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ELECTRON PARAMAGNETIC RESONANCE STUDIES ON THE FLUIDITY AND SURFACE DYNAMICS OF EGG PHOSPHATIDYLCHOLINE VESICLES CONTAINING GANGLIOSIDES

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The influence of different gangliosides (G_{M1}, G_{D1a}, G_{T1b}) on the fluidity and surface dynamics of phosphatidylcholine small unilamellar vesicles was studied by electron paramagnetic resonance. 5- and 16-nitroxystearic acid, sounding respectively the region close to the surface and that close to the hydrophobic core of the vesicle, were employed as spin-label probes. The signals released by 5-nitroxystearic acid showed that the presence of gangliosides reduced the mobility of the hydrocarbon chains around the probe. The effect increased by increasing ganglioside concentration, and diminished from G_{M1} to G_{D1a} and G_{T1b}. The decrease of membrane fluidity was also monitored by the 16-nitroxystearic acid probe. On addition of Ca^{2+} the fluidity of ganglioside-containing vesicles (as signalled by the 5-nitroxystearic acid probe) promptly decreased, thereafter returning slowly to the original value. It is suggested that gangliosides cause strong side-side head group interactions on the bilayer surface – between ganglioside oligosaccharide chains and between ganglioside and phosphatidylcholine polar portions – which lead the lipid chains to assembly in a more rigid fashion. The influence of Ca^{2+} is interpreted as due to lateral phase separation in the vesicle membrane. This phenomenon can be related to the formation or stabilization of ganglioside clusters on the vesicle surface.

Introduction

Gangliosides, sialic acid-containing glycosphingolipids, are normal components of the mammalian cell plasma membranes and are particularly abundant in the neuronal plasma membranes [1]. They are asymmetrically located in the outer membrane surface [2] and contribute to the formation of the carbohydrate-rich layer – the glycocalix [3] – surrounding the cells.

Gangliosides are assumed to play a role in a variety of surface events such as recognition phenomena, ion binding and biotransduction of membrane-mediated information [4–10]. These events are viewed as due to specific interactions between the ganglioside carbohydrate moiety, protruding from the membrane surface, and the external ligand. This is true in terms of individual interacting groups. However, the specificity and the ability to interact may well reside in the intrinsic properties of the membrane surface, modified by the presence of gangliosides.

Relatively little is known about the structure and dynamics of the external, carbohydrate-rich, membrane layer [11,12].

The approach used in the present study was to rebuild isolated membrane components, namely phosphatidylcholine and gangliosides, into artificial lipid bilayers (small unilamellar vesicles) and to monitor the dynamics of their interactions by means of two different stearic acid spin-label probes sounding, respectively, the region close to the polar surface and

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This paper follows the ganglioside nomenclature of Svennerholm [16] and the IUPAC-IUB recommendations [17] G_{M1}, II^3NeuAc-GgOse$_4$Cer; G_{D1a}, II^3NeuAc, IV^3NeuAc-GgOse$_4$-Cer; G_{T1b}, $II^3(NeuAc)_2$, IV^3NeuAc-GgOse$_4$Cer.

that close to the hydrophobic core of the lipid bilayer.

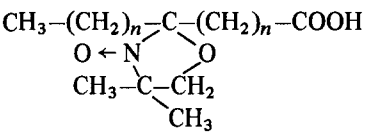
The use of spin-label spectroscopic probes, localized in the inner or more superficial region of the membrane lipids was shown to be a good tool for investigating this problem [13–15].

Materials and Methods

Chemicals and other products

Commercial chemicals were of analytical grade or of the highest purity available. Solvents were distilled before use. The water routinely used was freshly distilled using a glass apparatus. Egg phosphatidylcholine and egg phosphatidylethanolamine were purchased from B.D.H. and used without further purification provided that both of them showed a single spot when assayed by high performance thin-layer chromatography (silica gel thin-layer plates, HPTLC 60, Merck GmbH). The solvent system was chloroform/methanol/water, 60 : 35 : 4, v/v (1 h run at 20°C), and spots were revealed by exposure to iodine.

