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Structural organization of plasma membrane lipids isolated from cells cultured as a monolayer and in tissue-like conditions

Galya Staneva [a,*], Teodora Lupanova [a], Claude Chachaty [b], Diana Petkova [a], Kamen Koumanov [a], Roumen Pankov [c], Albena Momchilova [a]

[a] Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria
[b] Universite Paris 6, INSERM U893, CHU St. Antoine, 27 rue Chaligny, 75012 Paris, France
[c] Biological Faculty, Sofia University, 8 Dragan Tsankov Str., Sofia 1164, Bulgaria

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ABSTRACT

Complementary biophysical approaches were used to study the structural organization of plasma membrane lipids obtained from fibroblasts cultured as two-dimensional (2D) monolayer and in tissue-like three-dimensional (3D) conditions. Fluorescence microscopy experiments demonstrated different domain patterns for 2D and 3D plasma membrane lipid extracts. ESR demonstrated that 3D lipid extract is characterized with lower order parameter than 2D in the deep hydrophobic core of the lipid bilayer. Higher cholesterol and sphingomyelin content in 3D extract, known to increase the order in the glycerophospholipid matrix, was not able to compensate higher fatty acid polyunsaturation of the phospholipids. The interfacial region of the bilayer was probed by the fluorescent probe Laurdan. A higher general polarization value for 3D extract was measured. It is assigned to the increased content of sphingomyelin, cholesterol, phosphatidylethanolamine and phosphatidylserine in the 3D membranes. These results demonstrate that cells cultured under different conditions exhibit compositional heterogeneity of the constituent lipids which determine different structural organization of the membranes.

1. Introduction

Cell cultures are a widely used convenient model for investigation of a large number of processes in living cells. The conventional culture method (two-dimensional (2D) monolayer cultures), compared to the model using three-dimensional (3D) extracellular matrix, has some disadvantages coming from the highly unnatural geometric and mechanical constraints imposed on cells [1,2]. Thus 2D cell cultures only approximate properties of normal tissues and this approximation is always limited to single cell types and does not take into account the impact of the other cells and the environment. Some reports in the literature have demonstrated that the adaptive processes of the cells in two-dimensional culture conditions cause dramatic changes in their functional and structural properties [3,4]. Since the cellular plasma membrane plays a key role in essential processes such as transmembrane signal transduction, it is likely that any alterations in its composition and structural organization would affect certain cellular functions. In addition, the impact of the extracellular environment and culturing conditions on the plasma membrane lateral heterogeneity and particularly on the formation of raft-microdomains which are recognized as membrane signaling platforms [5], represents a great interest in this context. The great variety in the structure of the native membrane phospholipids is a prerequisite for a marked membrane heterogeneity and coexistence of different types of membrane domains induced by the non-ideal miscibility of lipids.

The investigations on multi-component bilayers composed of plasma membrane lipids are still rare because, on one hand, it is difficult to isolate enough quantities and, on the other hand, to ascribe the observed physicochemical properties to specific membrane lipids, or to particular lipid interactions. For this reason we have addressed this question by combining three complementary biophysical methods to characterize and visualize the heterogeneous structure of the plasma membrane lipids obtained from cells cultured under well-controlled conditions. Fluorescence microscopy of giant unilamellar vesicles (GUVs), Laurdan fluorescence spectroscopy, and electron paramagnetic resonance were used. Usually, studies with GUVs are performed on definite lipid mixtures and there are very few reports on GUVs composed of natural lipid extracts [6–9]. While micrometer domain formation in giant vesicle is based on long-range ordering of the phospholipids, spectroscopic methods such as electron spin resonance are sensitive to short-range interactions and are appropriate for measurement of the molecular order and dynamics of the membrane bilayer at different levels. In addition to this structural information concerning

* Corresponding author. Fax: +359 2 9712493.
E-mail address: gstaneva@obzor.bio21.bas.bg (G. Staneva).

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the hydrophobic core of the bilayer, the monitoring of the polar part of the bilayer was also necessary because of the large variety of polar groups in the used types of plasma membrane lipids. Such a useful fluorescent probe is Laurdan, known to be located at the hydrophilic–hydrophobic interface of the bilayer with the lauric acid tail anchored in the phospholipids acyl-chain region.

In the present study we used the above mentioned biophysical approaches to characterize and visualize the lipid organization of plasma membranes isolated from cells cultured in different conditions: as a monolayer (2D) and in three-dimensional matrix (3D).

2. Materials and methods

2.1. Cell culture

Fibroblast cell line GD25β1 [10] was grown in DMEM containing 10% fetal bovine serum (FBS) and a mixture of antibiotics – antimycotics: penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). The GD25β1 cells were chosen because they lack contact inhibition and can be cultured either as a monolayer, or grown beyond confluency – as a multilayered three-dimensional culture. The stock culture was propagated as conventional monolayer and the cells were split and passaged after reaching 80–90% confluency. To obtain monolayer 2D and 3D cell cultures for plasma membrane preparation, fibroblasts were seeded at a concentration of 0.7×10^5 cells per cm^2. 2D cells were collected after 24 h when fibroblasts have reached 100% confluency and had cell density of about 1×10^5 cells per cm^2. Three-dimensional cultures were obtained as described elsewhere [4]. Briefly, the cells were cultured for five consecutive days after seeding which allowed them to produce their own matrix and form multilayered culture with cell density of about 9×10^5 cells per cm^2.

2.2. Isolation of plasma membrane fractions

Plasma membranes were obtained according to the procedure described elsewhere [11] which involves differential centrifugation. Briefly, the post – nuclear supernatant was loaded on a discontinuous sucrose gradient and centrifuged at 100,000*g* for 2,5 h. The plasma membrane fraction was obtained at a density of 45% (w/v) suspended in ice-cold 100 mM Tris buffer, pH 7.4 and used immediately for lipid analysis.

