

Membranes and Fluorescence Microscopy

Luis A. Bagatolli

Abstract Fluorescence spectroscopy-based techniques using conventional fluorimeters have been extensively applied since the late 1960s to study different aspects of membrane-related phenomena, i.e., mainly relating to lipid-lipid and lipid-protein (peptide) interactions. Even though fluorescence spectroscopy approaches provide very valuable structurally and dynamically related information on membranes, they generally produce mean parameters from data collected on bulk solutions of many vesicles and lack direct information on the spatial organization at the level of single membranes, a quality that can be provided by microscopy-related techniques. In this chapter, I will attempt to summarize representative examples concerning how microscopy (which provides information on membrane lateral organization by direct visualization) and spectroscopy techniques (which provides information about molecular interaction, order and microenvironment) can be combined to give a powerful new approach to study membrane-related phenomena. Additionally along this chapter, it will be discussed how membrane model systems can be further utilized to gain information about particular membrane-related process like protein(peptide)/membrane interactions.

Keywords Giant unilamellar vesicles · Membrane lateral structure · Membrane-peptide interaction · Polarity sensitive probes · Fluorescence microscopy

Introduction

Fluorescence spectroscopy-based techniques using conventional fluorimeters have been extensively applied since the late 1960s to study different aspects of membrane related phenomena, i.e., relating to lipid–lipid, lipid–protein (peptide), and lipid–DNA interactions. These types of studies encompass measurements of

L.A. Bagatolli (✉)

Membrane Biophysics and Biophotonics Group/MEMPHYS – Center for Biomembrane Physics, Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55 DK-5230, Odense M, Denmark
e-mail: bagatolli@memphys.sdu.dk

fluorescence excitation and emission spectra, fluorescence time decays (lifetimes) and fluorescence polarization (or anisotropy) using of a large variety of fluorescent probes. The last also includes applications of Förster resonance energy transfer (FRET) or fluorescence quenching techniques [1–9]. In these types of studies, liposomes have had a significant role as model systems for cellular membranes. Liposome studies generally involve aqueous suspensions consisting of small unilamellar vesicles (SUVs, mean diameter 30–50 nm), large unilamellar vesicles (LUVs, mean diameter 100 nm) and multilamellar vesicles (MLVs) being the most popular model systems. The composition of these membranes can range from single lipid components to mixtures of lipids (synthetic or natural lipid extracts), both with and without membrane proteins, including in some cases closed vesicles obtained from native biological membranes. Even though the aforementioned fluorescence spectroscopy approaches provide very valuable structural and dynamical information on membranes, they generally produce mean parameters from data collected on bulk solutions of many vesicles and lack information on membrane organization at the level of a single vesicle, a quality that can be provided by microscopy related techniques. The lack of information regarding direct imaging of the (local) spatial organization of membranes can also be extended to other experiments performed with other spectroscopy and thermodynamics related techniques such as differential scanning calorimetry, IR spectroscopy, NMR, and X-ray diffraction to mention a few [10–16].

The first experiments reporting visualization of lipid domains in bilayers by means of fluorescence microscopy provided valuable new information (such as shape and size distribution of different lipid domains), not available before in the membrane field [17–19]. However, few reports explored the local fluorescence properties of the probes in any detail, using the classical fluorescent spectroscopy approaches mentioned above. The continued development of confocal microscopy, both one-photon and two-photon approaches, which has greatly increased the information available through imaging, has allowed for rapid advances in the biophotonics field. For example, fluorescence correlation spectroscopy (FCS), three dimensional particle tracking methods, including polarization, lifetime and emission spectral information based techniques can now be performed in the microscope environment [20–26]. At the present time, there are a number of laboratories actively advancing this concept, i.e., performing spectroscopy in a microscope for a variety of protocols and generating exciting results from studies ranging from cell physiology to the mechanics of polymer motion on surfaces.

The advantages of using a microscope as the optical arrangement are clear. The light collection efficiency of a well-designed microscope is greatly enhanced over other optical arrangements. In addition, the flexibility of present fluorescence microscopes creates for the spectroscopist a malleable optical compartment which can be designed and readily re-designed as needed. Of equal importance as the sensitivity and flexibility of a microscope is the addition of spectroscopy to the ability to collect spatially resolved information. With a properly designed system, it is possible at present to perform quantitative spectroscopic studies at each pixel and build an information image related to the sample at hand, be it a living cell, an extended surface polymer, or a membrane model system.

