (2)$$

where the T_1's are the spin–lattice relaxation times of nitroxides in deoxygenated membranes measured in the presence and absence of 2 mol% $CuKTSM_2$ [43]. Profiles of the oxygen transport parameter (oxygen diffusion–concentration product) and the $CuKTSM_2$ transport parameter ($CuKTSM_2$ diffusion–concentration product) across the membrane provide useful information on the membrane organization and dynamics (Fig. 3) [43,50,51,58–63]. These molecules are more soluble in the membrane phase than in water ($CuKTSM_2$ is practically insoluble in water).

The major results obtained by Subczynski et al. [43] can be summarized as follows.

(2.1). In the liquid-crystalline phase of saturated PC membranes (e.g., DMPC), the incorporation of cholesterol decreases the collision rate between the nitroxide moiety and $CuKTSM_2$ at all depths in the membrane (Fig. 3, left).

(2.2). In *cis*-unsaturated PC membranes (DOPC and EYPC), virtually no effect of cholesterol on the $CuKTSM_2$ diffusion–concentration product (transport rate) was observed, either with cholesterol-type or phospholipid-type spin labels (Fig. 3, right). This is in clear contrast with the results obtained with the conventional EPR spectroscopy as described in the previous section (1.1 in particular).

Apparently, the second result (2.2) is contradictory to the previous finding made by using conventional EPR spectroscopy (1.1). How is it possible that the $CuKTSM_2$ transport rate in the cholesterol-rich domain is the same as that in the bulk domain, and yet the reorientational diffusion of cholesterol-type spin labels is greatly reduced in the cholesterol-rich domain? Since conventional EPR spectroscopy indicates that the rotational correlation time of the cholesterol-type probes after the addition of cholesterol is on a time scale longer than 1 ns, and since the $CuKTSM_2$ transport rate is on the order of $3 \times 10^6\ s^{-1}$, (or the characteristic time of ≈ 100 ns), this controversy can be resolved if the lifetime of the cholesterol-rich domains (or cholesterol oligomers) is between 1 and 100 ns and/or if the size of the domain is so small that all of the molecules in the domain can contact the bulk domain (see the model displayed in Fig. 2b and c). Perhaps these two characteristics, their short lifetimes and small sizes, are coupled. The models illustrated in Fig. 2c show that the fluid-phase immiscibility is prevalent in *cis*-unsaturated PC-cholesterol membranes, but where the cholesterol-rich (cholesterol-oligomeric) domains are small (several molecules) and/or short-lived (1–100 ns, for a previous review, see Refs. [5,64]). Of

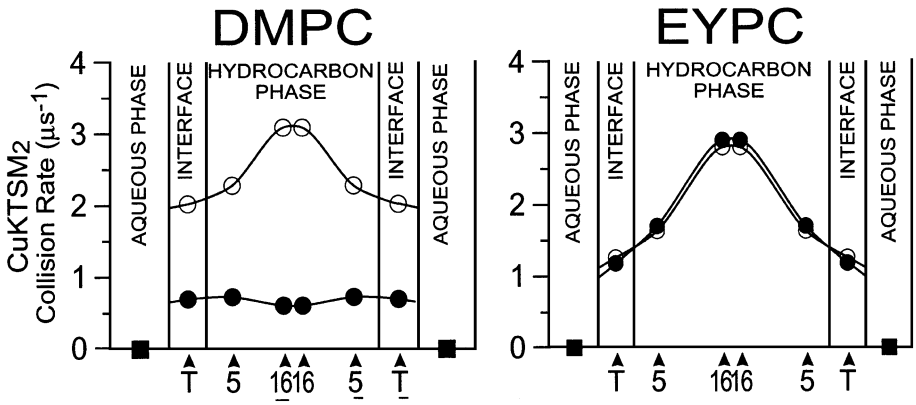

Fig. 3. Profiles of the rate of collision of the nitroxide probe attached to various places of phospholipid-type spin labels with $CuKTSM_2$ ($CuKTSM_2$ transport rate, Eq. (2)), observed at 40 °C across DMPC and EYPC membranes in the absence (open circle) and presence (closed keys) of 30 mol% cholesterol. Approximate locations of the nitroxide moiety of the spin labels are indicated by arrowheads. The underlined numbers for *n*-SASL in DMPC indicate that these SASLs are intercalated in the right half of the bilayer, but the nitroxide attached to the C16 may pass through the center of the bilayer and stay in the other leaflet of the membrane (SASLs are longer than the host DMPC). T indicates Tempo-PC. The closed squares indicate the value in the aqueous phase. Data adapted from Subczynski et al. [43].

course, due caution is required for the results obtained with unnatural probes, but these results obtained with various probes are all consistent.

