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New EPR Method for Cellular Surface Characterization

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Abstract. An electron paramagnetic resonance (EPR)-based membrane surface characterization method is presented to detect the properties of the carbohydrate-rich part of membrane surfaces as well as carbohydrate interaction with other membrane constituents and water-soluble molecules. The proposed method relies on the spin-labeling and spectral decomposition based on spectral simulation and optimization with EPRSIM software. In order to increase the sensitivity of characterization to the carbohydrate-rich part of the membrane surface, the sucrose-contrasting approach is introduced. With this method, which was established on model membranes with glycolipids and tested on erythrocyte membrane, we were able to characterize the surface and lipid bilayer lateral heterogeneity. Additionally, some properties of the interaction between glycocalyx and lipid bilayer as well as between glycocalyx and sucrose molecules were determined. The experiments also provided some information about the anchoring and aggregation of the glycosylated molecules. According to the results, some functions of the glycosylated surface are discussed.

Key words: EPR — Glycocalyx characterization — Membrane surface — Spin probe — Erythrocytes

Introduction

In the last few years a significant effort has been made to establish a more reliable and accurate characterization of the glycosylated outer part of the mammalian membrane surfaces, sometimes indicated as glycocalyx [10, 13, 18, 19, 28]. So far the majority of research concerning glycocalyx explores the active parts of the glycosylated molecules and their functional implications. However, the mechanisms of the interaction of the glycocalyx with other membrane parts are still not completely understood. Many studies indicate that interactions between sugar moieties in the glycocalyx [4, 5, 11, 20, 21] and the neighboring near-surface compartments [6, 9, 14] are specific, weak and cooperative. Many experiments also suggest that only a group or aggregate of glycosylated molecules can be active [4, 5, 20, 26]. Therefore, the idea of the existence of glycolipid microdomains (or functional rafts) is no longer only a theoretical construct [13, 21, 26, 28]. Since such microdomains may have an important impact on the proper function of glycosylated membrane enzymes, channels and receptors, we present here a new method to study the properties of the carbohydrate-rich part of membrane surface, as well as the interaction between the glycocalyx and lipophilic part of the lipid bilayer. The method is based on electron paramagnetic resonance spectroscopy (EPR) in combination with spin labeling [3, 15, 21, 22] and sucrose-solution contrasting. The spectroscopic approach enables us to measure intact cell membranes in an almost unperturbed environment, thus providing reliable information about the coexisting lateral domain structures [16, 17, 23, 24, 29]. The sucrose-based contrasting method gathers some additional information in terms of detected variation in dynamics and aggregation of the glycocalyx oligosaccharide chains. These are reflected in the lineshape of the EPR spectra of the applied spin probes. Choosing sucrose for contrasting agent, we perturb the glycocalyx [27], namely, the degree of freedom and the speed of the conformational motions of the carbohydrate chains. This enables an additional check of the reported characterization of the spin probes residing in the carbohydrate-rich part of membrane surface. The sucrose molecules could also mimic the

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polar molecules gathered in the vicinity of a receptor or a membrane channel.

With this approach we introduce a new way to probe the membrane surface, i.e., its carbohydrate-rich part, and its dynamics as well as its interaction with other membrane constituents and water-soluble molecules. Since many important processes involving the glycocalyx participate in various clinical problems, including the deficiencies of some active substances, genetic diseases, drug functionality, etc., we believe that the presented method is of broad interest.

Materials and Methods

Model and Biological Membranes

To characterize the glycocalyx regions by EPR spectra, we investigated model membranes as well as the membrane of bovine erythrocytes.

Liposomes with Gangliosides

Liposomes with gangliosides were prepared from 16 mg of Emulmetic 320 (Lucas Meyer, Hamburg; hydrogenated soy mixture of phospholipids: phosphatidylcholine 18–26%, phosphatidylethanolamine 15–22%, phosphatidylinositol 8–14%; approx. 22 μmol) and gangliosides (Sigma; GM1 20%; 6.2 mg—approx. 5 μmol—in 180 μl of phosphate buffered saline (PBS, pH 7.4, 290 mOsmol/kg).