2.3. Lipid extraction and analysis

Lipid extraction was performed with chloroform/methanol according to the method of Bligh and Dyer [12]. The organic phase obtained after extraction was concentrated and phospholipid fractions were separated on silica gel G60 plates (Merck) in a solvent system chloroform/methanol/2-propanol/triethylamine/0.25%KCl (30/9/25/18/6 v/v). The location of different phospholipid species was determined by iodine staining and by standards run in parallel. The spots were scraped and quantified by determination of the inorganic phosphorus [13]. Cholesterol content was assayed by gas chromatography using a medium polarity RTX-65 capillary column (0.32 mm internal diameter, length 30 m). Calibration was achieved by a weighted standard of cholestane.

2.4. Laurdan fluorescence measurements

Multilamellar vesicles (MLV) were prepared by mixing the appropriate amounts of stock solutions (in chloroform) of plasma membrane lipid extracts and Laurdan (Molecular Probes Inc., Eugene, OR) in a glass tube and then the solvent was evaporated under nitrogen. The dried samples were resuspended in PBS (pH 7.4). The final concentration of the probe and phospholipids were 0.8 μM and 0.4 mM, respectively (probe/phospholipids = 1/50). Multilamellar vesicles suspensions were heated at 37 °C and vortexed. The samples containing the fluorescent probe were protected from light during all steps. The emission spectra were recorded between 390 nm and 500 nm, whereas the samples were excited at 360 nm. In the other case the excitation spectra were recorded from 300 nm and the emission – at 440 nm. The excitation generalized polarization (GP) at 340 nm was calculated using fixed emission wavelengths of 435 nm and 470 nm.

$$GP_{ex} = (I_{435} - I_{470})/(I_{435} + I_{470})$$

where I_{435} and I_{470} are emission intensities at the characteristic wavelength of the gel phase (435 nm) and the liquid crystalline phase (470 nm), respectively.

The fluorescence measurements were performed on Perkin Elmer spectrofluorimeter at room temperature.

2.5. Fluorescence microscopy

2.5.1. Preparation of giant unilamellar vesicles

The traditional electroformation method (AC field, 1 V, 10 Hz) [14] was used for preparation of giant unilamellar vesicles (GUVs) from cell plasma membrane lipid extracts. The vesicles were always formed at 45 °C at which a high yield was consistently obtained. The vesicles were prepared in a temperature – controlled chamber in 1 mM Hepes buffer, 2 mM NaCl, pH 7.4. The higher NaCl concentration caused aggregation of the lipids on the electrodes and the GUVs yield was significantly reduced. At 3 mM NaCl, GUVs formation was completely impeded. As alternative was used a protocol (2 V, 500 Hz) for high salinity GUV formation reported by Montes et al. [9] and Pott et al. [15] by achieving up to 10 mM NaCl. Although the lipid aggregation on the electrodes was expressed at lower degree, the yield of giant vesicles was not high enough and the mean vesicle diameter was largely reduced (5 μm). The formation of domains in the presence of 10 mM NaCl appeared at slightly lower temperatures ($\Delta t \approx -4$ °C) than those at low salt concentration. This fact might be rather ascribed to smaller vesicle size which hinders accurate detecting of the temperature of domain formation than the salinity effect. Other possibility might be the exclusion of some lipid species from the plasma membrane extract and their aggregation on the electrodes caused by the salt presence. That is why we chose to mark the average temperature of domain formation in the presented data.

2.5.2. Imaging of GUV

The vesicles were observed using a Zeiss Axiovert 135 microscope equipped with 40× and 63× long working distance objective lens (LD Achroplan Ph2). Observations were recorded using Zeiss AxioCam HSm CCD camera connected to an image recording and processing system (Axiovision, Zeiss). The phase morphology transformation of the heterogeneous GUV membranes were followed in phase contrast and in fluorescence by Zeiss filter set 15 (Ex/Em = 546/590 nm). The headgroup-labeled lipid analog egg PE–Rhodamine (0.8 mol%) (Avanti Polar Lipids) was used as a fluorescent marker.

2.5.3. Microinjection of methyl-β-cyclodextrin

The microinjection was carried out with an Eppendorf FemtoJet. The micro-capillary for performing local microinjection to a GUV had 0.8–1 μm inner diameter. Injection was performed from a distance of about 50 μm from the GUV. Injected volumes of methyl-β-cyclodextrin stock solution were of the order of a few picoliters and required the use of very concentrated stock solution. Methyl-β-cyclodextrin (50 mM) was prepared in 1 mM Hepes

buffer, pH 7.4, identical to the buffer in which the vesicles were formed. All experiments were carried out at 24 °C (room temperature). The observations presented below are based on at least 5–10 experiments of the same kind.

2.5.4. ESR measurements

The ESR experiments have been performed at 9.3 GHz (Bruker, ER200D ESR, Wissembourg, France) using the doxyl-16-stearic spin-probe denoted hereafter as 16NS. In the whole temperature range of our experiments (7–47°), the reorientation of this probe is under the fast motional regime with reorientation correlation times $\tau \ll 2$ ns [16].

Plasma membranes lipid extracts were doped with 0.1 mol% of 16NS (Avanti Polar Lipids). After evaporation of the solvent the dry lipid extracts were hydrated with a large excess 500 µl of a buffer consisting of 25 mM Hepes, 150 mM NaCl, pH 7.4 at 45 °C. The lipid dispersion was centrifuged and 20 µl of the pelleted liposomes was transferred to an ESR measurement capillary cell and sealed. The ESR spectra were recorded after an equilibration time of 10 min at each temperature set by the variable temperature device (Bruker ER4111VT). The signal was digitized by the EPRWARE software (Scientific Software Service, Bloomington, IL61701, USA).