These conclusions are consistent with the data by Radhakrishnan et al. [65]. They demonstrated that cholesterol and sphingolipids form small complexes consisting of 15–30 molecules.

4. Submillisecond exchanges of lipids between the raft and the bulk domains and/or formation/dispersion of rafts in the influenza viral envelope

One of the simplest paradigms for biomembrane studies is the influenza virus (IFV) envelope membrane, which is made of a lipid bilayer containing only two glycoproteins, hemagglutinin and neuraminidase with a single transmembrane α-helix [66,67], which represent ~ 80% and ~ 20% of IFV membrane proteins, respectively [68]. Hemagglutinin exists as trimers [69] and neuraminidase forms tetramers [70] in the membrane. The lipid composition may be similar to that of the plasma membrane of host animal cells: IFV membranes are rich in cholesterol, the mole fraction of (total lipid-minus-cholesterol): cholesterol: (hemagglutinin-plus-neuraminidase) is 60:40:2 [44,64,68,71,72]. IFV membranes contain large amounts of detergent-insoluble raft lipids [73], and hemagglutinin preferentially interacts with the sphingolipid-cholesterol raft domains via its transmembrane α-helical fragment [9,74]. Biological membranes with high concentrations of cholesterol are likely to contain a liquid-ordered phase [26,75].

Initial experiments using the oxygen transport parameter (collision rate of molecular oxygen and the nitroxide moiety of the spin label) showed that IFV membrane contained two membrane domains with slow and fast oxygen transport rates [44]. The domain showing slow oxygen transport was termed the SLOT domain [63]. Although the SLOT domains have been found in artificially reconstituted membranes [63], this was the first case where the SLOT domain was detected in a biological membrane. The oxygen transport rate in the SLOT domain is smaller than that in the bulk domain by a factor of 16 (Table 1), which is a very large factor in such experiments. It is smaller than that in the purple membrane, which is a two-dimensional crystal of bacteriorhodopsin trimers with very small amounts of lipids intercalated in the space between protein molecules, and which showed a lower oxygen transport rate than any other membrane before IFV measurements were made (Table 1). The oxygen transport rates in purple membrane (measured with lipid probes) are greatly reduced because the transport rate within rhodopsin (protein itself) is very low, smaller than those in the fluid-phase lipid bilayer by a factor of ~ 100 (Table 1, Ref. [60]), and thus bacteriorhodopsin is a major barrier for oxygen transport in purple membrane.

Based on these observations, Kawasaki et al. [44] proposed that the SLOT domain in the IFV membrane may be a protein-rich and cholesterol-rich domain (see Fig. 4), because packing proteins at densities higher than that found in purple membrane appears to be difficult and the presence of cholesterol in the membrane is known to reduce the oxygen collision rate [50,51]. The SLOT domain in the IFV membrane may be a raft domain that is rich in cholesterol and stabilized by the presence of clustered proteins (Fig. 4). Another important possibility is that the clustering of hemagglutinin and neuraminidase may be assisted or even mediated by cholesterol. In fact, Kawasaki et al. [76] found that cholesterol was required for the successful functional reconstitution of hemagglutinin into artificial membranes, just as it was required for caveolin reconstitution [77].

When the oxygen concentration in the IFV sample was varied (by varying the partial oxygen pressure of the gas mixture in which the sample is equilibrated) during the initial series of experiments, it was found that the observed values of the oxygen collision rate were not proportional to the partial oxygen pressure (local oxygen concentration) in either the raft or the bulk domain. This result suggests the presence of rapid exchanges of lipids between the raft and the bulk domains (or rapid formation and dispersion of rafts) at a rate greater than $10^4\ s^{-1}$ (approximately the slowest rate measurable with pulse EPR T_1 experiments using nitroxide spin probes). Such a short lifetime of the lipid residency in a raft or of the raft itself was totally unexpected. In the following, for the ease of presentation, we use terms like "exchange of lipids" to indicate both simple exchanges of

Table 1
Oxygen transport parameter (the collision rate of molecular oxygen with the nitroxide spin probe in a sample equilibrated with atmospheric air) observed at 30 °C using 5-SASL (the hydrophobic region near the surface) and 16-SASL (the central part of the bilayer)