Liposomes without Gangliosides

For control experiments liposomes without gangliosides were prepared from 20 mg of Emulmetic 320 (approx. 27 μmol) in 180 μl phosphate buffer saline (PBS, pH 7.4, 290 mOsmol/kg).

Liposomes, prepared using vortexing and weak sonication, were left 12 hours for stabilization. This "hand-shaking" procedure provided multilamellar liposomes. Their dimensions were between 150 nm and 1000 nm (determined by measurement of the internal volume with EPR, photon correlation spectroscopy and transmission electron microscopy).

Erythrocytes

Erythrocytes were isolated from fresh bovine blood by washing the samples three times with PBS. Finally the 100 μl pellet (approx. 1.5 μmol of lipids, calculated from the mean surface area of an erythrocyte) was mixed with 100 μl PBS and transferred to a glass tube whose inner walls were coated with a thin film of the spin probe. The fact that the final lipid concentration in the erythrocyte suspension was lower than in the liposome suspension, was taken into consideration for achieving appropriate spin probe to lipid molecular ratios.

Spin Probes, Labeling and EPR Measurements

Lipid Bilayer

For characterization of the lipophilic part (lipid bilayer) of the membranes, spin-labeled methyl ester of the palmitic acid (5-doxyl-methyl palmitate, MeFASL (10,3)) was used. This lipophilic molecule incorporates easily into the membrane (*see* Fig. 1).

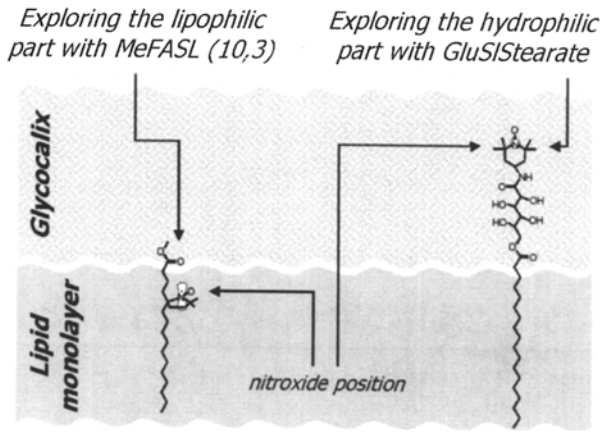

Fig. 1. Schematic presentation of the incorporation of the two spin probes used in the experiment into membrane. The outer membrane leaflet is presented together with the glycocalyx. The positions of the nitroxide groups are indicated with schematic orbitals and arrows.

MeFASL (10,3) of 4 nmol as a solution in ethanol was deposited in the glass tube by evaporation of ethanol. A cell suspension was added to the tube and the whole suspension was vortexed for five minutes at room temperature. Finally, the sample was centrifuged at 1,100 × *g* for 5 min and transferred to a glass capillary to perform EPR measurements. The label-to-lipid ratio was about 1:300 (determined from measurement of the EPR intensities).

Similarly, liposome membranes (27 μmol of lipids per 180 μl of PBS) were labeled with 100 nmol MeFASL (10,3), deposited as a thin film in the glass tube. The label-to-lipid ratio was about the same as in the erythrocyte case.

Hydrophilic Surface

For characterization of the hydrophilic surface of the membranes, the newly synthesized spin probe GluSlStearate (6-octadecanoyloxy-N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-2,3,4,5-tetrahydroxyhexanoic acid amide) was used. The synthesis, the characterization and the purity are described in [1]. This amphiphilic molecule has a long lipophilic tail, which enables the molecule to anchor into the membrane (Fig. 1). Since it has also a polar part, it either is partially dissolved in the water or it forms compact micelles. EPR spectra of the corresponding populations can be easily distinguished from other spectral components.