The least-squares fittings of simulated spectra to experimental ones (Fig. 1) have been performed by methods reported in Chachaty and Soulie [17] (see also www.esr-spectsim-softw.fr), yielding two parameters of interest in the present study: the order parameter S_{zz} and the reorientation correlation time τ of the *Z*-axis of the nitrogen hyperfine coupling tensor parallel to the C_{15}–C_{17} vector of the hydrocarbon chain of the probe.

3. Results and discussion

The functions of cellular membranes depend significantly on the membrane bilayer structure and physicochemical properties which in turn can be affected extensively by the extracellular environment. The GD25β1 fibroblast cells lack contact inhibition, which allows them to grow as a conventional monolayer cell culture (2D), or, if left to grow beyond confluency, they can form a three-dimensional, multilayered culture, embedded in naturally-synthesized extracellular matrix (3D). Plasma membranes were isolated from fibroblasts, cultured as a monolayer and in three-dimensional matrix and used for phospholipid extraction.

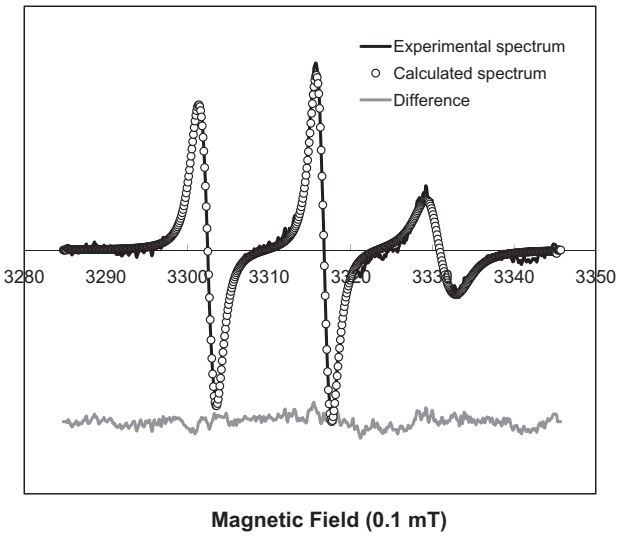

Fig. 1. Experimental and simulated spectra of the 16NS probe in the 3D plasma membrane extract at 37 °C. The fitting yields $S_{ZZ} = 0.082$ and $\tau = 0.76$ ns.

2D plasma membranes contained higher levels of phosphatidylcholine (PC) and lower levels of sphingomyelin (SM), phosphatidylserine (PS) and phosphatidiylethanolamine (PE) compared to 3D (Fig. 2A). Besides the lipid identification according to their head-groups, another factor for complete understanding of the lipid structural organization and phase state is the level of saturation of their acyl chains (Fig. 2B).

The analysis of the fatty acid composition of the separate plasma membrane phospholipids demonstrated differences in the ratio between saturated and unsaturated fatty acids in 2D and 3D fibroblasts (Fig. 2B). It should be pointed out that SM which was increased about two fold in 3D cells, exhibited twice as a high level of fatty acid saturation compared to 2D fibroblasts. This was due mainly to the presence of long C20:0 and C22:0 saturated fatty acids (Fig. 2E). On the other hand, as evident from Fig. 2A the most abundant plasma membrane phospholipid fractions were PC, PE and PS + PI and their sum amounted to about 70 mol% from the total plasma membrane phospholipids. These phospholipids showed a higher level of saturation in 2D plasma membranes (Fig. 2B) which could be attributed to the lower content of polyunsaturated fatty acids than monounsaturated ones (Fig. 2C and D). Overall, the lipid analysis predicted a more ordered lipid bilayer in the hydrophobic core of the lipid bilayer composed of 2D plasma membranes compared to 3D. The cholesterol/total phospholipids ratio was also different in 3D and 2D cells. The amount of CHOL was increased up to about 34 mol% in 3D plasma membrane lipid extract vs. 21 mol% in 2D. Our previous studies revealed a similar trend for SM [18]. All these results suggest that the differences in structural components of 2D and 3D plasma membranes as well as in their ratios could underlie eventual specificities in their phase behavior.

Thus, studies were performed to analyze the processes of phase separation of membrane phospholipids extracted from plasma membranes of fibroblasts cultured as a monolayer (2D) and in a 3D matrix. For this purpose, we used as an experimental model GUVs to visualize the phase behavior and ability of plasma membrane lipids to form domains in the temperature range from 37 °C to 4 °C (Fig. 3). GUV bilayers were composed of cellular plasma membrane lipid extracts. The visualization of the phase behavior of 2D and 3D lipid extracts was achieved by different fluorescent probe partition into the coexisting phases. Egg PE–Rhodamine exhibits a greater partition coefficient for the fluid phase. Thus, the bright vesicle area represents the more fluid phase and the microdomains with more ordered structure occur as dark regions. In the 37–22 °C temperature interval two lipid extracts exhibited homogeneous appearance (Fig. 3A (a and b)) and no formation of dark domains in the micron-scale were observed for both types of plasma membranes. Especially for 3D plasma membranes, a population of multilamellar giant vesicles was observed as shown in Fig. 3A (b and c). The indication of multilamellarity was the brighter vesicle contour in fluorescence as well as darker and thicker vesicle contour in phase contrast (data not shown) compared to the majority of vesicles. The presence of twice as high levels of negatively charged lipids such as PS in 3D plasma membranes could be a reason for the increased multilamellarity in 3D lipid extracts. The increasing proportions of PS in simple binary mixtures of phosphatidylcholine/phosphatidylserine confirms this tendency to form multilamellar giant vesicles which was verified by us (data not shown) and other authors [19]. It is quite likely that the presence of negative charges prevents the hydration and the separation of the lamellas in the deposited lipid film.