Membranes	5-SASL (5-PC)	16-SASL (16-PC or 14-EASL)	References
DMPC	1.2	3.7	[63]
DMPC + 50% CHOL[a]	0.26	3.1	[50]
DOPC	1.5	2.8	[51]
DOPC + 50% CHOL	0.61	5.0	[51]
EYPC	1.8	2.8	[51]
EYPC + 50% CHOL	0.49	3.9	[51]
POPC (20 °C)	1.1	2.2	[62]
POPC + 20% CHOL (20 °C)	0.49	ND[b]	note[c]
POPC + 10% L_{24} (20 °C)	0.90	2.1	[62]
POPC + 10% $(LA)_{12}$ (20 °C)	0.73	1.4	note[c]
BR/DMPC = 1:80	0.93	2.4	[63]
BR/DMPC = 1:40, BULK	1.2	2.1	[63]
BR/DMPC = 1:40, SLOT	0.50	0.80	[63]
Purple membrane	0.37	0.30	[44]
Gel-phase DMPC at 20 °C	0.39	0.39	[62]
Rhodopsin (at β-ionone ring of retinal)	0.02	0.02	[60]
IFV, BULK	ND	2.2	[44]
IFV, SLOT	ND	0.14	[44]

[a] The percentage represents the molar percentage. CHOL = cholesterol.
[b] ND = not determined.
[c] Unpublished data (Subczynski).

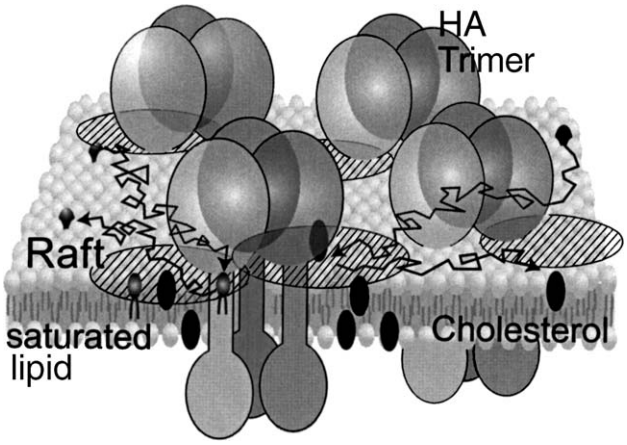

Fig. 4. Small and transient raft domains may be formed in the vicinity of (and include) hemagglutinin trimers (and neuraminidase tetramers, not shown in this figure) due to the slight suppression of thermal motion around the hemagglutinin trimers. The lifetimes of the rafts or the residency times of the raft-associated molecules in the rafts may be on the order of 100 μs or less. On average, the 14-EASL molecule spends one-third of the time in raft domains. Since the oxygen transport rate is very low in these raft domains, they are likely to contain high concentrations of hemagglutinin trimers and cholesterol.

lipids and exchanges of lipids due to the formation and dispersion of the raft domains themselves.

Kawasaki et al. [44] developed a method to measure the exchange rates, and found that the exchange rates from and to the SLOT (protein- and cholesterol-rich raft) domain are 7.7 and 4.6×10^4 s^{-1}, respectively. Such fast exchange rates indicate that the molecules in one domain can reach the other domain within this time scale, and thus suggest that both the SLOT and the bulk domains are small. Therefore, it is concluded that the raft and the bulk domains form fine, intricate mosaics with constantly changing patterns.

Such short lifetimes and small sizes of rafts are consistent with the very delicate balance for molecular partitioning between the bulk and the raft domains. Quite small changes in the oligomerization states of membrane proteins or in the interactions of proteins with cholesterol, due to protein oligomerization or conformational changes, could shift the delicate balance, leading to large changes in the stability and the size of the rafts. In this sense, for future investigations of rafts, both concepts, the protein-stabilized raft domains and the raft domains formed by cholesterol-induced clustering of proteins, would be useful.

One might wonder about the difference between such a raft domain formed near the clustered transmembrane proteins and the boundary lipids, which are lipids in direct contact with transmembrane proteins. While these rafts may also be located near the clustered proteins and stabilized by them, the exchange rates between the SLOT-raft domain and the bulk domain are a factor of ~ 300 smaller than those for the lipids between the bulk and the boundary regions around transmembrane proteins [63,78–81]. The longer residency time in the SLOT domain suggests that it may play an important role in the function of the plasma membrane. The SLOT domain, either the protein-stabilized raft domain or the raft domain formed by the cholesterol-induced clustering of proteins, may be important in packing the IFV membrane proteins during the budding process and in increasing the probability of successful fusion events by concentrating hemagglutinin in the raft domain [73,74,82].