The main aim of this contribution is to elaborate and provide a concise review on the different approaches that use fluorescence spectroscopy tools in a microscope to explore membrane related phenomena. In other words, I will attempt to summarize representative examples concerning how microscopy (which provides information on membrane lateral organization by direct visualization) and spectroscopy techniques (which provides information about molecular interaction, order and microenvironment) can be combined to give a powerful new approach to study membrane related phenomena. Additionally, I will discuss how the membrane model system can be further utilized to gain information about particular membrane related process like protein(peptide)/membrane interactions.

Suitability of Different Model Membrane Systems for Fluorescence Microscopy Studies

The most popular membrane model systems (SUVs, LUVs, MLVs) may not be fully suitable to perform fluorescence microscopy experiments, particularly if the collection of structural details at the level of single (unilamellar) vesicles is the main purpose of the experiment. Even though a fraction of the population of MLV (micrometer size) can already be observed under a microscope (see Fig. 1A), SUVs and LUVs have sizes below the resolution of an optical microscope and the visualization of, for example, membrane lateral structure at the level of single vesicles cannot be obtained. It is interesting to mention, however, that some attempts to use small liposomes (LUVs) under a fluorescence microscope have been recently reported [27]. In these studies, determination of the full size distribution of different liposomes preparations (either extruded or non-extruded lipid vesicles) was successfully achieved. The last study opens a novel application involving membrane structures under the resolution limit of the fluorescent microscope with interesting potential applications using fluorescence to study membrane related phenomenon. By using small vesicles and the abilities of a fluorescence microscopy for instance, future ensemble-based assays investigating vesicle-size-related effects will benefit from the ability to perform accurate measurements in real time on a great number of single objects [27].

GUVs as Membrane Model Systems: Advantages and Disadvantages

From a historical perspective, the first documented report involving membranes and optical microscope related techniques comes from the mid-eighteenth century. Although the credit for discovery and characterization of unilamellar lipid vesicles (SUVs and LUVs) was given to Bangham and Horne during the early 1960s (using electron microscopy as an experimental technique) [28], reports about the colloid behavior of lecithin and other phospholipids can be found much earlier in the literature [29, 30]. In fact, hydration of dried extracts of lecithin from egg-yolk or brain and the subsequent formation of myelin figures have been documented in 1854 and

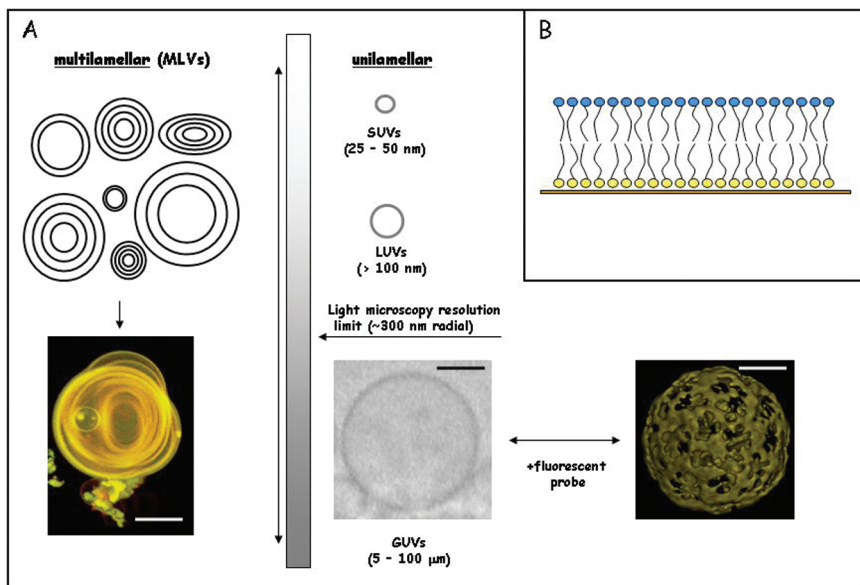


Fig. 1 Sketch of different membrane bilayer model systems. (A) A fraction of the population of MLVs (above 1 μm size) can be observed by fluorescence imaging (*left* fluorescent image, MLV composed of POPC, DiIC₁₈ probe). Only GUVs can be observed by using light microscopy based techniques (notice that SUVs and LUVs have dimensions below the resolution limit of the microscope). Incorporation of fluorescent probes and application of confocal fluorescence microscopy techniques provide information about the 3D structure of the GUV including the lateral organization of the membrane (*right* fluorescent image, GUV composed of Ceramide/Sphingomyelin mixture, DiIC₁₈ probe [118]). (B) Sketch of planar membrane bilayers on mica (atomically flat substrate). The different colors on the lipid polar head groups illustrate the possibility to generate compositionally asymmetric membranes (generally using Langmuir–Blodgett technique). The bars on the fluorescence microscopy images represent 10 μm