Gangliosides G_{M1}, G_{D1a} and G_{T1b} were extracted and purified from bovine brain according to Tettamanti et al. [18]. Their identification, structure analysis and purity were assayed as described by Sonnino et al. [19]. The final purity was over 99% for all three gangliosides. The composition of the ganglioside lipid portions, analyzed according to Sonnino et al. [19] is reported in Table I. The used spin probes were the *N*-oxyl-4,4′-dimethyloxazolidinenitroxy derivatives of stearic acid, having the following general formula:



In particular we employed 5-nitroxystearic acid and 16-nitroxy stearic acid purchased from Syva Assoc. (Palo Alto, CA, USA).

TABLE I

LIPID COMPOSITION OF GANGLIOSIDES USED G_{M1}, G_{D1a} AND G_{T1b}

	G_{M1}	G_{D1a}	G_{T1b}
Long chain base (mol%)			
C18 : 0	6.3	8.1	4.7
C18 : 1	59.6	53.4	47.9
C20 : 1	2.7	1.0	4.0
C20 : 1	31.4	37.5	43.4
Fatty acid (mol%)			
C16 : 0	1.0	2.6	1.1
C16 : 1	0.0	0.8	0.0
C18 : 0	97.0	87.9	89.3
C18 : 1	0.0	1.3	0.1
C20 : 0	2.0	5.2	9.3
C22 : 0	0.0	2.2	0.2

Preparation of small unilamellar vesicles

Phosphatidylcholine or phosphatidylcholine-ganglioside small unilamellar vesicles were prepared following the procedure of Barenholz et al. [20], already applied to ganglioside-containing systems [21]. The integrity of small unimellar vesicles was monitored according to Barenholz et al. [20] by: (a) turbidity measurements; (b) assaying the percentage of amino groups (carried by phosphatidylethanolamine inserted as a marker in the vesicle; about 5% in molar terms) available to 2,4,6-trinitrobenzenesulphonic acid (TNBS). The percentage of outer-sided phosphatidylethanolamine animo groups was reported to be about 65% in the case of small unilamellar vesicles [20]. All mixtures were prepared in Tris-HCl or sodium phosphate buffer (25 mM, pH 7.0).

Preparation of spin-labelled small unilamellar vesicles

The incorporation of spin labels into lipid unilamellar vesicles was accomplished as described elsewhere [13]. The concentration of the spin-label probe in the suspension was approx. $1 \cdot 10^{-4}$ M; the molar ratio of label to total lipids was made about 1/100 in order to avoid formation of liquid lines due to the incorporation of the spin label [22]. The penetration of the fatty acid spin probes into the vesicles was not influenced by the presence of gangliosides.

Electron paramagnetic resonance (EPR) spectra determinations

EPR spectra were recorded by a Varian E-4 spectrometer at room temperature (microwave frequency, 9.52 GHz; amplifier gain, $1.6 \cdot 10^3$; modulation amplitude, 1 G; time constant, 0.3–1.0). For spin labels having fast, nearly isothropic motion, an empirical

motion parameter, τ_0, can be derived from the equation [23,24]

$$\tau_0 = KW_0[(h_0/h_{-1})^{1/2} - 1]$$

where $K = 6.5 \cdot 10^{-10}$ s is fixed arbitrarily at its limiting value in the case of rapid isothropic tumbling, W_0 is the width of the central line and h_0 and h_{-1} are the heights of the center and high-field derivative lines, respectively, measured as indicated in Ref. 24. The order parameter, S, corrected for the polarity of the environment of the probe, is obtained from the anisothropic hyperfine splittings $T_\parallel$ and $T_\perp$ measured as shown in Fig. 2 by using the equation [25]:

$$S = \frac{T_\parallel - T_\perp}{T_{yy} - T_{xx}} \cdot \frac{a_n}{a_n'}$$

where T_{yy} and T_{xx} are the rigid lattice principal hyperfine values obtained from single crystal spectra; a_n' and a_n are the isotropic hyperfine coupling constants for the probe in the membrane and crystal states respectively:

$$a_n' = \tfrac{1}{3}(T_\parallel + 2T_\perp) \quad \text{and} \quad a_n = \tfrac{1}{3}(T_{zz} + 2T_{xx}) .$$