Labeling procedures of the erythrocyte suspension with GluSlStearate were comparable to those of MeFASL (10,3). However, due to a slower GluSlStearate incorporation into the membranes in comparison to the MeFASL (10,3), the amount of GluSlStearate deposited was increased to 20 nmol and the time for labeling of erythrocytes was increased to 20 minutes (previously 5 minutes). The label-to-lipid ratio was determined to be 1:150 (measurements of the EPR intensities). With this labeling procedure it can be assumed that GluSlStearate stays in the outer membrane leaflet within the time of measurement.

Labeling of liposomes with GluSlStearate was done in the preparation step, i.e., the dry spin probe was added to the dry lipid mixture before addition of PBS. The label-to-lipid ratio was the same as in the MeFASL (10,3) case, around 1:300. It should be stressed that both the spin probe as well as the glycolipids were added in the preparation step to assure their coexistence in various membrane parts and both leaflets.

EPR measurements were performed on a Bruker ESP 300E X-band spectrometer. The spectra were taken at a microwave

frequency of 9.6 GHz, a power of 20 mW, a modulation frequency of 100 kHz and an amplitude of 1.5 G, a field sweep of 120 G, and a single-scan time of 80 s. In order to increase the signal-to-noise ratio, up to 10 spectra were accumulated in each measurement.

Sucrose Contrasting Method

To check and test the effect of the polysaccharide chains on the EPR spectra, we tried to enhance it with the addition of sucrose to the cell or liposome suspension. Because sucrose is similar to the sugar moieties of the polysaccharide chains of the glycocalyx, it can provide a similar type of interaction with our spin probe as does the glycocalyx. Due to sugar-sugar interactions based on hydrogen bonding, the sucrose molecules could stick to the glycocalyx and increase the probability of detecting a hindrance effect of surface carbohydrate chains on GluSlStearate motion. Therefore, the sugar-contrasting method increases the sensitivity of EPR spectra to glycosylated surface.

We believe that in a model system such as liposomes the glycolipids are distributed on both sides of the membrane. Since in this case, too, the sucrose and the spin probes were added during the preparation, all three components, namely the sucrose, the glycolipids, and the spin probes were on both sides of the liposome membrane. A buffer solution of 180 µl of 30% sucrose was used instead of pure buffer solution.

For erythrocytes, 100 µl buffer solution containing 30% sucrose was added to the 100 µl of washed pellet instead of pure buffer solution. Since the amphiphilic GluSlStearate cannot flip-flop fast enough between bilayer leaflets and since the GluSlStearate was incorporated into the external leaflet, the spin probes, sucrose, and most probably also the glycolipids were found predominantly in the external leaflet of the erythrocyte membranes. It should be also noted that the hyperosmotic conditions, which arise due to the addition of the sucrose to the extracellular solution, caused only a small perturbation to the membrane domain structure in the lipophilic part, as was determined by the lipophilic spin probe.

Simulation of EPR Spectra

To characterize the lipophilic and hydrophilic part of the membrane, experimental spectra of spin-labeled membranes were simulated in the model described in detail in reference [24]. In this model the multi-domain structure of the membrane is taken into account [16, 17] with a slow exchange of various membrane constituents between the domains (on the EPR time scale), and at the same time a fast and anisotropic rotational motion is supposed [25] (on the EPR time scale). These assumptions are based on several experimental facts about lateral heterogeneity of the cellular membrane [12, 23, 29], as well as on the experimental setup, which includes the small size of the spin-labeled molecules and physiological temperatures.

In the model used, each membrane's domain type is characterized by its proportion d, order parameter S, rotational correlation time τ_c, additional broadening W, polarity correction factor on tensor components of the hyperfine coupling p_A and polarity correction factor on tensor components of the Zeeman coupling p_g. The proportion of the particular domain type implies the proportion of the spin probe, which reports particular characteristics of its motion and its environment. As a consequence of the heterogeneous distribution of spin probes, the actual proportion of domain types can be different from the measured one.