1867 by R. Virchow and C. Neubauer respectively [31, 32] using light microscopy. These membranes structures have dimensions of tens of micrometers and are the natural precursor of giant unilamellar vesicles (GUVs) when rehydration of dried lipid layers is performed. GUVs were first reported as alternative membrane model systems in 1969 by Reves and Dowben [33]. In recent years, GUVs have become very popular objects on which to perform fluorescence microscopy related experiments (see Fig. 1A). One of the reasons is that the dimensions of GUVs (mean diameter around $\sim 25 \mu\text{m}$) are higher than the intrinsic resolution limit of light microscopy related techniques ($\sim 250 \text{ nm}$ radial), allowing observation of structural details in membrane organization practically above $\sim 300 \text{ nm}$. Also the average size of GUVs is similar to that of the plasma membrane of a variety of cells. The last circumstance allows us to perform experiments at the level of single vesicles on the same size scale (curvature) as natural membrane systems (for example, cell

plasma membrane). Since the experiments are performed at the level of single vesicles, heterogeneity in shape and size or the presence of multilamellar vesicles are ruled out.

One of the significant aspects in using giant vesicles as model systems is the ability to control the molecular composition of the membrane as well as the environmental conditions. For instance, studies of the lateral structure of membranes using giant vesicles as model systems and fluorescence microscopy as experimental technique were normally restricted to giant vesicles composed of single lipid species or mixtures with few components [17–19, 34–44]. However, as recently reported in the literature, it is also possible to form giant vesicles from natural lipid extracts [36, 45–47] and native membranes [45, 48–50]. Additionally, GUVs containing membrane proteins can also be generated [51–56] often involving an electroformation method using proteoliposomes as starting point. Curiously and even though the description of giant vesicles was done almost 40 years ago, it was not until 1999 that a wide application of fluorescence microscopy related techniques was practically tested in this model system. Seminal reports however about the use of GUV, fluorescence microscopy, and detection of lipid lateral heterogeneity were reported in the late 1980s from the group of M. Glaser [57].

Regarding the generation of these giant lipid structures, there is no general agreement about the experimental conditions required to obtain GUVs. This lack of consensus may be due to the fact that the mechanism underlying giant vesicle formation still remains obscure. As a consequence, there are many different methods described in the literature to prepare giant vesicles [33, 45, 52, 54, 58–61]. Although the aforementioned scenario may look complex, most of these methods are based on two main experimental protocols: the gentle hydration method, originally described by Reeves and Dowben [33], and the electroformation method introduced by Angelova and collaborators [59, 60]. Of these two experimental protocols, the electroformation method has the advantage that it provides the more homogeneous population of GUVs, with sizes between 5 and 100 μm in diameter. Additionally, the electroformation protocol requires less time compared to the gentle hydration method ($\sim 1\text{--}2$ h vs $12\text{--}24$ h, respectively) and provides a high yield of giant unilamellar vesicles ($\sim 95\%$) [62, 63].

Perhaps the main drawback of GUVs and their applications as model systems for biological membranes is the fact that low salt concentrations (generally below 10 mM NaCl) are normally required for their preparation using the above-mentioned protocols. This problem has recently been overcome by the introduction of a new electroformation protocol by Pott et al. [64]. This method, originally developed for single phospholipid species was successfully tested for preparing GUVs from compositionally complex lipid mixtures, including native biological membranes [49]. This protocol produces membrane objects that practically keep the composition and organization of biological membranes (i.e., a more complete model system respect to those composed only for lipids). This protocol offers another choice with respect to that reported by Baumgart et al. [50], in which cell blebbing is used to produce GUVs. This last method is limited to cell plasma membranes, precluding the use of membranes from internal cell organelles to generate GUVs.

Planar Membranes

Even though it is not the aim of this chapter to extensively review fluorescence microscopy applications in other model membranes other than GUVs, it is fair to mention that planar bilayer membranes are very interesting membrane models to perform fluorescence microscopy experiments (Fig. 1B). An interesting example regarding the combination of planar membranes and fluorescence microscopy is that recently reported by Simonsen [65], where the evolution of the lateral structure of planar membranes upon changes in molecular composition induced by PLA₂ is explored. This last approach allows simultaneous correlation of membrane lateral pattern and enzyme kinetics. In fact many planar membranes systems were investigated (lipid–lipid but also lipid–protein interactions) using fluorescence microscopy as is nicely reviewed by Crane and Tamm [66].