Each SLOT domain may be small, but the entire SLOT domain occupies a substantial area in the IFV membrane. From the ratio of the inbound (K_2) to the outbound (K_1) rates of the lipid in the SLOT domain, the fraction of the SLOT domains as a whole can be estimated, and it turns out that the SLOT domains may occupy about one-third of the membrane $\{K_2/(K_1+K_2) = 0.046/(0.046+0.077) = 0.37\}$. Similarly, Mayor and Maxfield [83a] concluded that raft-domains may occupy half of the total plasma membrane area of cultured cells.

5. Diffusion of a GPI-anchored protein, CD59, defines small transient lipid rafts in the resting cell membrane: an approach by single-molecule optical microscopy

Lipid rafts are often defined by detergent extraction, but their size in unperturbed plasma membranes is unknown [15]. Pulse EPR provided some insights as described above. However, one would want to more directly observe the movement of raft molecules in and out of raft domains (and/or the formation and dispersion of raft domains). Since such events occur stochastically, i.e., they do not occur synchronously, approaches using single molecule techniques may be required for direct observations of such events.

Using single molecule techniques (both single particle tracking at a 25 μs resolution and single fluorophore imaging at video rate), Suzuki et al. [41,42] tracked on the cell membrane movement of CD59, a GPI-anchored protein and a receptor for the eighth component of compliment (C8), at the level of single molecules using anti-CD59 Fab conjugated with colloidal gold or fluorescent dyes. Biochemically, over 85% of CD59 was recovered in the DRM fractions after cultured ECV cells were extracted with cold Triton, indicating that, biochemically, CD59 is a typical raft-preferring protein. They compared diffusion of CD59 and that of DOPE, an unsaturated phospholipid excluded from rafts.

Previously, using single particle tracking at a 25 μs resolution and single fluorophore imaging at video rate, Fujiwara et al. [83b] found that DOPE undergoes hop diffusion in compartmentalized cell membrane: in NRK cells, DOPE molecules are confined within 230-mm ϕ compartments for 11 ms on average before hopping to an adjacent one, and by repeating such temporary confinement and hop movement, they undergo macroscopic diffusion over many compartments. Such compartmentalization depends on the actin-based membrane skeleton, but not on extracellular matrix, extracellular domains of membrane

proteins, and rafts. They proposed that the various transmembrane proteins anchored to the actin membrane skeleton meshwork act as rows of pickets which temporarily confine phospholipids, due to the steric hindrance as well as hydrodynamic friction-like effect of transmembrane picket proteins anchored to the membrane skeleton. The compartment boundaries do not have to be totally closed off by picket proteins because the friction-like effect of immobile proteins propagates quite far over several protein distances. Here it is important to realize that transmembrane proteins that are not immobilized (via anchoring on the membrane skeleton) will not make effective diffusion obstacles.

Carrying out similar high-resolution diffusion measurements, Suzuki et al. [41,42] found almost identical dynamics for a GPI-anchored raft protein, CD59, and for DOPE, a typical non-raft phospholipid. Both diffused rapidly while confined within 110-nm compartments (Suzuki et al. used ECV cells, and their compartment size is smaller than that in NRK cells found by Fujiwara et al. [83a]). On average, each hopped to a new compartment every 25 ms. The nearly identical scales of confinement and hop frequencies indicate that CD59 can only be associated with small rafts with lifetimes as short as a few milliseconds or less (because CD59 monomers hopped across the picket line once every 25 ms on average, the lifetime of the raft must be much shorter than 25 ms). The transient nature of the association of GPI-anchored proteins (GFP–GPI) has also been reported [84]. Further analysis of the FRET data of Varma and Mayor [30] by Mayor's group supports the model of small rafts, containing a very small number of GPI-anchored protein molecules (S. Mayor, personal communication). This conclusion is also consistent with the results of Kenworthy and Edidin [32] and Kenworthy et al. [33].

Small and short-lived rafts may nevertheless be important in membrane function, particularly in the assembly of signaling complexes after receptor cross-linking [13]. In the case of transmembrane proteins in the plasma membrane, molecular complexes and monomers often exist in a very delicate balance [65,85]. Therefore, an input that shifts the balance slightly, such as small conformational changes, could induce large changes in the aggregation states of the transmembrane receptor, which in turn may work as a switch to turn on downstream signaling pathways. Likewise, receptor clustering could work as another type of switch: by slightly reducing the thermal movement of lipids in and around the receptor clusters, the ordering effects of the transbilayer domains of the transmembrane receptor molecules, saturated anchoring chains of GPI-anchored proteins, and cholesterol win out over the thermal randomization. This may lead to the coalescence of numerous small rafts, each containing one or two intracellular signaling molecules, into large and stable rafts around the receptor clusters. Therefore, experiments to add CD59 ligands (C8) or to cross-link CD59 were performed as described below.