Since the determined number of domain types is usually 3 or 4 and each corresponding spectral component is calculated using 5 + 1 parameters (S, τ_c, W, p_A, p_g and d), we have to provide 17 or 23 spectral parameters to characterize the best fit to each of the experimental spectra. In order to resolve this task we use hybridization and combinations of local search optimization routines (Simplex Downhill) and evolutionary-based algorithms (Genetic algorithms), which minimize the fitness function describing the goodness of fit. The effectiveness and accuracy of the procedures in solving the problem of characterization of membranes by EPR spectra simulation are presented in the articles [7, 8, 24], where it was shown that hybrid evolutionary optimization is capable to uniquely resolve more than 20 spectral parameters. In this study even larger statistics was used. Each set of best-fit parameters was determined as the best of 200 sets from 200 runs of the hybrid evolutionary optimization method (usually 20 runs are performed).

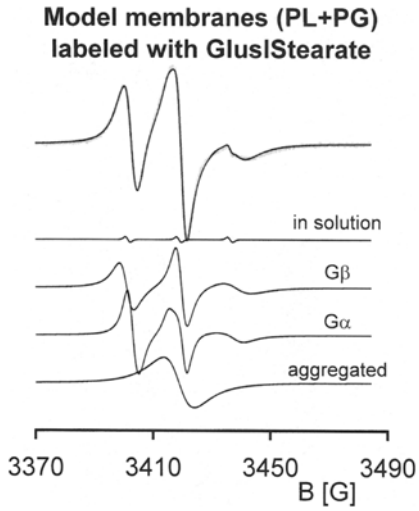

Fig. 2. The EPR spectrum and its resolved spectral components of the GluSlStearate incorporated into the model membrane. Spectral components arising from spin probes in the solution, surface domains without steric hindrances, surface domains with large steric hindrances and components arising from aggregated spin probes, are denoted with "*in solution*", *Gβ*, *Gα*, and "aggregated", respectively (*G* Stands for glycocalix).

Results

Experiments were done on model membranes and membranes of bovine erythrocytes labeled with GluSlStearate and MeFASL (10,3). According to the physical properties of both spin probes, it is thought that the spin probes anchor in the membrane, as it is schematically presented in Fig. 1. GluSlStearate reports on the motional characteristics imposed on the nitroxide group, in the region above the membrane surface. Therefore, it is capable to monitor the aggregation and dynamics of the polysaccharide chains in the membrane surface (hydrophilic part of biological membranes). The addition of sucrose should increase the sensitivity of restrictions imposed by the glycocalyx and therefore provide a contrasting picture of the glycosylated surface. However, the lipophilic domain structure can be directly monitored with MeFASL (10,3), which detects the influence of the sucrose added to the membrane dispersion.

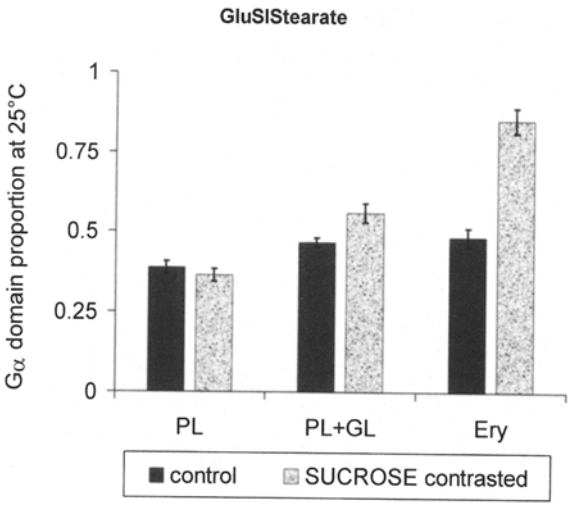

Fig. 3. Proportion of the domain type with large steric hindrances as determined by the spin labeling with GluSlStearate. The comparison between the model membrane without glycolipids (*PL*), model membranes with glycolipids (*PL + GL*) and erythrocyte membranes (*Ery*) measured at 25°C is shown for the pure membrane suspension (*control*) as well as for the membrane-sucrose suspension (*sucrose contrasted*).

