The Sensing of Membrane Microdomains Based on Pore-Forming Toxins

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Abstract: Membrane rafts are transient and unstable membrane microdomains that are enriched in sphingolipids, cholesterol, and specific proteins. They are involved in intracellular trafficking, signal transduction, pathogen entry, and attachment of various ligands. Increasing experimental evidence on the crucial biological roles of membrane rafts under normal and pathological conditions require new techniques for their structural and functional characterization. In particular, fluorescence-labeled cytolytic proteins that interact specifically with molecules enriched in rafts are of increasing interest. Cholera toxin subunit B interacts specifically with raft-residing ganglioside GM₁, and it has long been the lipid probe of choice for membrane rafts. Recently, four new pore-forming toxins have been proposed as selective raft markers: (i) equinatoxin II, a cytolyxin from the sea anemone Actinia equina, which specifically recognizes free and membrane-embedded sphingomyelin; (ii) a truncated non-toxic mutant of a cytolytic protein, lysenin, from the earthworm Eisenia fetida, which specifically recognizes sphingomyelin-enriched membrane domains; (iii) a non-toxic derivative of the cholesterol-dependent cytolyxin perfringolysin O, from the bacterium Clostridium perfringens, which selectively binds to membrane domains enriched in cholest erol; and (iv) ostreolysin, from the mushroom Pleurotus ostreatus, which does not bind to a single raft-enriched lipid component, but requires a specific combination of two of the most important raft-residing lipids: sphingomyelin and cholesterol. Non-toxic, raft-binding derivatives of cytolytic proteins have already been successfully used to explore both the structure and function of membrane rafts, and of raft-associated molecules. Here, we review these four new derivatives of pore-forming toxins as new putative markers of these membrane microdomains.

Keywords: Cholesterol, equinatoxin, fluorescent probe, liquid ordered phase, lysenin, membrane microdomains, membrane raft, ostreolysin, perfringolysin O, sphingomyelin.

INTRODUCTION

Cell membranes are composed of a great variety of different lipids and proteins [1]. According to the fluid mosaic model [2], it has been long assumed that all of these molecules are relatively randomly distributed in the homogeneous fluid lipid bilayer. The current model of cell membrane structure, however, presumes that the different lipids are de-mixed in the membrane plane according to their various chemical and physical properties, to form distinctive membrane microdomains. In particular, sterols and sphingomyelin (SM) are responsible for the separation of the membrane lipid domains into separate co-existing liquid-disordered (LD) and liquid-ordered (LO) domains [3]. The domains characterized by the LO phase, in which the lipids are tightly packed but can still undergo fast lateral mobility, are the basis for the formation of membrane rafts, cell membrane entities that are enriched in sphingolipids, cholesterol, and specific proteins [4, 5].

Membrane rafts are believed to be transient, dynamic and unstable membrane microdomains that can be stabilized on binding ligand molecules [6-8]. Their composition is also believed to change on a sub-millisecond scale [8]. They have been shown to exist as nanoscale clusters [6, 9] of various sizes [10, 11]. Experimental evidence of the crucial biological roles of membrane rafts has dramatically increased in the last 15 years. Rafts have been shown to be involved in several important biological functions, including exocytosis and endocytosis, signaling, pathogen entry, and the attachment of a variety of molecular ligands [1, 4, 12-15]. In particular, their involvement in the transduction of various signals has been shown to be important under a variety of disease conditions; e.g. Alzheimer’s disease, Parkinson’s disease, cardiovascular and prion diseases, systemic lupus erythematosus, and acquired immunodeficiency syndrome [16]. All of these findings have made membrane rafts interesting targets for further structural and functional studies of cell membranes and their constituents, as well as the for the cure and prevention of diseases by pharmacological approaches. The development of new techniques and approaches that would allow suitable visualization of these membrane microdomains will therefore be of great use.

Detergent insolubility has long been considered as the main basis for membrane raft isolation. In contrast to membrane domains in the LD phase, lipids in the LO phase are resistant to solubilization by many detergents [17], and therefore membrane rafts have often been operationally called ‘detergent-resistant membranes’ (DRMs) [4]. However, DRMs should not be assumed to describe biological rafts in size, structure, composition or even existence, as detergents themselves have been shown to be able to induce the formation of ordered domains [17, 18].

Membrane rafts are not easily visualized, due to their temporal instability and small size [11]. Several modern microscopic methods have been used recently for the visualization of membrane microdomains, including scanning probe techniques, such as near-field scanning optical microscopy and atomic force microscopy, and optical techniques, like fluorescence correlation microscopy, fluorescence resonance energy transfer (FRET), stimulated emission and depletion microscopy, and stochastic reconstruction microscopy [19]. Other recent attempts to visualize particular membrane lipidic and/or lipid domains have used fluorescent lipid analogs or lipid-binding probes, which have included proteins and antibodies [20]. In particular, non-toxic recombinant fluorescence-labeled derivatives of natural toxins are gaining interest, which include pore-forming toxins (PFTs), that interact specifically with molecules enriched in membrane rafts.