6. Stimulation-induced formation of temporal but stabilized rafts

Suzuki et al. [41,42] further found that CD59 binding with its natural ligand C8 or cross-linking with an anti-CD59 IgG antibody induced intracellular signaling events, such as intracellular Ca^{2+} spikes and activating phosphorylation of Src family kinases (SFK). A very clear perspective was obtained when the movement of CD59 that are engaged in signaling was observed, using gold particles abundantly coated with anti-CD59 IgG antibody molecules as probes.

First, when these gold particles were attached to the cell membrane, they formed raft-like domains beneath them in the plasma membrane by cross-linking CD59 and concentrating cholesterol.

Second, like the monomeric CD59 described in the previous section, the cross-linked CD59 (and the associated raft domain) also showed temporal confinement within compartments of the same size (110 nm). On average, each CD59 cluster hopped to a new compartment an average of once every 200 ms, an eight-fold slower than that for monomeric CD59.

Third, the cross-linked CD59 often exhibited a different type of confinement in 90-nm membrane zones (in diameter), with CD59 visits every 3.2 s (about 20 times a minute) for 0.7 s on average, in addition to the hop-type movement over 110-nm compartments (on average, this longer confinement took place once every 12 hops). Such zones were detected as TCZs, which were first identified and defined by Jacobson's group [29,86,87], using their statistical method. The formation of TCZs that cross-linked CD59 exhibited required cholesterol, intact actin filaments, and SFK activities, and their appearance correlates well with the occurrence of intracellular calcium signals, suggesting the essential roles that such domains may play in the downstream signaling events.

Taken together, we propose that at least three types of rafts may be present in the plasma membrane (Fig. 5). The first type (a, a′, a″ in Fig. 5) is a small, unstable kind of raft that monomeric GPI-anchored proteins (or signaling proteins with saturated alkyl chains located in the inner leaflet of the membrane) may be associated with in the absence of extracellular stimulation. It may consist of only a few molecules and its lifetime may be shorter than 1 ms.

The second type of raft (b in Fig. 5) may appear when an extracellular signal is received by a GPI-anchored receptor or transmembrane receptor with some affinity to cholesterol and saturated alkyl chains. When these receptor molecules are liganded (or when the clustering of the receptor molecules is induced by cross-linking ligands, like multivalent antigens), these activated receptors form oligomers, which then induce small but stable rafts around them, perhaps due to the slight reduction of thermal motion around the cluster and the subsequent