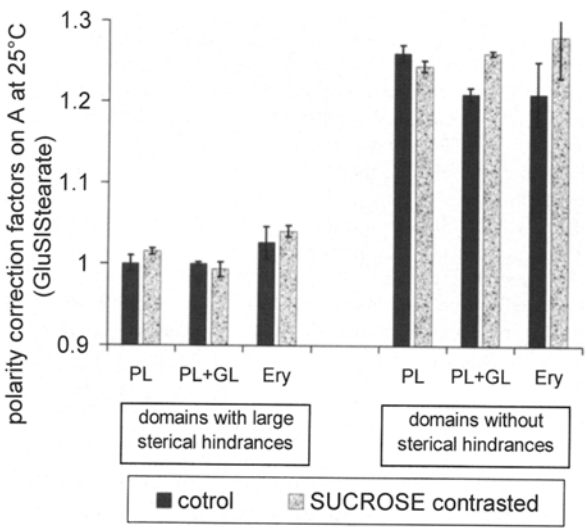

Fig. 4. Polarity correction factors of the hyperfine tensor coupling of the domain type with and without steric hindrances as determined by the spin labeling with GluSlStearate. The comparison between the model membrane without glycolipids (*PL*), model membranes with glycolipids (*PL + GL*) and erythrocyte membranes (*Ery*) measured at 25°C is shown for the pure membrane suspension (*control*) as well as for the membrane-sucrose suspension (*sucrose contrasted*).

The experiments were done with phospholipid vesicles (PL), mixed phopholipid-glycolipid vesicles (PL+GL), and finally with bovine erythrocytes (ERY). In each case, membranes were labeled with either GluSlStearate or MeFASL (10,3) and were measured without sucrose or with sucrose. Therefore

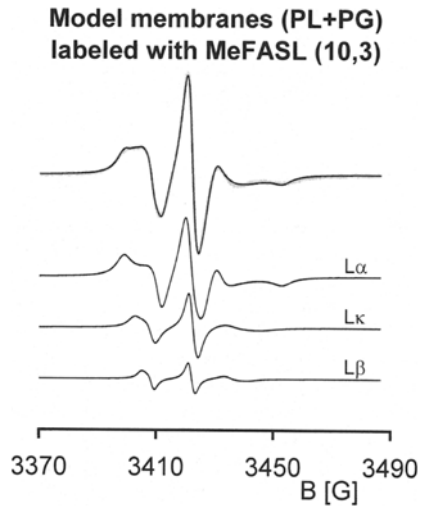

Fig. 5. The EPR spectrum and its resolved spectral components of the MeFASL (10,3) incorporated into the model membrane. Spectral components arising from spin probes in the ordered domains, intermediate, and disordered domain types are denoted with *Lα*, *Lκ*, and *Lβ* respectively (*L* stands for lipophilic part of membrane, not for liquid phase).

four experiments were performed for each membrane.

GluSlStearate

The results of the GluSlStearate experiments are shown in terms of resolved spectral components in the surface layer in Fig. 2 and the proportions of domains with large steric hindrances in Fig. 3 at 25°C. Additionally to these data it should be noted that the proportion of domains with large steric hindrances increases slightly when temperature is raised from 25°C to 35°C and is then constant up to 45°C (*data not shown*). Polarity correction factors of the Zeeman coupling constants for domains without steric hindrances and domains with large steric hindrances are shown in Fig. 4. The mean values of the rotation correlation times of the GluSlStearate in domains with large steric hindrances are between 1 and 2 ns, in domains without steric hindrances, around 0.6 ns. The latter results of rotation correlation time possess a relatively high error of about 0.6 ns and do not change significantly with temperature.