Upon interaction with a membrane, the PFTs spontaneously change their native conformation to enable the formation of transmembrane pores that consist of several protein monomers [21]. In several cases, membrane rafts have been suggested to act as concentrating platforms that can promote the oligomerization of these PFT proteins. Several PFTs have been found to interact with different molecules that are enriched in DRM domains that fulfill the criteria of membrane rafts; e.g. SM, cholesterol, ceramides, gangliosides, or the glycans core of glycoprophosphatidylinositol (GPI)-
anchored proteins [7, 20, 22-30]. Among the non-toxic fluorescent derivatives of such natural toxins, the cholera toxin B-subunit (CT-B) binds to the raft-enriched ganglioside GM1, and it has long been the only such probe for membrane rafts [31]. Recently, however, four new derivatives of PFTs that can bind to membrane SM and/or cholesterol have been explored as new putative markers of membrane microdomains, and these are reviewed here (Table 1).

SPHINGOMYELIN-BINDING PORE-FORMING TOXINS

Sphingomyelin (N-acylsphingosine-1-phosphorylcholine, SM) is a relatively abundant sphingolipid constituent of vertebrate cell membranes that is mostly located in the outer leaflet of the plasmalemma [32]. In some cells, the SM concentration can account for up to 60 mol% of their total phospholipid content; e.g. in erythrocytes, and ocular lens, peripheral nerve tissue and brain cells [33]. SM shares a phosphocholine moiety with phosphatidylcholine (PC), whereas its ceramide backbone is specific for SM and glycolipids. As well as its structural role in cell membranes, SM is important in intracellular signaling pathways. Hydrolysis of membrane SM by activated sphingomyelinas leads to the formation of ceramide, which serves as a secondary messenger in sphingomyelinase-dependent signal transduction [34], and can induce membrane endocytosis and blebbing [35]. Membrane SM has also been shown to be involved in some disease conditions, e.g. in Niemann-Pick disease type A, an autosomal recessive disorder that arises due to sphingomyelinase deficiency and that results in an over-accumulation of SM in cell membranes [36]. Membrane SM has also been suggested to act as a non-receptor-based gate for the entry of low-density lipoprotein, and thus to have a role in atherosclerosis [37]. All of these observations make SM an interesting target for specific probes that would allow further functional studies of cellular SM and its behavior in cell membranes under conditions of health and disease. As SM is significantly enriched in membrane rafts, specific probes that would sense higher local distributions of SM would also be very important in structural and functional studies of membrane microdomains. In this regard, the PFTs from sea anemones and earthworm coelomic fluid are the most promising candidates for development of SM-specific protein-based probes.

The actinoporins are potent PFTs that have been purified from sea anemones [38-40]. They are 20-kDa cysteine-less proteins that have permeabilisation activities towards membranes that contain SM. The most studied of the actinoporins are equinatoxin II (EqtII), from the sea anemone Actinia equina, and sticholysins I and II, from Stichodactyla helianthus [41]. The three-dimensional structure of the actinoporins shows a tightly folded β-sandwich that is flanked on two sides by α-helices. The N-terminal region of approximately 30 amino-acid residues contains one of the α-helices, and this is the only part of the molecule that can be dislocated from the body of the molecule without disrupting its general folding [42-44]. This N-terminal flexibility has been shown to be crucial for the pore-forming activities of the actinoporins [45].

The structure of sticholysin II in a complex with phosphocholine, the headgroup of both SM and PC, allowed the definition of the amino acids that participate in the binding of this headgroup (labeled in green on Fig. 1) [44]. These amino acids are conserved in the actinoporins and thus it appears that the mode of headgroup binding is conserved across the actinoporins family [46].

Formation of actinoporin pores proceeds through several steps, which include membrane binding, transfer of the N-terminal region to the membrane, oligomerization of several monomers, and the pore formation itself, which brings the N-terminal regions of the individual monomers across the membranes to form the final pore [38-40, 45].

The membrane-binding region of the actinoporins was defined by functional studies [45, 47-51], which also provided insight into the molecular recognition of SM by these PFTs. Surface plasmon resonance and lipid dot-blot analyses have shown that EqtII binds SM directly and specifically, and not any of the other related lipids, like PC, dipropionylPC, or lysyPC [46]. The labeling of EqtII with 31P on trytophans indicated for the first time that the tryptophan at position 112 in EqtII participates in the direct recognition of SM [52]. Mutagenesis of this tryptophan, and of its neighboring Tyr113 residue, has shown that these two residues are the most important ones for SM recognition [46]. Tyr113 and Trp112 of EqtII are located on a broad loop at the ‘bottom’ of the molecule (see Fig. 1), in close vicinity to the phosphocholine binding site, and this structure might represent a generic three-dimensional structural motif that can bind to a single SM molecule. Thus it appears that the discrimination between SM and PC occurs in the region directly below the phosphocholine headgroup [46]. The preferential interaction of EqtII with SM has also been inferred from 31P and 2H solid-state nuclear magnetic resonance studies that used large unilamellar vesicles composed of various mixtures of PC, SM and cholesterol [53, 54].