Most of the fluorescence microscopy studies reported in planar membranes focus on measurements of fluorescence intensity of particular selection of probes with almost no practical combinations of fluorescence spectroscopic parameters (such as lifetime [67], polarization or emission spectra shift [68]) and microscopy. In fact, the use of probe partitioning into the different membrane regions as a criterion to assign lipid phases was questioned recently for this model system in experiments that combine atomic force microscopy (AFM) and fluorescence microscopy [69]. The last criterion was also stressed for GUV systems comparing and discussing the information obtained from various “partition-like” fluorescence probes with polarity sensitive fluorescent dyes [70] (see next section).

In summary, planar bilayer membrane systems are excellent candidates to apply fluorescence spectroscopy related studies [67] as well as fluorescence fluctuation analysis similar to those done in GUVs systems [24, 25]. In particular, this model system offers the possibility to easily generate asymmetric membranes, something that is possible, but practically difficult using GUVs [71]. Another advantage of planar membranes over free standing bilayers models (i.e., GUVs) is related to the geometrical features of the model system. For example determination of size and area analysis of different lipid domains in membranes displaying lateral phase separation is much simpler in planar membrane systems [72] than in GUVs (where quasi-spherical membrane structures are present) [73].

Fluorescent Probes and Lateral Structure of Biological Membranes

From the work reported using fluorescence spectroscopy (microscopy) and membrane systems, it is very obvious that a large number of options regarding lipid-like fluorescent probes are presently available (to have an idea one can refer to the catalog of Invitrogen/Molecular probes, for example). With the attempt to summarize the available information I decided to divide the lipid-like fluorescent probes into two main families. The last takes into account the partition properties among coexisting lipid phases and the particular mode of excitation under a fluorescence microscope. The first family contains those probes where excitation is viable by

one photon excitation mode, particularly in the visible range. These probes are the most used in epi-fluorescence and confocal fluorescence microscopy, particularly given the availability of the equipment in common laboratories. Generally, these probes have a two-chain lipid-like structure of variable size and unsaturation (even though some fluorescent moieties can themselves be used without further chemical modifications, i.e., without attachment of alkyl chains). For this group of probes, the fluorescent moiety can be attached either to the lipid chain or to the polar head group (Fig. 2A). One of the common features of these fluorescent probes is the uneven

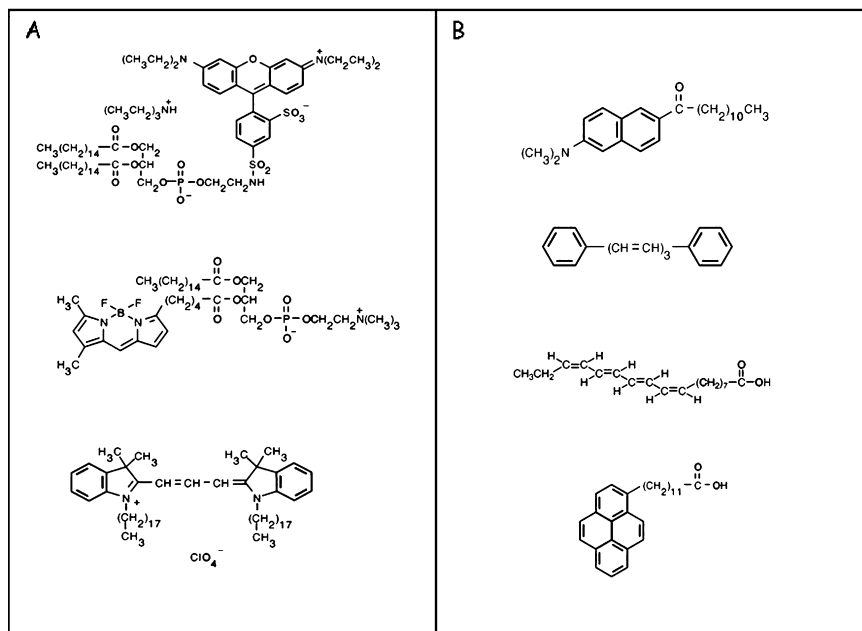


Fig. 2 Chemical structure of representative fluorescent probes: (A) Rhodamine-DPPE (top), Bodipy-PC (center), DiIC₁₈ (bottom). (B) From top to bottom: LAURDAN, DPH, Parinaric acid, C12 Pyrene