MeFASL (10,3)

With MeFASL (10,3) we show the resolved spectral components in the lipophilic part of the membrane in Fig. 5 as well as the proportions of domain type characterized by the highest order parameter in Fig. 6. These results reflect the influence of the added sucrose to the lipophilic part of the membrane.

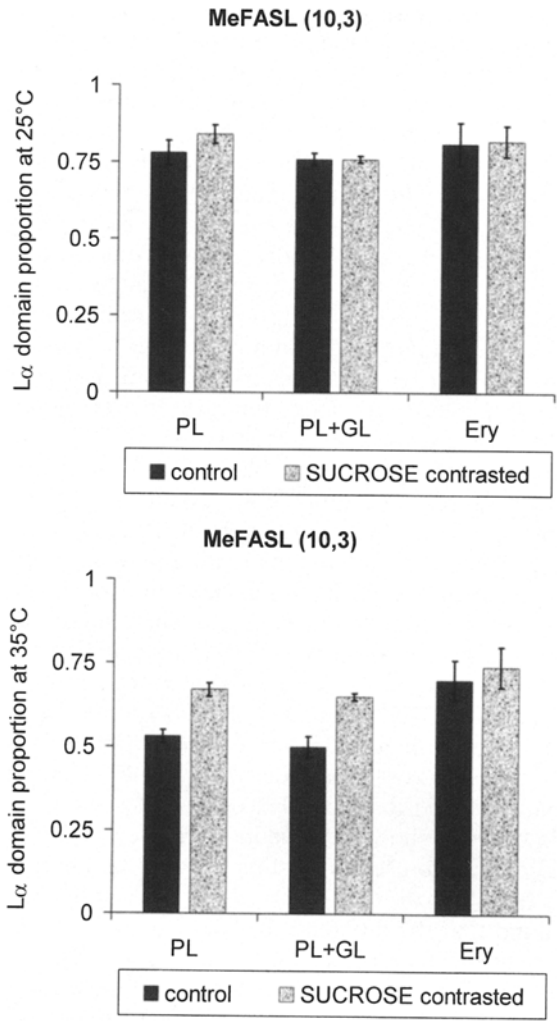

Fig. 6. Proportion of the ordered domain type as determined by spin labeling with MeFASL (10,3). The comparison between model membranes without glycolipids (*PL*), model membranes with glycolipids (*PL + GL*) and erythrocyte membranes (*Ery*) measured at 25°C (upper graph) and at 35°C (lower graph) is shown for the pure membrane suspension (*control*) as well as for the membrane-sucrose suspension (*sucrose contrasted*).

Other parameters, which are not shown, do not exhibit a significant difference between different membrane composition or temperature dependencies. The proportions of the ordered domains decreases by 60% when the temperature is increased from 25°C to 45°C, independently of sucrose addition. The order parameters decrease from 0.7 to 0.4 for most of the order domains and from 0.3 to 0.2 for the disordered domains when the temperature is increased from 25°C to 45°C. The mean values of the rotation correlation times of the MeFASL (10,3) are between 0.3 and 1 ns, the polarity correction factors are between 0.9 and 1 for different membrane domains.

Discussion

Heterogeneity of the Surface and Interaction between the Surface and the Lipophilic Part of the Membrane

The structure of GluSlStearate is characterized by three parts: the hydrophobic acyl residue bound to the hydrophilic tetrahydroxy spacer group, and the piperidine moiety containing the nitroxide EPR-responding group. In our experiments, the amphiphilic GluSlStearate meets the highly anisotropic structure of the membrane, which can be described in short by the transmembrane heterogeneity in terms of molecular organization, gradients of segmental motions as well as the lateral organization in domains. Besides the differences between the outer and inner leaflets of the plasma membrane, the outer leaflet is covered by the glycocalyx.

To separate and distinguish the various contributions of the applied EPR approach, which furnishes the spectra, we decided to explore the lipid bilayer of the membrane by the spin-labeled ester of the fatty acid MeFASL (10,3), which reports about the hydrophobic portion. On the other hand, GluSlStearate is anchored in the hydrophobic portion of the membrane but reports about the glycocalyx.