Below 22 °C, in the temperature interval 22–10 °C, the plasma membrane lipids of 2D fibroblasts exhibited a clear bi-phasic behavior, demonstrated by the presence of bright and dark regions (Fig. 3B (a and b)). Two coexisting phases were identified as fluid ones since the observed domains had always a round shape. When fluid domains are embedded in a fluid environment, circular

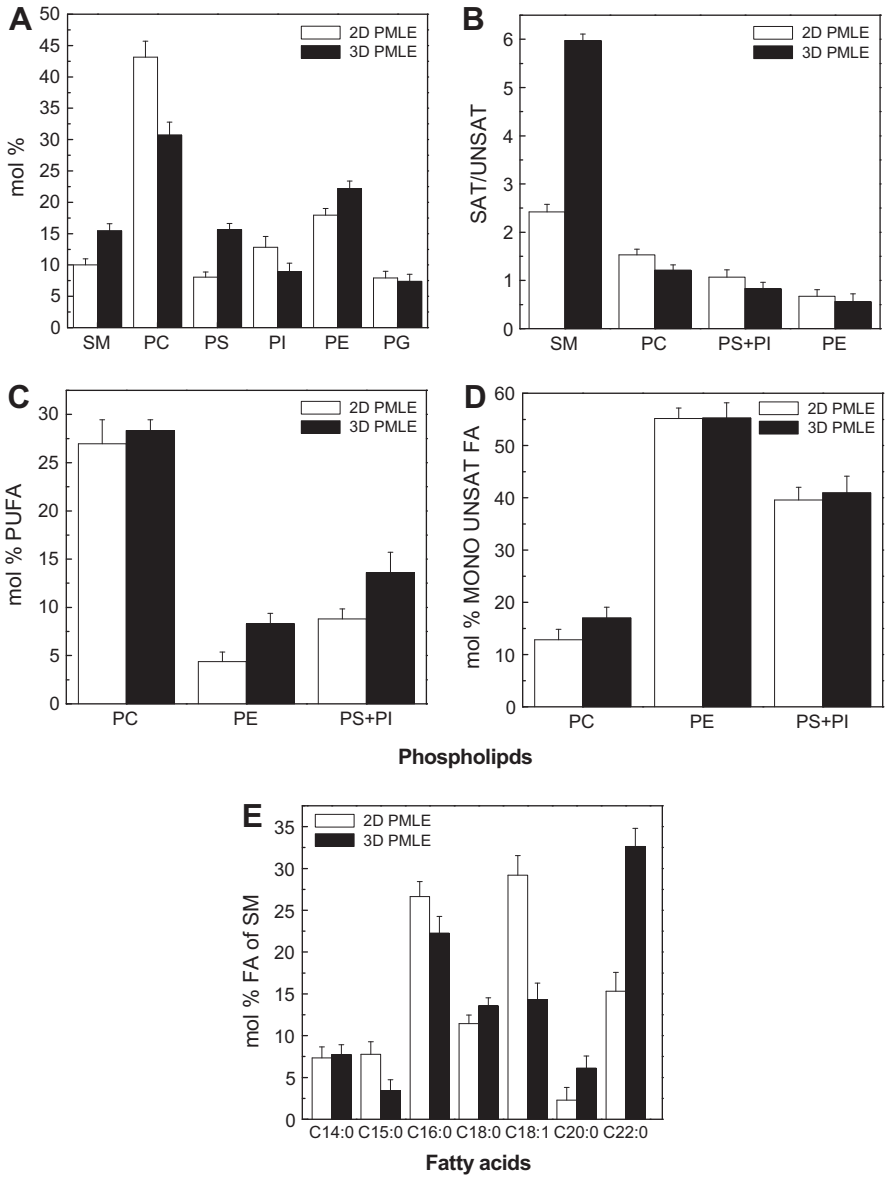

Fig. 2. Identified phospholipid species (mol% of the total lipids) in plasma membranes of fibroblasts cultured as a monolayer (2D conditions, white bars) and in 3D matrix (black bars) (A). PMLE: plasma membrane lipid extract. Coefficient of saturation of fatty acids (B); percentage of polyunsaturated species from total plasma membrane fatty acids in the major phospholipids (C); percentage of monounsaturated species from total plasma membrane fatty acids in the major phospholipids (D); fatty acid composition of sphingomyelin (mol%) in plasma membranes of 2D and 3D fibroblasts (E). The presented values are averages of three independent determinations.

domains will form because both phases are isotropic and the line tension associated with the interface of the two demixed phases is minimized by the optimizing area-to perimeter ratio [20]. These domains showed a tendency to merge and to form larger round-shaped structures moving within a light background (Video S1). Poor Rhodamine–PE spots appearing as dark domains correspond to the liquid-ordered phase mimicking the lipid matrix of cellular "lipid rafts" whereas the light background represents the liquid-disordered phase.

The plasma membrane lipids of 3D fibroblasts in contrast to 2D, exhibited a homogenous appearance in this temperature interval. Such phase behavior is not surprising in view of the cholesterol content in both samples. Studies on model membranes with artificial lipids (unsaturated phosphatidylcholine/sphingomyelin or saturated phosphatidylcholine/cholesterol) demonstrated single phase existence in liquid-ordered state at cholesterol mole fraction 0.35–0.4 [21]. Indeed, if cholesterol was gradually removed from GUVs, composed of 3D lipid extracts, by using methyl-β-cyclodextrin (local microinjection around target vesicle), formation of round-shaped domains in GUV was initiated at 24 °C (Fig. 4 (a–e)). Thus, cholesterol concentration of 3D plasma membrane lipid extract (34 mol%) would approach that of 2D extract (21 mol%) which correlates with the observed bi-phasic behavior for these extracts. At lower temperatures (below 15 °C) formation of more structured regions was detected. The clusters of dark domains with irregular shape were observed in 3D lipid extract (Fig. 3B (c–e)). These domains did not fuse to yield a larger domain which is an intrinsic property of gel-like phases (Video S2). At 4 °C the plasma membranes of 2D lipid extract exhibited three-phasic behavior as the dark fraction became dominant. Within this background bright round-shaped and darker irregular domains (marked by white arrows) were visible (Fig. 3C (a–c)) (Video S3). The fraction of irregular domains in 3D lipid extract, however, was larger compared to 2D (Fig. 3C (d and e)) (Video S4). The composition of these domains is not known and could be assigned to the greater content of saturated species of the charged lipid PS.