To test the use of EqtII as a marker for cellular SM, a green fluorescent protein fusion protein (EqtII-GFP) was produced and expressed as a His6 protein in the Escherichia coli Rosetta (DE3) pLysS strain. Addition of GFP to the C-terminus of EqtII did not alter its SM-specific membrane activity. The EqtII-GFP fusion protein was found to bind to the plasma membrane of MDCK II cells when added extracellularly. Furthermore, the apical side of the polarized MDCK cells was stained, but not the basolateral, in agreement with the canonical cellular SM distribution [55]. When EqtII-GFP was expressed intracellularly within the same cells, a punctate perinuclear staining was revealed (Fig. 1). EqtII-GFP co-localized with markers for the Golgi apparatus, mainly at the cis-Golgi, and did not co-localize with markers for the nucleus, endoplasmic reticulum, or mitochondria [55]. Recent investigations [56] have shown that membrane binding of EqtII is rapidly accompanied by extensive membrane reorganization into microscopic domains that resemble coalesced membrane rafts, with the concomitant blocking of endocytosis. Cholera toxin subunit B (CT-B) was also seen to co-localize within these domains. In addition, sticholysin II co-localized with raft-associated membrane fractions, and its lytic activity was shown to be abolished by incubating CT-B with raft-like lipid vesicles (SM: dioleoylphosphatidylcholine [DOPC]; cholesterol; 1:1:1) [22]. Furthermore, with large unilamellar vesicles

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Table 1. Representative Overview of the Cytolytic Proteins and Their Lipid Binding Components that are Enriched in Membrane Rafts

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>MW (kDa)</th>
<th>Binding Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equinatoxin II</td>
<td>Actinia equina</td>
<td>20</td>
<td>SM</td>
<td>Bakrač et al., 2008.</td>
</tr>
<tr>
<td>Lysenin</td>
<td>Eisenia fetida</td>
<td>33.4</td>
<td>SM</td>
<td>Ishitsuka et al., 2004.</td>
</tr>
<tr>
<td>Perfringolysin O</td>
<td>Clostridium perfringens</td>
<td>54</td>
<td>Cholesterol</td>
<td>Ohno-Iwashita et al., 2004.</td>
</tr>
<tr>
<td>Ostreolysin</td>
<td>Pleurotus ostreatus</td>
<td>15</td>
<td>Cholesterol/SM</td>
<td>Sepčić et al., 2004.</td>
</tr>
<tr>
<td>CT-B</td>
<td>Vibrio cholerae</td>
<td>11.5</td>
<td>GM</td>
<td>Baca et al., 2004.</td>
</tr>
</tbody>
</table>
and lipid monayers, the co-existence of the $s_{c}+b_{c}$ and $l_{c}+b_{c}$ phases was found to favor the membrane insertion of EqtII, and epifluorescence measurements showed that the interfaces between co-existing lipid phases can function as preferential binding sites for EqtII [57]. The binding of fluorescently labeled EqtII to the domain boundaries was shown also in a giant unilamellar vesicle model system [58].

Lysenin and the homologous proteins LRP-1 and LRP-2 are secreted through the dorsal pores of the earthworm, and they are believed to act in the protection of *Eisenia foetida* from predatory vertebrates [65]. Recombinant lysenin has also been shown to be active against *Bacillus megaterium* (an *E. foetida* pathogen) in the nematode range, but not against other soil bacteria. The lysis of bacterial cells was shown to be independent of pore-formation [66]. Specific binding of lysenin to SM has been confirmed using several approaches, including enzyme-linked immunosorbent assays, thin-layer chromatography immuno-staining, FRET, and SM-containing lipid vesicles [60, 66]. Interactions of lysenin with related SM analogs (e.g. sphingosine, ceramide, sphingosinephosphorylcholine), or with other phospholipids or glycosphingolipids, were not observed [60, 67]. Experiments with planar lipid membranes also showed the formation of voltage-dependent large conductance channels in a SM-dependent manner [68, 69].

The importance of membrane SM for lysenin activity is also reflected in its maximal activity towards sheep erythrocytes, which contain a very high content of SM [64]. Several immunofluorescencel studies of cell-bound lysenin have been performed to determine its potential as a specific SM marker. Lysenin binding to fibroblasts from a Nieman-Pick disease type A patient yielded strong uniform staining by lysenin, and this was impaired by pre-incubation of lysenin with SM-containing lipid vesicles [60]. Furthermore, sphingomyelinase-pretreated CHO cells, and cells with defective sphingolipid synthesis, were resistant to the cytolytic action of lysenin, which suggests that lysenin can be used as a tool for isolation of SM-deficient cell mutants; e.g. cells deficient in sphingolipid synthesis or in ceramide transport from the endoplasmic reticulum to the Golgi apparatus [70]. Lysenin has also been proposed as a tool in de/remyelinization studies in the brain, due to its binding to the surface of oligodendrocyte lineage cells that show increased membrane SM content during cell development [71].