partition into coexisting membrane regions [17, 34, 38, 70, 74, 75]. Examples of these probes are amphiphilic derivatives of rhodamines, fluoresceins, dialkylcarbocyanine (DiI, DiO), dialkylaminostyryl (DiA), and coumarins, including bodipy, perylene, and naphthopyrene (see Fig. 2 for representative example). As was mentioned above, the partition of this family of fluorescence probes has been used as a criterion to assign lipid phases in GUVs displaying lipid phase coexistence [70]. Even though an extensive characterization of the partition of several fluorescent dyes on selected lipid mixtures was reported [74], the aforementioned criterion to define the nature of a lipid phase is still risky. Changes in the partition properties of the probes on the different membrane regions are highly dependent on the chemical composition of the local membrane domain and not on the phase state [18, 34, 70]. A practical solution to strength the data interpretation when fluorescent images are

used to assign lipid phases is the use of fluorescence correlation spectroscopy [17, 38]. With this last approach the diffusion of the probe can be related to the nature of the existing lipid phases. Alternative measurements of lifetime [76] or polarization under the microscope are also strong alternatives to explore which type of lipid phase is present.

As mentioned in the introduction section, the vast majority of the studies on the characterization of lipid membrane's lateral structure using fluorescence spectroscopy techniques (cuvette experiments) exploits other fluorescent parameters such as fluorescence lifetime, rotational correlation time (obtained by anisotropy measurements), and position of the emission spectral maximum. Several fluorescent probes, particularly those UV-excited fluorescent probes (such as Pyrene, DPH and their derivatives, parinaric acid, LAURDAN, PRODAN), are used in regular fluorescence cuvette experiments [7, 77–84]. These dyes represent a second family of membrane probes and have not been fully exploited yet using fluorescence microscopy related techniques. Two main reasons for the dearth of such studies are (i) the inaccessibility of these advanced microscopy techniques, i.e., lifetime microscopy or polarization fluorescence microscopy (expensive and specialized equipment is required along with significant user expertise) and (ii) it is practically difficult to perform fluorescence microscopy experiments (one photon excitation, i.e., epifluorescence and confocal) with UV-fading fluorescent probes, since the extent of photobleaching is high and is often technically difficult to obtain reliable fluorescence images. An alternative solution is the use of multiphoton microscopy experiments. In this way an extensive application was reported with the probe LAURDAN under multiphoton excitation microscopy [25, 70, 85, 86]. Perhaps one of the most remarkable features of these probes (particularly the ones that keep a fatty acid like structure, i.e., DPH, LAURDAN, parinaric acid) is the fact that they show an even distribution in membranes displaying phase coexistence. In this last situation correlation of the probe's spatial distribution and fluorescence parameters such as spectral shift, polarization, or lifetimes is ideal to relate the membrane region of interest with lipid phases. Other recently reported polarity sensitive probes to be fully exploited under fluorescence microscopy are 3-hydroxyflavone derivatives [87], C-LAURDAN [88], and di-4-ANEPPDHQ [89]. These probes show different emission spectra depending to the membrane's phase scenario and also show even distributions on membranes displaying lateral phase separation.

Lateral Structure of Compositionally Simple and Complex Membrane Model Systems

As mentioned above, it was not until the end of the 1990s when several papers appeared applying fluorescence microscopy related techniques (epifluorescence, confocal, and two-photon excitation fluorescence microscopy) to bilayer membrane systems. These reports showed for first time images of the temperature-dependent lateral structure of giant vesicles composed of different phospholipids, phospholipid binary mixtures, ternary lipid mixtures containing cholesterol, natural lipid extracts,

and native membranes [17–19, 34, 36–38, 40–42, 45, 46, 85, 90, 91]. These papers presented a correlation between micron-size (visual) lipid domain structures and local lipid dynamics under different environmental conditions.