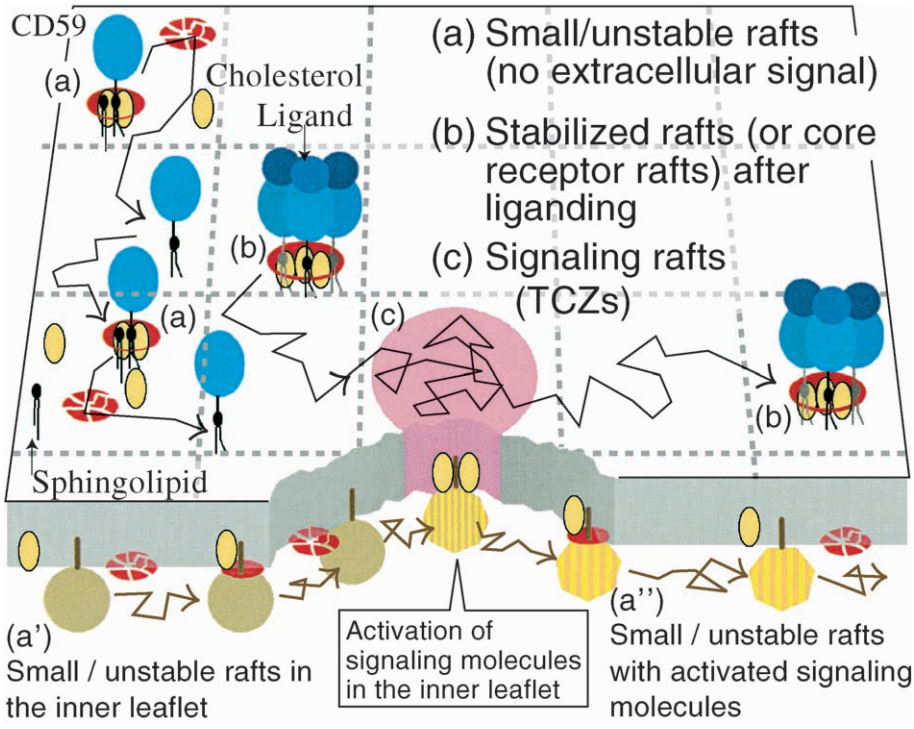

Fig. 5. Three types of rafts found thus far in the plasma membrane. The first type (a) is prevalent in the absence of extracellular stimulation. They are small (perhaps consisting of several molecules) and unstable (the lifetimes may be less than 1 ms), and may be the kind of raft that monomeric GPI-anchored proteins associate with. The second type of raft (b) may appear when receptor molecules form oligomers upon liganding or cross-linking. The receptors may be GPI-anchored receptors or transmembrane receptors with some affinity to cholesterol and saturated alkyl chains. Oligomerized receptors may then induce small but stable rafts around them, perhaps due to the slight reduction in the thermal motion around the cluster and the subsequent assembly of cholesterol. Given the rather stable oligomerization of the receptor molecules, the second type of raft may be stable for minutes, although the associated raft-constituent molecules may be exchanged frequently between the raft and the bulk domains. We call such receptor-associated rafts "core receptor rafts". The third type of raft (c) may be formed around these core receptor rafts (although the core receptor rafts may be undergoing diffusion). Here, they are called "signaling rafts", because they are likely to be directly involved in downstream signaling from receptor molecules, by assembling signaling molecules through the (transient and/or more stable) coalescence of rafts that may contain one or two signaling molecules. Small/unstable rafts are also likely to exist in the inner leaflet of the membrane (a′) and could coalesce with the core receptor rafts (b and c), where the signaling molecule in the inner leaflet is activated, which might also leave from the signaling rafts (c and a″). In CD59 experiments after liganding or cross-linking, the clustered CD59 are often found in TCZs, and we propose that these TCZs are representative of signaling rafts. The downstream signaling may occur by the recruitment of other raft-preferring signaling molecules. In the case of CD59, the formation of signaling rafts also depends on actin filaments and SFK activities.

assembly of cholesterol. We will call these rafts as "core receptor rafts". The lifetime of the core receptor rafts tends to be long, perhaps on the order of minutes, although the lipid molecules associated with the core receptor rafts may be exchanging with the bulk lipid.

The third type of raft (c in Fig. 5) may form around these core receptor rafts, and it is called a "signaling raft" here, because it is likely to be directly involved in downstream signaling from receptor molecules by assembling signaling molecules through the (transient and/or more stable) coalescence of small/unstable rafts containing a signaling molecule. Such coalescence of rafts between two halves of the membrane, i.e., the core receptor rafts in the outer leaflet may recruit small/unstable rafts in the inner leaflet beneath them although the mechanism for the coalescence between outer–inner rafts is not clear. In CD59 experiments after liganding or cross-linking, clustered CD59 was found in TCZs, and we believe that these domains are representative of signaling rafts, because the appearance of TCZs closely correlates with the downstream signaling events.

Consider the recruitment of Lyn kinase upon the cross-linking of the Fcε receptor [88–90]. The receptor itself does not have any enzymatic activities, and in the steady state, only a small portion of the receptor (less than 30%) is bound by Lyn kinase [91], and Lyn has to be and is recruited to the sites of cross-linked FcεR for the downstream signaling [88,92,93]. How does receptor clustering induce the recruitment of Lyn? What makes the clustered receptor molecules different from monomeric receptor molecules? We think one of the major consequences of receptor cross-linking is the decrease of thermal motion in and around the receptor clusters. This could make possible assembly of cholesterol (and possibly other raft-preferring lipids like glycosphingolipids and cholesterol) with the aid of small/unstable rafts (the first type of raft), inducing the "core receptor rafts" (the second type of raft) in/around the receptor clusters. The lipids in the core receptor rafts may still be exchanging with