However, it soon became clear that the presence of other lipids, for example glycosphingolipids, can significantly influence lysenin membrane activity. Epithelial cell lines (e.g. MDCK cells) that are enriched in glycosphingolipids in their apical domains are highly sensitive to lysenin when added from the basolateral side, although they are resistant if lysenin is applied apically. Model membrane systems have indicated that glycolipids alter the local density of SM, and consequently decrease lysenin binding [72]. Another molecule that can significantly affect lysenin binding is cholesterol. The binding of lysenin to SM: cholesterol vesicles, and their permeabilization, were significantly enhanced when the vesicles contained at least 30 mole% cholesterol [60], which indicated the importance of a specific membrane phase for the activity of lysenin. Ishitsuka and Kobayashi [73] further studied the effects of cholesterol in terms of the membrane activity of lysenin on SM-containing membranes. The incubation of lysenin with SM-based liposomes enriched in cholesterol inhibited the hemolytic activity of lysenin. However, the presence of cholesterol in SM-rich membranes did not affect lysenin binding, and even promoted its oligomerization. The authors thus suggested that the effects of cholesterol might be linked to the fluidization of membrane SM, and consequently to a decreased number of membrane-bound lysenin monomers, which would result in facilitated oligomerization. Similar observations were also made when lysenin binding to SM-rich membranes was studied using a surface plasmon resonance technique. Addition of 50 mole% cholesterol to SM-membranes increased lysenin binding in comparison to pure SM membranes, but...
did not affect the kinetic parameters (with $K_D$ values of 5.3 nM and 8.6 nM, respectively), which suggests that cholesterol can change the distribution of SM in the membrane, and thus enhance its accessibility to lysenin [60]. Furthermore, surface plasmon resonance analyses have indicated that only wild-type lysenin binds efficiently to raft SM (e.g. that the binding is enhanced on SM/DOPC, SM/DOPC/cholesterol and SM/dipalmitoylphosphatidylcholine [DP PC]/cholesterol membranes, where phase separation occurs), while the Trp20 mutant of lysenin loses this ability, and does not discriminate between free and clustered SM molecules [63].

The use of a fluorescent fused recombinant lysenin (His-Venus-lysenin) further confirmed that lysenin not only specifically ‘senses’ SM molecules, but is particularly sensitive to the lateral distribution of SM within the membrane. The binding of His-Venus-lysenin to SM plus C18:1-PC liposomes, where lipid immiscibility is observed and SM clusters are formed, is much higher compared to its binding to SM plus C16:0-PC liposomes, where the lipids are more or less homogeneously mixed. The fluorescence was observed in aggregates, which suggests that lysenin binding depends on the local concentration of SM (Fig. 2). Isothermal titration calorimetry revealed that one lysenin molecule can bind five SM molecules [72]. This was also the first study in which lysenin was proposed as a probe for the investigation of membrane rafts and their heterogeneity. Studies that used a truncated lysenin mutant confirmed that this can also be used. This mutant lysenin, which consists of the minimal amino-acid fragment necessary for SM recognition, was tagged with GST and shown to be non-toxic [74]. Double staining of Jurkat T cells with this GST-tagged truncated lysenin and CT-B showed no co-localization of these two fluorescent probes, which suggested that SM-rich domains are spatially distinct from GM1-rich domains [74]. Indeed, in SM-containing liposomes, the presence of GM1 abolished the binding of Venus-lysenin, while the binding of CT-B was not affected by the presence of SM [74], which implies that CT-B recognizes its target (GM1) irrespective of the lipid distribution, while lysenin does not. The absence of lysenin and CT-B co-localization was further confirmed by immuno-fluorescent studies. Similar results, indicating different locations of cholesterol- and SM-rich microdomains on the plasma membrane, were obtained using non-toxic derivatives of lysenin and perfringolysin O as respective probes for membrane SM and cholesterol. Both proteins were fused with a photoswitchable fluorescent protein, Dronpa, which allows the visualization of stained membrane microdomains by using super-resolution microscopy, PALM [75]. As well as shedding some light on the structural heterogeneity of membrane rafts, the use of fluorescent lysenin has provided evidence that structurally different rafts also show functional diversity. In Jurkat T cells, the cross-linking of SM showed that SM-rich domains provide a functional signaling cascade platform that is distinct from those characterized by T cell receptors or by GM1 [74]. Observations performed by using the superresolution microscopy and fluorescently labeled lysenin revealed the involvement of the SM-rich domains in the progression of cytokinesis [76]. Very recently, the structural and functional heterogeneity of SM membrane pools was inspected by comparatively staining COS-1 cells with lysenin and EqII [77]. The use of both SM probes revealed the existence of at least three different SM pools in plasmalemma: the first stained only with lysenin, the second stained only with EqII, and the third that could be labeled by both probes. The same heterogeneity was found in intracellular membranes, where lysenin could be found in late endosomes, while EqII stained both late and recycling endosomes.