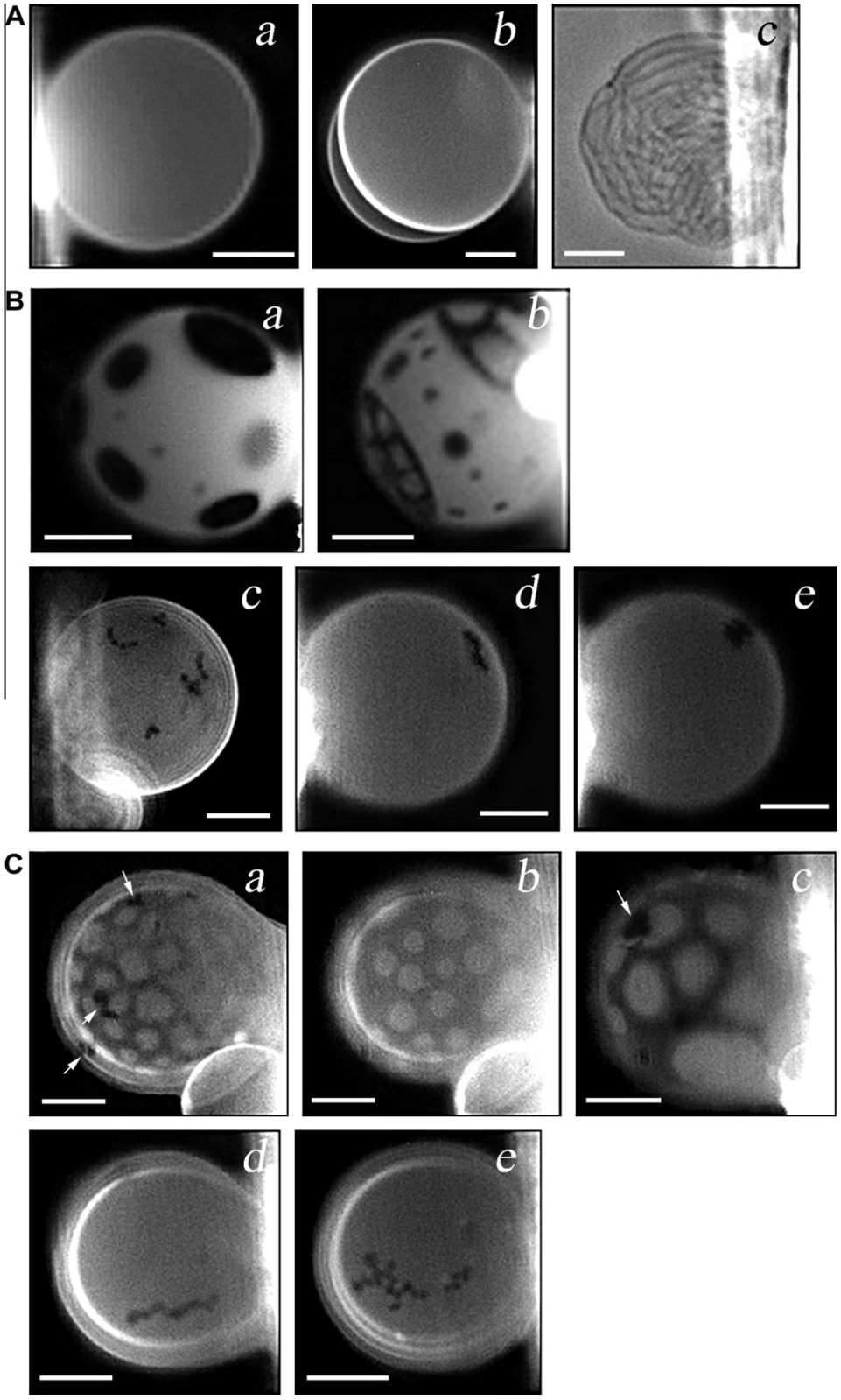

Fig. 3. Domains pattern of plasma membrane lipids from cells cultured in 2D and 3D conditions. Giant vesicles were composed of lipid extracts and observed in 37–4 °C temperature range. Homogeneous appearance was observed for both types of plasma membranes (A (a and b)) in 37–22 °C range. Population from multilamellar giant vesicles was noticed for 3D plasma membranes (A (b and c)). Well resolved bi-phasic behavior in 22–10 °C temperature interval: liquid/liquid coexistence (bright regions-L_d phase and dark domains-L_o phase) for 2D plasma membranes (B (a and b)) and liquid/gel for 3D (dark domains-L_β phase) (B (c–e)). In 10–4 °C temperature range, the plasma membranes of 2D lipid extracts exhibit three-phasic behavior (L_β-denoted by arrows, L_o, and L_d) as the dark fraction became dominant (C (a–c)). Liquid/liquid coexistence was not observed for 3D plasma membranes (C (d and e)). The fraction of irregular gel domains in 3D (C (d and e)) was larger compared with 2D plasma membranes (Fig. 2C (a and c)). Bar 20 μm.