The experimental data involving fluorescence microscopy and GUVs have also been used to construct lipid phase diagrams for artificial lipid mixtures (i.e., phase diagrams that include visual information about membrane lateral structure) [38, 41, 75, 92]. If this type of experiments are performed, it is very important to corroborate the local phase state in each particular membrane region with different approaches beside probe partition as mentioned above (i.e., FCS, LAURDAN GP, FLIM) [17, 25, 38, 70, 76]. Importantly, if phase diagrams are constructed with these data, careful evaluation of thermodynamic equilibrium is required (something that is not really demonstrated in the work reported until now). In fact, some differences in particular regions of the cholesterol containing ternary mixtures (canonical “raft” mixtures) phase diagrams was reported in the literature when phase diagrams were obtained either from GUVs experiments or bulk techniques involving solutions of LUVs (fluorescence spectroscopy in cuvette) [41, 93]. These discrepancies may be related to the resolution of the fluorescence microscope [9, 75]; however it is interesting to notice that in the GUV experiments reported on no additional fluorescence spectroscopy data beside the partition of the lipophilic probe is used to construct the phase diagram [75]. By confirming the existence of phases by fluorescence parameters other than imaging fluorescence intensity and validating basic thermodynamic rules in GUVs systems, one can be assured that the information obtained is suitable to construct a phase diagram. By applying image analysis, for example, quantitative area information and further tests of thermodynamics rules can be performed, including experiments to evaluate the presence of domains below the resolution of the microscope (LAURDAN GP, see [85]). Quantification of the area contribution of the different lipid phases in GUVs displaying lipid phase coexistence is presently under evaluation in our laboratory [73].

The information obtained from model systems can be utilized to explore biological membranes with the possibility to display lipid mediated lateral heterogeneity. Examples are reports studying, for example, GUVs composed of native lung surfactant [45], stratum corneum (SC) skin lipids membranes [94], brush border membranes [48], and red blood cells [49]. Figure 3 shows a representative example of skin lipid mixtures (extracted from stratum corneum of human skin tissue; a complex mixture of ceramide, fatty acids, and cholesterol) and pulmonary surfactant from pig lungs (containing a complex mixture of DPPC, cholesterol, and unsaturated lipids including surfactant proteins). Both mixtures at the correspondent biologically relevant temperature show coexistence of different lipid phases (gel/gel and liquid ordered/liquid disordered-like phase coexistence for skin lipid mixtures and lung surfactant respectively; Fig. 3A and B, left panel). Perturbations of either composition (removing of cholesterol) and temperature in lung surfactant membranes (Fig. 3 B) or temperature and pH for human SC skin lipid mixtures (Fig. 3 A) generate significant changes in the lateral structure of these different membranes [45, 94]. This behavior can be

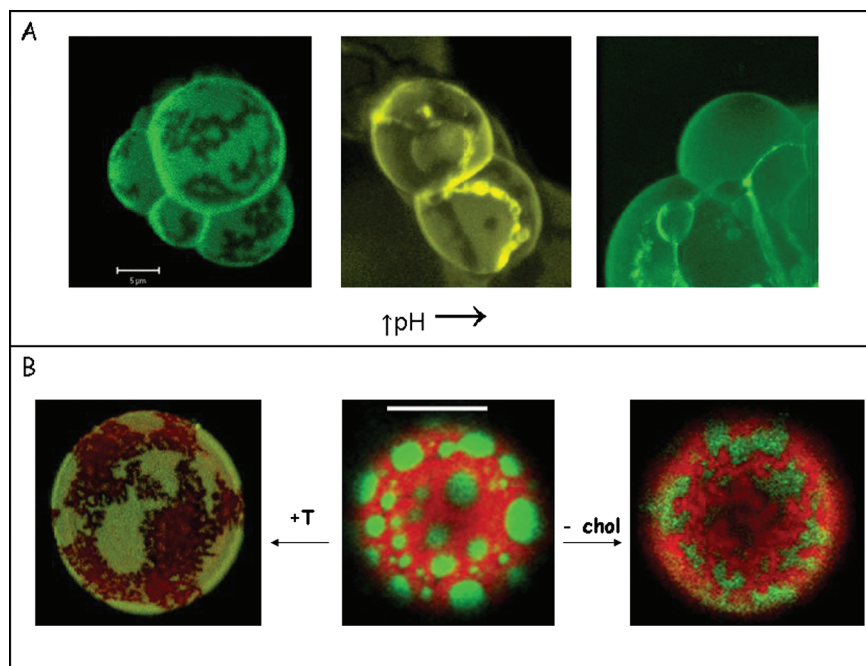


Fig. 3 Examples of giant lipid structures composed of compositionally complex membranes. (A) Effect of pH on giant lipid membranes composed of native lipid mixtures extracted from human skin stratum corneum membranes. The figure in the *left* show the particular lateral structure of this membrane (coexistence of two gel -like phases) at physiological conditions (pH 5, $T = 32^{\circ}\text{C}$, DiI C_{18} probe). Increasing the pH to 6 and 7 (center and right fluorescence microscopy image respectively) generates a change in the lateral organization of the lipids (for more details see [94]). (B) GUVs composed of pig native lung surfactant at physiological conditions (center fluorescence microscopy image showing presence of liquid ordered (*red*)/liquid disordered (*green*)-like phase coexistence; $T = 37^{\circ}\text{C}$). An increase in the temperature cause a phase transition on the native material (*left* fluorescence microscopy image, $T = 38^{\circ}\text{C}$). Extraction of cholesterol generates a lateral pattern that resembles coexistence of gel (*red*)/liquid disordered (*green*) phases (*right* fluorescence microscopy image). For additional details see [45]. The bars represent 15 μm