The data obtained from GluSlStearate point to at least two types of significantly different surface regions in liposomes containing glycolipids. In one region, large steric hindrances of motion are detected, while in the other, almost a free rotation of the piperidine moiety occurs. To our surprise, these two regions were also found in liposomes devoid of glycolipids (Fig. 2). However, the proportions of the domains with large steric hindrances of motion were different for the two liposome types—the proportion of the domains with large steric hindrances is greater when the membranes contain glycolipids. The possible explanation for the observation of domains in membranes devoid of glycolipids could be deduced from the comparison of the temperature dependencies of the domain proportions in both the hydrophilic and the lipophilic part (results mentioned in the previous section). We believe that this arises from the varying depth of spin-probe anchoring. However, when the acyl residues of GluSlStearate are anchored in the disordered domain type of the lipid bilayer, the anchoring is deeper and therefore the piperidine moiety comes closer to the polar groups of the phospholipids. Consequently, the phospholipid polar heads hinder the rotational motion of the nitroxide group of the GluSlStearate. At higher temperatures the proportion of the disordered lipophilic domains increases, causing more GluSlStearate molecules to be pulled deeper into the lipophilic part of the

membrane. In such case, the nitroxide moiety of the GluSlStearate could feel the restriction of the phospholipid heads. Therefore, the proportion of the spin probes experiencing large steric hindrances could increase slightly with temperature.

This result is also confirmed by the significant differences between the polarity correction factors of the hyperfine coupling for the corresponding domain types (Fig. 4). Formally, the polarity correction factor reflects the perturbation on the nitroxide electron structure, i.e., it reflects a moderate probability of finding the unpaired electron in the nitrogen nucleus of the nitroxide group of the spin probe due to polarity changes of the environment or more generally due to effects of the varying electric potential. In this context our result agrees with a different position of the nitroxide group relative to the membrane surface and its electric double layer. Accordingly, this anchoring dependence of the glycosylated molecules demonstrates only one of the possible mechanisms by which the interaction between glycocalyx and lipid bilayer might occur.

In membranes with glycolipids, labeled with GluSlStearate, we measured a significant increase in the proportion of domains with large steric hindrances with respect to the pure phospholipid membranes (Fig. 3). Therefore it can be concluded that in the glycolipid-containing membranes some of the GluSlStearate spin probes detect the restriction imposed by the glycosylated surface. In the domains with large steric hindrances the polarity correction factor is comparable to the polarity correction factor of the deeply-anchored spin probes in the pure phospholipid membranes (Fig. 4). This indicates that the glycolipids alter the membrane surface potential, which is in an agreement with some other studies [2]. Additionally, the polarity correction factor in the domains of liposome membranes containing glycolipids, where the steric hindrances are small, is significantly smaller than this factor for the same domain type in liposome membrane of pure phospholipids. Supposing that the typical distance of the potential changes on the membrane surface could be larger than the size of few lipids together, the glycolipids would change the membrane surface potential also outside the so-called glycolipid microdomains. Since this difference is not negligible according to our determination of the polarity correction factors, one might speculate that the size of the domains is comparable to the typical distance of the potential changes near the surface or even larger than that.

Sucrose Contrasting Method

The addition of sucrose (gray color and fills on all the figures) increases the detected proportion of GluSlStearate domains with large steric hindrances of motion only if the glycolipids are present in the membranes, otherwise the proportions do not change (Fig. 3). This enables us to explain the experiments within the following picture. GluSlStearate spin probes can detect the hindrances imposed by the carbohydrate chains on the membrane surface only if it is in contact with these chains. It can happen especially in real membrane systems that the GluSlStearate would not report about the steric hindrances, although it is anchored in a glycolipid microdomain if there is no carbohydrate chain in the very close neighborhood. Adding sucrose to such a system would increase the area in which one molecule of GluSlStearate could detect the carbohydrate chain on the surface, as a consequence of the sucrose sticking to the carbohydrate chains. This also means that with sucrose addition we are able to detect the proportion of the glycosylated part of the membrane surface more accurately than in non-contrasted samples. According to this result one can explore the glycocalyx in cell membranes in situ where no larger damage or modification to the membranes is necessary. Since many diseases are closely connected with the function of glycosylated molecules at the membrane surface, this contrasting method in combination with EPR could provide a powerful screening technique related to glycocalyx properties.