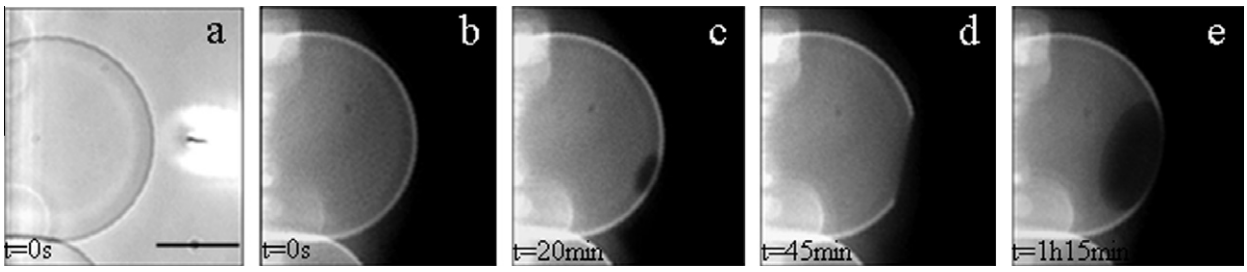

Fig. 4. Time series of cholesterol depletion by methyl-β-cyclodextrin from GUV composed of plasma membrane lipids, extracted from cells cultured in 3D conditions, at 24 °C. Bar 20 μm.

Fluorescence microscopy experiments demonstrated different domain patterns for 2D and 3D plasma membranes lipids. The growth, shape and size of the domains depend critically on variations in the lipid composition, especially the cholesterol content, and the ability of lipids to mix. Macroscopic L_o/L_d phase separation in plasma membrane lipids has never been visualized at physiological temperature but this does not preclude the existence of domains in the sub-micron region. These findings do not contradict with the current definition of "lipid rafts" at cellular level – nanometer-range in size, highly dynamic sphingolipid–cholesterol enriched domains [22]. In addition, the majority of the studies using plasma membrane extracts detected macroscopic domain formation of the type L_o/L_d [8,23] or L_β/L_d [7] at room temperature rather than physiological.

It is noteworthy that the observed phase behavior of the plasma membrane lipid extracts in this model system might be different compared to physiological conditions not only because the natural lipid asymmetry is not preserved in the membrane bilayer but also because the natural membranes are not in thermodynamic equilibrium as it is in biomimetic systems. Another major factor which governs the intrinsic membrane organization in cell plasma membrane is the presence of proteins. Even though, the ability of these plasma membrane lipid extracts to form domains in liquid-ordered phase (lipid model of cell *rafts*) was tested taking into account the found differences in the lipid composition between two extracts. Currently, there are no proofs in the literature demonstrating that PS, PE, PI (the main lipids in the inner leaflet of the plasma membrane) and cholesterol are able to form micron-scale liquid domains in model systems. The raft-like domains are micron-scale sphingomyelin–cholesterol domains exhibiting round shape and growing by fusion. Therefore, the ability to form micron-scale domains with such properties in the given lipid extract might be mainly attributed to the SM/CHOL ratio. Of course, the presence of other lipids modulates the fraction of Lo domains. For example, recently, Wassall and Stillwell [24] have shown that polyunsaturated PE and CHOL possess a mutual aversion that drives the lateral segregation of polyunsaturated phospholipids into highly disordered domains away from CHOL. Thus more CHOL would be available to interact with SM and to form raft-like lipid domains. Hence, when the lipid asymmetry is not available in the membrane bilayer as it is in our case, the presence of more polyunsaturated PE than the natural levels in the outer leaflet would lead to the some overestimation of the fraction of Lo domains. The rest of the lipids, such as PS and PI might have similar or opposite effect but at present there is no information of their impact on the raft-like domain formation. That is why we state that the demonstrated phase behavior of 2D and 3D plasma membrane lipids mimics to certain degree the ability of present lipids to form raft-like domains.

To describe in more detail the changes in the plasma membrane structure of cells cultured in 2D and 3D conditions, we performed investigations with spin-labeled stearic fatty acid, the polar carboxyl group of which positions spontaneously at the water interface and the reporter group positions at the deepest hydrophobic part of the bilayer. 2D lipid extract was more ordered than the 3D at the level of the doxyl probe in the whole investigated temperature range (Fig. 5A). However, the difference was more pronounced at lower temperatures between 5 °C and 20 °C. Such behavior of molecular order for the two types of plasma membrane lipid extracts is predictable in view of the fact that the major fraction (about 70 mol%) from total phospholipids, composed of PC, PE and PS + PI, contained less polyunsaturated fatty acids in 2D lipid extract compared to 3D. Even higher CHOL and SM content in 3D extract, which are known to increase the order in the glycerophospholipid matrix, were not able to compensate the higher order imposed by higher fatty acid saturation of phospholipids in 2D extract. These results are consistent with some reports in the literature and correspond to the conception of homeoviscous adaptation [25]. This concept implies that the membrane lipid