**CHOLESTEROL-BINDING PORE-FORMING TOXINS**

Cholesterol is another abundant lipid in the membranes of vertebrate cells. Cholesterol can mediate membrane fluidity and can increase the ordering of membranes through the formation of the $L_n$ phase and the consequent formation of segregated lateral domains in the bilayer plane that have specific properties (i.e. membrane rafts). Cholesterol is also involved in several important cell functions, as for example mediation of signal transduction, and exocytosis and endocytosis [78]. It is also a precursor in the synthesis of steroid hormones, oxysterols, bile acids and neuroactive steroids [79]. Any alterations in cholesterol metabolism can thus be reflected in a plethora of pathological conditions. This can cause common diseases such as atherosclerotic cardiovascular syndrome, or result in rare genetic malformation diseases, like Smith-Lemli-Opitz syndrome, lathosclerosis, desmosterolosis, chondroplasia punctata, and congenital hemidysplasia [79]. Impaired cholesterol trafficking can also lead to hyper-accumulation of cholesterol within lysosomes and late endosomes, like in Niemann-Pick syndrome type C [80], and cholesterol also appears to be involved in the pathogenesis of Alzheimer’s disease and Parkinson’s disease [81]. Molecules that can be used to label membrane cholesterol are thus extremely interesting from several points of view, as they might allow studies of the distribution of cholesterol in the membrane plane, and to follow its intracellular trafficking and involvement in different cellular processes under normal and pathological conditions.

For a long time, the polyene antibiotic filipin was used as a membrane marker for cellular cholesterol. Filipin can form complexes with free and membrane-embedded cholesterol, which can be visualized upon excitation by UV light using fluorescent microscopy. The use of filipin as a membrane raft marker has several drawbacks, however. First, filipin is only suitable for staining of fixed cells, as otherwise it is cytotoxic. Secondly, filipin binds to membrane cholesterol indiscriminately [82, 83 Fig. 3], which is reflected also by the demonstration that after removing 30% cholesterol from membranes using methyl-$eta$-cyclodextrin (M$eta$CD), filipin binding is still significantly retained [82, Fig. 3].

Among other non-protein molecules that are suitable for direct imaging of cholesterol and its trafficking, there is also a series of fluorescent cholesterol analogs. Dehydrocholesterol is a naturally occurring fluorescent cholesterol derivative that can undergo rapid transbilayer movement, and it has been used for the study of trafficking of sterols [84, 85]. Currently, novel non-ionic amphipathic cholesterol analogs, poly(ethylene glycol)cholesterol esters (PEG-cholesterol) are gaining much interest for the labeling of membrane cholesterol. These PEG-cholesterol show cholesterol-like characteristics, but they are also water-soluble and only weakly cytotoxic. Among these, fluorescein-tagged PEG-cholesterol (iPEG-cholesterol) has been the most frequently used. iPEG-cholesterol preferentially partitions into cholesterol-rich membranes, and co-localizes with the filipin fluorescence, as well as with the membrane raft markers CD59 and GM1 [86]. iPEG-
cholesterol was thus shown to be suitable for the study of the dynamics of cholesterol-rich membranes. In living cells, it is distributed into the outer membrane leaflet [86], and can be slowly internalized by a clathrin-independent pathway into endosomes, although only at low concentrations and at 37 °C.

This specific interaction of perfringolysin O with membrane cholesterol has stimulated a series of studies on the use of perfringolysin O as a marker for membrane cholesterol [94]. To date, several non-toxic perfringolysin O derivatives with retained cholesterol binding have been produced. C0 and its methylated form, MC0, were obtained by limited proteolysis of perfringolysin O between the 144-145 amino-acid residues, and they exist as a complex of 38 kDa and 15 kDa proteins. The binding to erythrocytes of both C0 and MC0 is comparable to that of the native perfringolysin O; however, C0 and MC0 are not hemolytic, and do not oligomerize up to 20 °C and 37 °C, respectively [95, 96]. One of the most used of the perfringolysin O derivatives is known as BC0, which also has the same binding properties as the native protein, but which is lytic only above 37 °C. BC0 was obtained by proteolysis and biotinylation of perfringolysin O, and it is structurally comparable to biotinylated C0. When coupled with FITC-conjugated avidin, BC0 can be used for staining membrane cholesterol without the need for antibodies [97]. Analyses of BC0 binding to erythrocytes and to V-79 and HUVEC cells by flow cytometry and confocal microscopy have showed that BC0 is a very suitable probe for visualization of membrane cholesterol and for the evaluation of the topology and/or distribution of membrane cholesterol [97]. Further investigations of BC0 membrane binding have revealed that BC0 is not only a suitable probe for cholesterol, but also for the distribution of cholesterol in cell membranes, as it interacts with membrane domains that have higher local concentrations of cholesterol Fig. (3). Binding of BC0 was shown to be enriched in low-density membrane fractions that contain cholesterol, SM, and Src family kinases, thus fulfilling the criteria for membrane rafts. This binding was retained even when the raft-like domains were isolated without detergent extraction; however, depletion of approximately 30 mole% cholesterol using MJβCD abolished the binding of BC0 [82, Fig. 3]. Furthermore, BC0 co-localizes with the NAP-22 protein, which is a major component of brain-derived rafts [98], and which decreases in aged cells, suggesting that the density of cholesterol-rich membrane rafts decreases during cell aging [99]. The labeling patterns of NAP-22 and cavelolin do not coincide [100], which suggests that BC0 binds to non-caveolar rafts, and thus reinforces the hypothesis of membrane raft heterogeneity [5, 6, 10, 101, 102].