reproduced in model membranes using representative lipids. For example ternary mixtures composed of DOPC/DPPC/cholesterol have been utilized to mimic lung surfactant [45].

Peptide(Protein)–Lipid Interactions Studies in GUVs

As was emphasized above GUVs are micrometer size objects that allow one to perform fluorescence microscopy at the level of single vesicles. The additional visual information extracted from these experiments can be fully exploited to interrogate the effect of proteins and peptides on the structure of membranes. In this section

representative examples will be summarized with particular emphasis in the utilized experimental strategy.

Peptide–Membrane Interactions

A wide range of peptides and toxins are harmful to cells by altering the hydrophobic-hydrophilic seal of biological membranes. Generally this last effect is related to the composition of the membrane and the chemical structure of the peptide [95–99]. In fact the membrane damage can be exerted by different mechanisms: (i) *membrane lysis (or membrane solubilization)*: peptides that remain tightly bound to the membrane interface and promote bilayer damage via solubilization of membrane components (so-called detergent-like or carpet-like mechanism) [100–103] and (ii) *pore formation*: peptides that acquire a particular conformation across the lipid bilayer and consequently form size-defined permeating structures named as pores but do not alter the long-range interactions between membrane components [96–98, 100, 101, 104–108].

A common approach to study the aforementioned membrane perturbation is to observe leakage of fluorescent molecules entrapped inside liposome solutions. However, this fluorescent cuvette experiment lacks visual information, a feature that can be obtained using GUVs and fluorescence microscopy. The use of GUVs and fluorescence microscopy not only allows the direct observation of the leakage phenomenon caused by the interaction of the peptide with the membrane (pore formation or membrane lysis, see Fig. 4), but also can be potentially used to examine simultaneously lateral structure of the membrane and peptide distribution in the plane or across the membrane (if the peptide is fluorescently labeled). Few

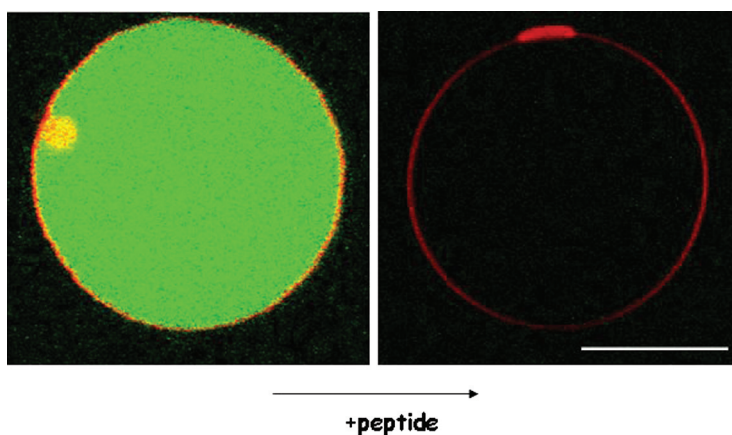


Fig. 4 GUV composed of POPC labeled with DiIc8 (membrane, *red*) filled with carboxyfluorescein (*green*). Upon peptide addition (mellitin in this case) a leakage of the fluorophore is observed due to formation of pores (see [104, 105]). The *bar* represents 20 μm