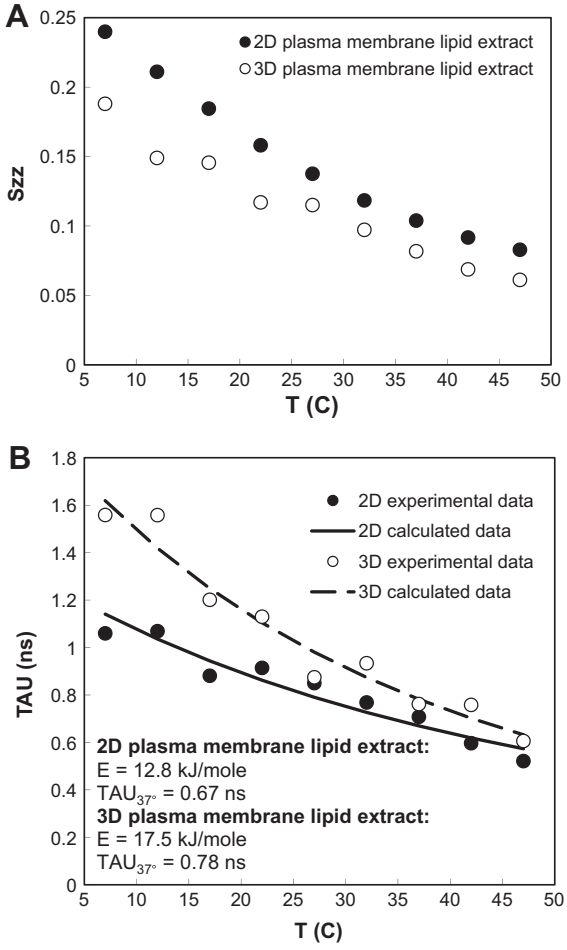

Fig. 5. Order parameter of the *Z*-axis of A and g tensors (A) and temperature dependence of reorientation correlation times fitted using the expression $\tau = \tau_0 \exp(E/RT)$ where E is the activation energy in kJ/mol (B).

composition varies in response to conditions that perturb the phospholipid acyl chain conformational mobility in such a way as to maintain this conformational mobility constant.

Lipid extracts from 3D plasma membranes were richer in cholesterol which would suggest higher values of the order of the bilayer compared to 2D which, unexpectedly, was not the case. Thus, adaptive changes in the phospholipid composition would be these which produce a decrease in the order parameter of acyl chains. One possible reason for the observed order of the ESR probe is most probably due to the increased fatty acids unsaturation in the major phospholipid species (Fig. 2C). Similar results were observed in mycoplasma and in Chinese hamster ovary cells, where the increase of the cholesterol/phospholipid ratio is related to a decrease in SAT/UNSAT ratio [26,27]. Another possible factor, related to the cell homeoviscous adaptation, could be the higher levels of PE in CHOL-enriched 3D lipid extract, generally known to comprise of polyunsaturated species. These *in vitro* studies are consistent with *in vivo* conditions in which a deep DPH fluorescent probe was used. The fluidity of the outer and the inner plasma membrane monolayers was lower in 3D (outer 0.2351 ± 0.0025; inner 0.2229 ± 0.0017) than 2D cells (outer 0.2423 ± 0.0019, inner 0.2208 ± 0.0021) [18]. Overall, these data demonstrate a generally lower order of the ESR spin-probe in 3D plasma membrane lipids in the deep hydrophobic core of the bilayer compared to 2D. The reorientation correlation time obtained from a spectral fitting is an effective parameter, function of the overall tumbling of the probe and of segmental motions. The latter involving some hundred thousand transient conformers give rise to the motionally averaged order parameter S_{zz}.

As $S_{ZZ}^{2D} > S_{ZZ}^{3D}$ (Fig. 5A), it is expected that the interactions of the doxyl chain of the probe with the substrate molecules are more restricted in the 2D lipid extract than in the 3D one. However, the reorientation correlation times exhibited a tendency to be a bit shorter in the 2D extract than in the 3D extract (Fig. 5B). The higher levels of long C20:0 and C22:0 saturated fatty acids of SM in 3D plasma membranes lipid extract (Fig. 2E) may induce length asymmetry in the lipid hydrocarbon chains. Thus, partial monolayers interdigitation would be a reason for the restricted movements (rotations) of the nitroxide radical in the middle of the bilayer in 3D membranes compared to 2D.

Native membranes exhibit a large compositional heterogeneity of the constituent lipids. As showed in Fig. 2 the alterations observed in the lipid composition between 2D and 3D plasma membrane extracts were related to differences in the percentage ratio of the studied fatty acids, the degree of saturation as well as percentage ratio of the polar residues. The acyl-chain region was probed using the doxyl ESR spin-probe attached to the carbon 16 of stearic acid. This probe undergoes a fast motional regime allowing an unambiguous determination of correlation times and order parameters in contrast with reporter groups located at more shallow and thus more ordered depths (C5 through C12) than the center of the bilayer (C16). For the latter reporter groups indeed, the apparent order parameter depends both on the "true" order parameter S_{ZZ} and on the reorientation correlation time (see for instance [16] and references therein). That is why another opportunity to examine the interfacial properties of the phospholipid bilayer of plasma membrane lipid extracts is to use the fluorescent marker 6-dodecanoyl-2-dimethyl-aminonaphthalene (Laurdan). Whereas spin-labeled fatty acids report on different packing properties in the deep hydrophobic core of the membrane bilayer, Laurdan is sensitive to polarity in the interfacial region of the membrane bilayer [28]. Laurdan is negligibly soluble in water, homogeneously distributed into the solid and liquid lipid phases and it shows a lipid phase-dependent emission spectral shift. The spectra are blue-shifted when the membrane bilayer is in ordered phase state and red-shifted in the liquid-disordered state. This effect is attributed to the reorientation of water molecules present at the lipid interface near the fluorescent moiety of Laurdan [29]. All these properties offer a great advantage over other fluorescent probes which show preferential localization in particular membrane regions.

Fluorescence excitation and emission spectra of Laurdan in multilamellar vesicles prepared from plasma membrane lipid extracts of fibroblasts, cultured in 2D and 3D conditions were recorded at intervals of 10 nm at room temperature (Fig. 6).