This hypothesis is further supported by electron microscopy analyses of membrane vesicles recovered in raft fractions of cells previously incubated with BC0, which has shown that not all of the raft-fraction vesicles associate with BC0 [28]. BC0 coupled with fluorescent avidin or colloidal gold-avidin has also been used as a probe to analyze the distribution of membrane cholesterol by fluorescence and electron microscopy [97, 100, 105]. However, due to the fluorescence of avidin, this was not suitable for real-time imaging of dynamic raft movements in living cells. Therefore, smaller constructs of perfringolysin O have been prepared and tested for their cholesterol binding and specificity.

The D4 domain is the β-sheet-enriched domain of cholesterol-dependent cytolysins that is located at the C-terminus, and it represents the minimal toxin fragment that binds to cholesterol with high affinity. Surface plasmon resonance has revealed that the membrane binding of D4 and perfringolysin O are similar. Thus, it has been shown that the D4 domain retains its stable structure, selectively binds cholesterol in membrane rafts, and is recovered in the low-density membrane fraction [106]. Finally, enhanced green fluorescent protein-tagged D4 (EGFP-D4) has been produced and used for staining of MOLT-4 cells. Here, the EGFP-D4 membrane binding was similar to that of Alexa-labeled D4, so the fusion with EGFP does not influence the binding of the D4 domain. Depletion of cellular cholesterol with MJβCD abolishes this binding. Thus, EGFP-D4 has been proposed for the monitoring of the distribution and movement of cholesterol in cell membranes [106].
Recently, new insights into the interactions of perfringolysin O with membrane cholesterol have been published. Nelson et al. [93] showed that the binding of perfringolysin O does not correlate with the ability of certain sterols to promote the formation of rafts. Using steady-state intrinsic tryptophan fluorescence with perfringolysin O and artificial lipid vesicles with different compositions, Nelson et al. [93] showed that tightly packed phospholipids and sterols in the l_s phase weaken the perfringolysin-O-membrane interactions. Similarly, phospholipids with smaller headgroups were shown to increase the perfringolysin O membrane activity. These two phenomena can be explained by the ‘umbrella model’ of lipid-raft structure; i.e. the shielding of cholesterol by the phospholipid headgroups. Overall, the insertion of perfringolysin O into the membrane was shown to be triggered in a more loosely packed lipid environment.

Further investigations on membrane binding of perfringolysin O to lipid vesicles have shown that the presence/absence of membrane rafts does not correlate with perfringolysin O binding (e.g. increased SM content in cholesterol-rich membranes decreases perfringolysin O binding) [107]. Perfringolysin O binding is triggered only when the cholesterol concentration exceeds the association capacity with phospholipids, whereby the excess cholesterol at the membrane surface is then free to associate with the perfringolysin O. Perfringolysin O thus appears to bind to cholesterol that is membrane embedded, yet surface exposed. Similar trends, e.g. the prerequisite of at least 30–40 mol% of membrane cholesterol for effective binding of perfringolysin O, have also been observed with listeriolysin O [91]. More recent investigations on the raft affinity of perfringolysin O have suggested that perfringolysin O binds to raft-associated domains, but is translocated to less-ordered membrane domains upon establishment of its transmembrane pore complex [108]. Very recently also, it was shown that mutations in the amino-acid residues around the conserved Cys459, which is located in the membrane-interacting domain D4, can alter the susceptibility of perfringolysin O in terms of the cholesterol threshold that is necessary for perfringolysin O binding. Engineered mutants can bind to artificial and biological membranes that have much lower cholesterol mole% than the native protein, reinforcing their future use as molecular probes for studying the distribution and dynamics of membrane cholesterol [109].