those in the bulk domain, but the core receptor raft itself would keep its integrity by always holding sufficient numbers of raft lipids in it. In our model, this would become possible because the thermal motion in/around the receptor clusters is decreased, and the affinity of cholesterol and saturated alkyl chains (this argument is all right with GPI-anchored proteins, but in the case of the FcεR, we have to assume a certain level of affinity between the receptor and cholesterol), coupled with nonconformability of cholesterol with the bulk unsaturated alkyl chains, would slightly win out over thermal randomization. In the case of FcεR, glycosphingolipids are also assembled in/around the core rafts [7]. After formation of core receptor rafts, due to similar balance between coalescence and randomization, the small/unstable rafts may coalesce with the core receptor rafts to form "signaling rafts" (which may also be transient). In the case of Lyn, (transient) coalescence must occur between the outer and the inner leaflets of the plasma membrane. Since FcεR is a transmembrane protein, the formation of core receptor rafts and coalesced rafts (signaling rafts) in the inner leaflet of the plasma membrane may occur in a way similar to that in the outer leaflet of the membrane.

In the case of GPI-anchored receptors, how the core receptor rafts formed in the outer leaflet of the membrane could be coupled with the rafts (small/unstable rafts containing a signaling molecule) in the inner leaflet of the membrane takes place, leading to formation of signaling rafts residing in both leaflets of the membrane, is not known. One could envisage two possibilities. One is that specific transmembrane proteins are first recruited to the core receptor rafts perhaps due to their affinity to the clustered GPI-anchored receptor and/or rafts, and their recruitment in turn induces assembly of signaling molecules on small/unstable rafts in the inner leaflet, perhaps due to the affinity between protein molecules and between the rafts (between the rafts formed around the transmembrane protein and the small/unstable rafts). The other possibility is the lipid interaction between the core receptor raft in the outer leaflet and small/unstable rafts in the inner leaflet, which might involve the interdigitated structure of the core receptor raft in the central part of the bilayer. Although such interaction is expected to be weak, *transient* inter-leaflet coupling of rafts may be sufficient to induce signal interaction. If, for example, Lyn is activated by autophosphorylation, when two Lyn molecules (on two separate rafts) are recruited beneath the same core raft at about the same time (if their residency periods there overlap), Lyn will be activated and this does not require a long stay of Lyn beneath the core receptor rafts. Such weak inter-leaflet coupling of rafts may also contribute to recruitment of signaling molecules in the inner leaflet even when transmembrane proteins are involved. In either case, it would be important to realize that molecular interactions involving rafts may be brief, perhaps taking place for a fraction of a second, rather than long and stable interactions often assumed in various biochemical/cell biological experiments.

Transient confinement of GPI-anchored proteins and other raft molecules was first found by Sheets et al. [29] using single particle tracking, and single particle tracking has been used since then to study various raft domains in cellular as well as artificial membranes [38,94–96]. In retrospect, the gold probes used for these single particle tracking experiments may have slightly cross-linked the raft-preferring molecules, which enhanced the appearance of the TCZs. However, these studies made important contributions in clearly showing the presence and the various characteristics of cholesterol-dependent rafts in the cell membrane. In addition, they revealed that very low cross-linking levels could change the properties of the TCZs, in agreement with the concept of delicate balance between large/stable rafts and small/transient rafts, which is influenced by the levels of thermal motion and protein oligomerization. Suzuki and Sheetz [97] also found that cross-linked GPI-anchored proteins tend to be associated with cholesterol-dependent, actin-associated domains. Somewhat consistent with these results, Friedrichson and Kurzchalia [31] found that after chemical cross-linking, GPI-anchored proteins form clusters of about 15 molecules. This clustering was specific for the GPI-anchored form of the molecule and was dependent on cholesterol. Pralle et al. [34] found 50-nm diameter raft domains using photonic force microscope. Meanwhile, Schütz et al. [98] found ≈ 700-nm domains using single dye tracing experiments employing Cy5-DMPE as a probe although the domain has not been fully characterized.

7. A thermal Lego model for rafts as building blocks for signaling pathways in the membrane

Recently, scaffolding proteins have been drawing extensive attention as key components in various signaling pathways [99]. The molecular interactions of signaling molecules and their specificities would be greatly enhanced when signaling molecules are assembled by scaffolding proteins. Likewise, the rafts in the plasma membrane would provide a platform to concentrate signaling molecules for more efficient and specific signaling. One advantage of a raft over scaffolding proteins could be its versatility. Since the raft is a structure partially based on lipid interactions, it can accommodate different lipid-anchored and transmembrane protein molecules, and thereby it could work as a platform that transduces various types of signals, processes signals in different ways depending on the kind and the history of the extracellular signals by assembling different molecules, and enhances crosstalk between otherwise separate signaling pathways.