applications were reported in the literature using slight variations of the aforementioned approach [102, 104, 105, 107, 109, 110]. For example, in those applications reported by Ambroggio et al, the lytic mechanism of different peptides (Alzheimer β -amyloid peptide 1–42, Citropin, Aurein, and Maculatin) has been studied [104, 105]. In these experiments GUVs are loaded with different-sized water soluble fluorophores including a membrane reporter (lipid-like fluorescent probe, Fig. 4) [104, 105]. For example, when GUVs are exposed to Maculatin peptides, a low molecular weight probe entrapped in the GUVs (Alexa⁶³³-maleimide in this case) first leaked out without significant leakage of a high molecular weight probe (Alexa⁴⁸⁸-dextran, 10 kDa molecular weight). Additionally it can be simultaneously observed that there is not any evident change in the overall three-dimensional structure and integrity of the GUVs during the leakage event (using the membrane probe DiIC₁₈, red) [104, 105]. The interpretation of the above results is that the Maculatin peptide generates pore-like structures in the membrane allowing the differential leakage of the soluble fluorescent probes from the inner side of the vesicles. A similar effect is observed for the well-known pore-forming peptide melittin and the Alzheimer β -amyloid peptide 1–42 [104, 105]. On the other hand, a different behavior is achieved by the small antibiotic peptides Citropin and Aurein at the same concentration as the one used for the Maculatin experiments. In the case of Citropin and Aurein concomitant leakage of both dyes and membrane destruction is observed when GUVs are exposed to the peptides. Consequently, quantitative information regarding leakage kinetics (fluorescence intensity vs. time) and pore size (leakage of entrapped fluorophores of different molecular weight) can be simultaneously obtained in this type of experiment using GUVs and fluorescence microscopy.

A similar approach was used to observe the interaction of the cell penetrating peptide pep-1 using GUVs composed of POPC or POPC/POPG (4:1 molar) [102]. In this case the GUVs were loaded with three different sized soluble fluorescent markers, Alexa Fluor⁶³³-maleimide (1300 MW), Alexa Fluor⁴⁸⁸-Dextran (3000 MW), and Alexa Fluor⁵⁴⁶-Dextran (10000 MW). In these experiments it was observed that pep-1 does not alter the bilayer integrity and does not provoke changes in membrane permeability at peptide to lipid ratios below 1. However, above this ratio liposomes suffer an absolute destruction with the simultaneous leakage of the three dyes, ending in deformed lipid aggregates. This experiment gives an idea of the cytotoxic mechanism of pep-1 when it is present at high concentrations [102]. Similar experimental strategies (but using a single water soluble fluorophore) were used by Tamba and Yamazaki [109] to observe the effect of magainin 2 on GUVs composed of PC/PG mixtures and Hasper et al. [107] to explore the bactericidal mechanism of lantibiotic peptides in lipid II containing GUVs.

In summary, these “leakage” experiments at the level of single vesicles allowing simultaneous determination of membrane-destabilization mechanisms mediated by different membrane-active peptides including estimation of pore size (if pore formation is present), spatial localization of peptides (if fluorescent labeled peptides are available), and membrane lateral structure upon peptide addition. The last approach allows more complete information than those based on fluorescent cuvette

measurements where visual information about the microscopic scenario is not available.

Protein–Membrane Interactions

The majority of the experiments reported using GUVs containing membrane proteins focus on studying the spatial correlation between certain membrane proteins and cholesterol enriched domains (liquid ordered phase). These experiments particularly involve the use of lipid-like probes and fluorescently labeled proteins in the GUVs. Imaging of the fluorescence intensity distribution in the GUV membrane and/or diffusion measurements (using FCS) were reported in the literature in order to ascertain the distribution of membrane proteins into particular lipid phases [45, 48, 53, 56]. Another type of fluorescence microscopy experiments involving GUVs is related to the potential interaction of proteins with the outer leaflet of the GUVs bilayer. Generally the effects reported in these experiments are related to changes in membrane lateral structure or eventually changes in the 3D structure of the GUVs [111–113]. Other reports that explored the aforementioned effects are related to proteins that cause lipid hydrolysis, i.e., lipids that change the membrane composition (lipases for examples) [114, 115].

Future Directions

A large amount of information relating to various membrane-related phenomena were obtained in the last 10 years using GUVs and fluorescence microscopy. However, development of more sophisticated membrane model systems is still required to achieve more realistic information concerning biological membranes. For instance, the potential effect of the cytoskeleton on membrane lateral structure has not been yet deeply explored. However, some papers reported polymerization of actin inside giant vesicles [116] and modulation on the lateral structure of the membranes displaying phase coexistence upon protein polymerization [117]. Last but not least the local dynamics of domain formation was recently described by Celli et al. [25]. These authors used a combination of imaging and fluctuation techniques to investigate the temporal evolution of gel phase domains at the onset of phase separation. These types of studies are extremely valuable and likely to be extended to cholesterol containing ternary mixtures. The full and concerted characterization of structural and dynamical aspects of lateral phase separation phenomenon in model membranes will establish the basis to evaluate if related processes happen in biological membranes. The last will also help to ascertain when lipid mediated phenomenon (or liquid ordered phases) are really relevant in particular biological membranes.

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