Ostreolysin is a 15-kDa acidic protein from the edible mushroom *Pleurotus ostreatus*, and it belongs to the aegerolysin protein family that currently includes over 300 similar proteins from bacteria, fungi and plants [110]; their exact biological roles are not known. Mushroom aegerolysins are suggested to be involved in the formation of mushroom fruit bodies [111, 112], while the aegerolysins from bacteria and filamentous fungi might be involved in host-pathogen interactions [110]. Some aegerolysins, including pleurotolyisin A [113] and its isoform ostreolysin (our unpublished data), are membrane binding components of binary cytolysins, along with another ca. 59 kDa protein with a membrane attack complex/perforin (MACPF) domain. Our studies on the membrane binding of ostreolysin [114, 115] have shown that ostreolysin specifically interacts with natural and artificial lipid membranes that are highly enriched in cholesterol and SM or, alternatively, cholesterol and a fully saturated glycerophospholipid. In contrast to other putative raft markers that bind the pure cholesterol, like perfringolysin [90] or SM, like the actinoporins [46] and lyssenin, [60], ostreolysin can recognize only mixtures of these two lipids, particularly with an excess of cholesterol, which suggests that it has a very specific interaction with membrane rafts. This has been reinforced by several studies that have shown specific interactions of ostreolysin with cholesterol. SM liposomes, ostreolysin binding to the DRMs of both cells and equimolar cholesterol. SM liposomes [115], and inhibition of ostreolysin binding after treatment of cell membranes with MJCaD [101]. Immunofluorescence studies on the binding of low concentrations of ostreolysin to the membranes of chondrocytes [116] and of CHO cells [101, Fig. 4a] have shown binding and clustering of ostreolysin, which further suggests the association of ostreolysin with distinct membrane microdomains. As in the case of lysenin [74] and perfringolysin [100], double immunolabeling experiments have shown that there is no co-localization of ostreolysin and other raft markers, like caveolin and CT-B [101], Fig. 4a which suggests that there are functionally and structurally different raft-like regions of the plasma membrane. These raft-like regions would comprise at least two lipid microdomain types: those containing (caveolae) or lacking caveolin [5].

Furthermore, by comparing the membrane activities of various raft-binding cytolysins, it can be seen that ostreolysin needs a higher local cholesterol concentration and a higher degree of membrane ordering for effective binding. In contrast to most other putative raft-binding cytolysins, which readily interact with vesicles composed of equimolar mixtures of SM, cholesterol and palmitoyl-oleoyl-PC (POPC) or DOPC, or with membranes composed of equimolar ratios of cholesterol and DOPC [22, 27], the presence of even lower ratios of monosaturated or disaturated PC in equimolar SM, cholesterol liposomes dramatically diminishes ostreolysin membrane activity [115]. Using lipid vesicles composed of equimolar ratios of SM and various natural and synthetic sterols, it has been further shown that the membrane binding of ostreolysin does not coincide with the ability of specific sterols to induce the formation of the l_s phase [117]. These combined data suggest that ostreolysin requires other structural/physical membrane features to fully exert its activity. To clarify the effects of membrane ordering on the ostreolysin membrane activity, detailed analyses of a series of ostreolysin-susceptible and ostreolysin-resistant lipid vesicles were carried out, using electron paramagnetic resonance and Fourier-transformed infrared spectroscopy [118]. Using GHOST analysis of these electron paramagnetic resonance spectra, which provides a condensation algorithm that filters and groups the solutions found in optimization runs, this has revealed that regardless of their lipid compositions, all ostreolysin-susceptible membranes have nearly identical domain-distribution patterns. The most ordered domain across all of these vesicle types, with an order parameter >0.72, covered 80% or more of the membrane area. The distribution and size of these domains, however, remain to be determined. It is also interesting to note that the addition of higher concentrations of ostreolysin to susceptible lipid vesicles did not induce any detectable changes in the electron paramagnetic resonance spectra [115], and did not modify the membrane fluidity [114]. This suggests that ostreolysin does not induce significant fluidity changes in the membrane upon binding.

As in the case of other raft-binding cytolysins, the membrane lipid composition needed for optimal membrane ‘ docking’ of ostreolysin remains to be clarified. One of the possible recognition sites might be small cholesterol or cholesterol/SM clusters arranged in the plane of the membrane. Such transient, nanoscale, lateral microheterogeneities of membranes where the stoichiometry varies with the cholesterol concentration were recently suggested by molecular dynamics simulations of cholesterol. DPPC [119] and cholesterol. SM membranes [120]; these microheterogeneities were found to be typical for cholesterol-and-SM-rich membranes and for membrane rafts [1, 11].

Ostreolysin has recently been used to study the association of the desmosomal cadherin desmocollin 2 (Dsc2) with cholesterol-enriched membrane domains, and the influence of cholesterol on desmosome assembly in MDCK epithelial cells. Biochemical analysis showed the association of Dsc2 in cholesterol-enriched fractions that contained the membrane raft marker caveolin-1, flotillin-1 and ostreolysin, and immunofluorescence microscopy confirmed this co-localization of Dsc2 and ostreolysin, which suggests that there is a pool of Dsc2 that is associated with membrane rafts [121, Fig. 4b]. In the T24 (malignant) urothelial cancer cell line, ostreolysin was also used to reveal the enrichment of cholesterol and SM membrane nanodomains along intercellular membrane
nanotubes [122]. These highly curved tubular structures that connect neighboring cells are highly fragile and very difficult to isolate, and therefore their exact molecular composition remains unknown.