The excitation spectrum of 2D lipid extract showed two peaks at 340 nm and 370 nm, the latter being of lower intensity (Fig. 6A). The spectrum of 3D lipid extract is characterized with one well pronounced peak at 355 nm (Fig. 6A). The Laurdan emission spectrum of 2D extract showed two well resolved peaks at 415 nm and 470 nm (Fig. 6B). The emission spectrum of 3D is characterized with one peak at 430 nm (Fig. 6B). Both spectra are broad as the 2D plasma membrane lipid extract exhibited clearly two, differing in polarity, environments (blue- and red-shifted peaks) unlike 3D extract, where a blue-shifted pick is preponderant. Such peak is typical for lipid mixtures enriched in CHOL (30–40 mol%), forming membranes in liquid-ordered state, where the presence of CHOL noticeably reduces the dipolar relaxation effect [30]. As discussed above, such phase behavior would be consistent with the fact that the 3D lipid extracts are comprised of higher amount of SM and cholesterol (34 mol%). The extent of water dipolar relaxation, which is related to the phase state of lipid membranes, is also characterized by the parameter general polarization (GP) which is defined by analogy to the fluorescence polarization

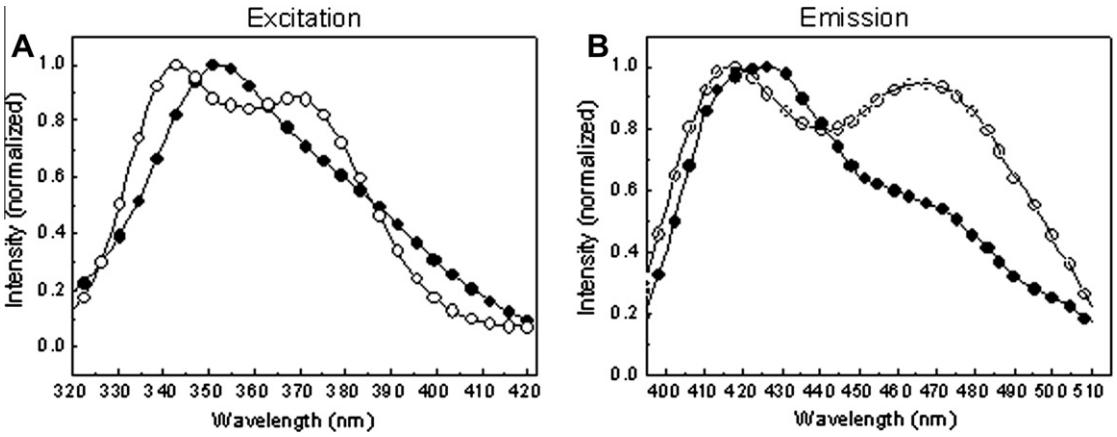

Fig. 6. Normalized excitation (A) and emission spectra (B) of Laurdan obtained at 20 °C for plasma membrane lipid extracts from fibroblasts cultured at 2D and 3D conditions. Open circles correspond to 2D and filled to 3D conditions.

function [31]. GP varies between −1.0 and 1.0 and has been interpreted to reflect the degree to which lipid molecules are ordered within the membrane. The calculation of GP showed values of 0.263 ± 0.016 for 2D and 0.330 ± 0.020 for 3D at room temperature. The higher GP value for 3D lipid extract means a lower hydration of the bilayer, which suggests stronger interactions between the neighboring phospholipids in the interfacial region of the membrane bilayer. The increased content of cholesterol and sphingomyelin in the 3D plasma membrane lipid bilayer as well the higher PE (low hydrated polar headgroup) and PS (charged polar headgroup) levels are factors which determine an increased GP value. The changes in GP are clearly correlated with the amount of cholesterol in the range of 0.2–0.4 mol% [32,33] as it is consistent with our data. In addition, the presence of anionic phospholipids leads also to blue-shifted specters and increased GP values as a function of the charged lipids concentration [34]. The higher order in the interfacial region of 3D extract is consistent with previously measured, by our group, surface properties of the lipid extracts cultured in 2D and 3D conditions [35]. These Langmuir monolayers studies stated that the elevated content of sphingomyelin in the plasma membranes of 3D fibroblasts is responsible for an increased rigidity and possibly reduced permeability of cells cultured in 3D environment.

It is well to emphasize that the interpretation of the results in Laurdan measurements using multilamellar vesicles (MLV) is not straightforward compared to Laurdan measurements in giant unilamellar vesicles (GUV, fluorescence of single vesicle), because in the bulk fluorescence measurements (cuvette technique), the additive property of the Laurdan GP function masks phase identification if more than one phase exist in membrane bilayer. An experimental obstacle emerged for using Laurdan measurements in GUV [36,37]. Giant multilamellar vesicles were systematically observed in the 3D plasma membrane lipid extracts contrary to 2D where mainly unilamellar ones were observed by epifluorescence as it was mentioned above. Despite the fact that the method of GUV electroformation is considered as the method with highest yields of unilamellar vesicles [38], the presence of charged lipids in the membrane bilayer changes this perception [19]. In order to interpret correctly the results, reliable method for evaluation of the unilamellarity of giant vesicles is required in case of use of lipid extracts. We therefore considered that MLVs are a compromise biomimetic system that is able to give an assessment of the average order in the interfacial region of bilayer composed of each lipid extract.

4. Conclusions

Our results indicate that cell culture conditions could influence various membrane functions which are supported by the observed changes in the membrane lipid composition, molecular order in the deep hydrophobic core and in the interfacial region of the membrane bilayer, and lateral membrane organization. The present studies also demonstrate how changes in the levels of two major microdomain-forming lipids (sphingomyelin and cholesterol) were able to induce formation of different domain patterns in the lipid bilayer of biomimetic systems. The organization of the lipid molecules in domains is considered as an important factor for protein sorting, dynamically regulating the association of protein targeting and so would support different biochemical functions and regulate whole cell physiology. Indeed, our group was able to establish differences in the chemical activity of cholesterol, assessed by its susceptibility to cholesterol oxidase in conventional and "matrix" cell cultures [18]. Thus, the reported differences in the physicochemical properties of plasma membranes in 2D and 3D cells imply that investigations of any membrane-related processes should be cautiously interpreted when using cells cultured as conventional monolayers.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcis.2011.03.085.

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