In order to show the benefits of the new method, we used the sucrose contrasting method to characterize the glycocalyx of the erythrocyte membranes. We found that the difference between the proportion of domains with large steric hindrances in the sucrose-contrasted membranes and uncontrasted samples is much larger for erythrocytes than the corresponding difference for model membranes (Fig. 3). This can be explained by the fact that in erythrocyte membranes, there are also larger and more branched oligosaccharide chains attached to erythrocyte glycoproteins beside the oligosaccharide chains of glycolipids applied in our experiments.

It is also very interesting to monitor the effect on the domain structure in the lipid bilayer core (lipophilic part) exerted by the sugar moieties from glycolipids and sucrose. This was done using the lipophilic spin probe, MeFASL (10,3). From our result obtained on liposome membranes it can be deduced that the glycolipids by themselves do not change significantly the proportions of existing domain types in the lipophilic part of the membrane, which is shown in Fig. 6. However, the addition of sucrose to the model membrane dispersion produces a significant increase in the proportion of the ordered domain type especially at higher temperatures (35°C), independently of the presence of the glycolipids (Fig. 6, lower graph). This result has two important implications. Firstly, since a group of diffusing molecules can at least locally alter the domain proportions, the activities of the involved membrane

active elements (channels, receptors) can also be changed. If these diffusing molecules could bind to membrane receptors or permeate through membrane, this could imply modified probabilities for binding or a changed diffusion rate. Secondly, according to previously discussed interference with lipid ordering, in the lipophilic part of membrane, related with the presence of the glycosylated molecules, the modified anchoring depth can influence aggregation of the glycosylated molecules. Since only aggregates of glycolipids are immunoactive [4, 20], a changed aggregation could be a very important phenomenon perturbing the glycosylated molecules on the cellular surface.

On the other hand, there is almost no significant effect of sucrose on the lipophilic domain structure in the erythrocyte membrane at both temperatures shown (Fig. 6), despite the high concentration of the sucrose used. This could be the effect of various chemical composition (phospholipids, glycolipids, cholesterol, proteins, ...) that could eliminate the sucrose effects on the lipophilic domain structure. Additionally, as a consequence of the more abundantly expressed glycocalyx in the case of the erythrocyte membrane, it is also possible that the sucrose molecules simply do not get close enough to alter the lipid bilayer. This explanation would also point to an important function of the carbohydrate chains at the cellular surface, involving shielding or preventing the changes in the lipophilic domain structure due to the diffusion of polar molecules. In this context it would be even more interesting to test the idea that the varying glycosylation across the membrane is in favor of local protection of the crucial membrane structure.

Conclusions

With the new spin probe GluSlStearate, which we introduced in this study, we were able to detect lateral heterogeneity of the membrane surface on the model membrane as well as the membranes of bovine erythrocytes. Since this spin probe reveals the hindrance of motion of molecules also in membranes without glycosylated constituents, it was concluded that this should be a consequence of its structure, which enhances anchoring in the more fluid domain type. However, it was clearly shown that the proportions of the spin probe molecules experiencing sterically-hindered motion increases in the presence of glycosylated molecules. This was further amplified with the sucrose-contrasting method, which enables the detection of interactions between sucrose, glycocalyx and lipid bilayer. Such interactions play an important role in membrane processes, which involve the glycocalyx constituents. This method therefore provides some new insight in the understanding of the glycocalyx involvement in some physiological processes.

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