GANGLIOSIDE-BINDING TOXINS

Cholera toxin subunit B (CT-B) is a part of cholera toxin from *Vibrio cholerae*, and it has been used regularly as the probe of choice for membrane rafts. CT-B assembles with the A subunit for their ADP-ribosyltransferase and NAD’glycohydrolase activities, which leads to the disruption of the intestinal epithelial barrier, and to heavy diarrhea, as seen in the disease cholera [31]. CT-B is composed of five 11.5 kDa polypeptides that assemble into a stable pentameric ring. By itself, CT-B is not toxic, and it binds with high affinity (Kd <1 nM) to the ganglioside G M1, which is enriched in membrane rafts. CT-B has been used for the detection of membrane rafts in several studies. Kenworthy et al. [7] used FRET microscopy, which can measure molecular proximity on a scale <100 Å between donor-labeled and acceptor-labeled antibodies. They compared the organization of three endogenous, raft-associated GPI-anchored proteins labeled with antibodies (the folate receptor, CD59, and 5’-nucleotidase) with GM1 labeled with CT-B. In most cases, the FRET correlated with the surface density of CT-B.

Bacia et al. [123] used fluorescence correlation spectroscopy to trace the mobility of CT-B (as a lδ-phase marker) and dialkylcarbocyanine (as a lδ-phase marker) in the outer leaflet of unilamellar vesicles (GUVs). This technique traces temporal fluorescence fluctuations from the diffusion of single fluorescent molecules, and it allows raft-associated and non–raft-associated molecules to be distinguished without the use of detergents. In GUVs composed of a 1:1 equimolar mixture of DOPC, SM: cholesterol, which allows phase separation, CT-B and dialkylcarbocyanine showed different diffusional mobilities Fig. (6). On the other hand, in homogenous artificial GUVs composed of DOPC, the mobilities of CT-B and dialkylcarbocyanine were almost the same. When the cholesterol was depleted using MβCD, this reduced the mobility of dialkylcarbocyanine, and increased the mobility of CT-B. In HEK 293 cells, the same system saw no enhanced mobility of CT-B, which suggests that cytoskeleton disruption is required for higher CT-B mobility.

Further studies on CT-B-induced phase separation in GUVs have defined the dynamics of membrane phase separation, demonstrating it to be a highly dynamic process, and showing that GM1 appears to reside in the lδ phase, and to be associated with the lδ phase only upon addition of CT-B [124].

CONCLUSIONS AND PERSPECTIVES

Membrane microdomains, including rafts, have been a subject of several investigations over the last 15 years. Emerging evidence of the implications of rafts in crucial biological functions and under pathological conditions have required the development of new techniques and markers that allow their visualization. However, in addition to their heterogeneity, the spatial and temporal instabilities of these membrane microdomains have hampered these efforts, especially when studied in living cells. Non-lytic mutants or chemical derivatives of natural toxins, and particularly of the PFTs, either fused with a fluorescent protein or dye, or labeled with a fluorescent antibody, represent tempting tools for membrane-raft visualization. These can specifically target and bind to a raft-residing lipid, or lipid combination, or they can ‘sense’ a specific membrane physical phase (e.g. the lδ phase). We must, however, be careful in our interpretation of data obtained with these external raft probes.
as their binding can influence the membrane structure itself and can induce local rearrangements of membrane microdomains. This has been shown for the binding of the CT-B pentamer, which induces clustering of five GM1 molecules [31], and for listeriolysin O, which promotes the aggregation of several raft-associated molecules [125]. The use of antibodies, with their characteristic larger size, can lead to further clustering, as has been shown in the case of CT-B [126]. Such adverse effects can lead to mis-interpretation of the spatial and temporal architecture of these membrane microdomains. In this regard, PFTs tagged with a fluorescent dye, fluorescently fused PFTs, and engineered smaller binding domains of PFTs (as in the case of perforin-glycosyl O) might provide a more correct choice. Furthermore, it is important to distinguish between direct interactions of a putative raft marker with a certain lipid, and its recognition of a certain physical state of the membrane (e.g. the l phase) that derives from the use of particular lipid mixtures. It has been shown that the lipid phase can promote membrane insertion and pore formation of EqTII even in membranes without SM [57]. However, EqTII binding was still low compared to that with membranes with very low levels of SM.

**CONFLICT OF INTEREST**

The author(s) confirm that this article content has no conflicts of interest.

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Fig. (5). Phase separation in model membranes using with Alexa-488-labeled cholera toxin B subunit. GUVs were prepared with equimolar contents of DOPC, SM, cholesterol, with the addition of diacetylcarnocyanine-C18 (as a marker for l phases), and Gm in trace amounts. Cholera toxin B subunit (CtxB-488), which binds to G31, was added after the preparation. Diacetylcarnocyanine-C18 fluorescence is shown in red and cxTxB-488 fluorescence in green. Three-dimensional reconstruction of GUVs from confocal slices is shown. Scale bar, 10 μm. Reproduced from [123], with permission of Elsevier.
Pore-Forming Proteins As Raft Markers

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