The experimental detection of small/unstable rafts in unstimulated cells is probably a manifestation of the presence of ingredients in the plasma membrane for the

ready formation of core receptor rafts and signaling rafts (Fig. 5). These larger, more stable rafts are always on the verge of forming from the small/unstable rafts, i.e., the formation and the dispersion of these more stabilized rafts are always very delicately balanced. Therefore, if the thermal motion of the raft-associable receptor molecules is slightly reduced after extracellular ligands are received, due to oligomerization or cross-linking after liganding, cholesterol, short-lived cholesterol clusters, and small/unstable rafts may become associated with the oligomerized receptor molecules (these receptors must have sufficient affinity to cholesterol like GPI-anchored molecules), and form core receptor rafts. Namely, a slight reduction of the thermal motion in and/or around the (GPI-anchored) receptor may be sufficient to shift the delicate balance for the formation of core receptor rafts (therefore, in this sense, small/unstable rafts are the ingredients for formation of greater rafts). Cholesterol molecules in the core receptor rafts may be exchanging rapidly with the bulk region, but when the receptor molecules are oligomerized, there would always be a sufficient number of cholesterol molecules associated with the receptor oligomers to maintain the raft organization in/around the receptor oligomers. These core receptor rafts would form coalesced rafts with other small/unstable rafts, each containing perhaps one signaling molecule, forming TCZs or signaling rafts, which may be further stabilized by interactions with actin-based membrane skeleton/cytoskeleton. Such coalescence might occur from the inner leaflet of the membrane, although how the rafts in the outer leaflet may be coupled to the rafts in the inner leaflet remains unknown.

The small/unstable rafts are like Lego blocks for building the signaling pathways, the signaling molecule being the central part of the Lego block to which cholesterol and possibly saturated alkyl chains are attached as pegs (connecting parts) of the Lego blocks. The difference between normal Lego blocks and small/unstable nanometer-level Lego blocks in the membrane is that the Lego blocks in the nanoworld membrane are forming and dispersing continually, and that the pegs do not work until the thermal motion is somewhat suppressed, like at the places in and around the oligomerized receptor molecules. We call this model for small/unstable rafts the "thermal Lego model" here.

The functions of the pegs of the raft Lego blocks would be controlled by receptor oligomerization (by liganding or cross-linking), and possibly the binding of receptor molecules to the membrane skeleton/cytoskeleton. In the case of trafficking from the Golgi to the apical plasma membrane, unknown lectin-like molecules present in the lumen of the Golgi may be involved in cross-linking proteins to be transported to the apical membrane, and thus in inducing rafts around cross-linked proteins. The beauty of the raft hypothesis is that since lipid interactions are used as the basic mechanism for assembling rafts, the signaling platforms based on raft assembly could be very versatile, allowing for rapid switching of the downstream signaling pathways and various crosstalks, depending on the cellular environments and history. The raft-based signaling platforms could also be built and disintegrated very easily for turning on and off the pathway very quickly, like Lego models of Ninja (makeshift) beachheads, although the control mechanisms for such versatile constructions are as yet unknown. Therefore, one of the most important aims of raft studies at the next level along this line would be, first, to understand the selectivity of raft coalescence at the time of signaling, and, second, to find out the trick of how the pegs couple the rafts (core receptor rafts) in the outer leaflets with those (small/unstable rafts) in the inner leaflet of the plasma membrane.

8. Additional related points

The cholesterol concentration in the plasma membrane is continuously regulated by the fusion and fission of cytoplasmic vesicles and monomeric exchanges with cholesterol located in the cytoplasm and in lipoproteins in the extracellular space. Therefore, the cholesterol level in the plasma membrane may be determined by the steady-state equilibration. In addition, Haynes et al. [101] argued that there are two distinct pools of cholesterol in the plasma membrane.

Intracellular membranes may also contain rafts, which we did not discuss in this review. Lipid rafts were in fact first postulated as relatively stable structures in the Golgi which are involved in protein sorting and trafficking, and the general view is still that such rafts would greatly contribute to segregation of sphingolipids and GPI-anchored proteins in the Golgi [5,13,102]. Similar mechanisms appear to function in the outer leaflet of the plasma membrane and in the endosomal system [103]. The domains in the lumenal leaflet of the membrane of the Golgi and endosomes may be stabilized by some sort of triggering/signal, like that proposed here for the plasma membrane receptors. Since clustering of proteins and lipids are the key point for formation of stabilized rafts, the presence/production of lectin-like proteins in the lumen of the Golgi and possibly endosomes may play important roles in raft-based sorting and trafficking in intracellular membranes.

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