The corrected order parameter, S, is a measure of the amplitude of motion of the molecular long axis about the average orientation of the fatty acid chain in the lipid bilayer. Its maximum value is 1.0 for perfect order while complete disorder results in a value of $S = 0$. The error limit to measurements of $2T_\parallel$ and $2T_\perp$ was lower than 0.3 G. Therefore differences in $2T_\parallel$ and S respectively from 0.3 G and 0.01 were considered significant.

Effect of Ca^{2+} on EPR spectra of ganglioside-containing unilamellar vesicles

Ca^{2+} (as $CaCl_2$) was added to the vesicle suspension at two concentrations: 5 mM and 50 mM (final). The effect of the cation was followed by recording the EPR spectra at fixed times (chosen from zero time to a maximum of 5 h after addition).

Results

In order to define our experimental model we preliminarly established (a) the maximum amount of ganglioside (G_{M1}, G_{D1a} and G_{T1b}) in the mixture which was compatible with the maintenance of a vesicular structure; (b) the time stability (at 20 and 37°C) of ganglioside-containing vesicles.

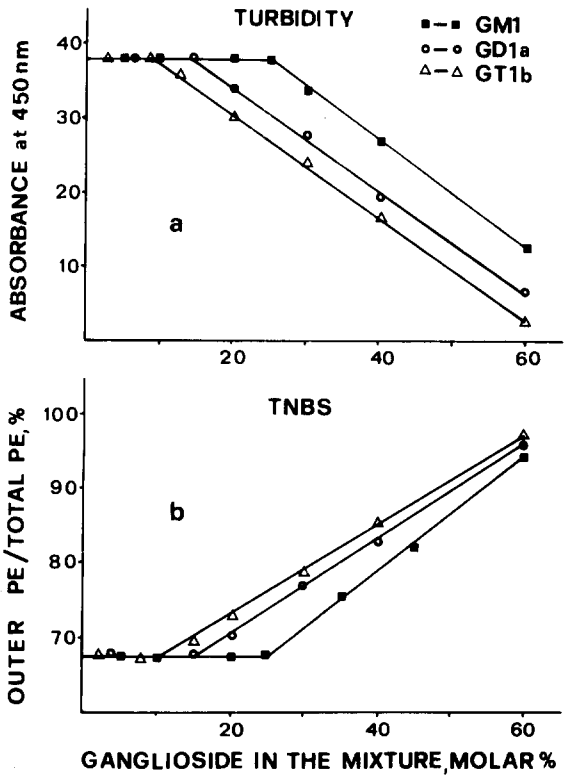


Fig. 1. Physicochemical features of mixed aggregates of egg phosphatidylcholine, phosphatidylethanolamine (used as sidedness marker) and gangliosides (G_{M1}, G_{D1a}, G_{T1b}) at increasing proportions of ganglioside. (a) Turbidity measurements; (b) determination of outsided aminogroup available to TNBS. The data exposed are the mean values of three experiments. The S.D. values were less than 5% of the mean.

As shown in Fig. 1a, on increasing the molar content of ganglioside in the vesicle the turbidity level (measured as absorbance at 450 nm) remained constant up to a certain ganglioside concentration, above which it started gradually diminishing. The percentage of outer-sided phosphatidylethanolamine (See Fig. 1b) persisted unchanged at a value of about 65% up to the same ganglioside concentration, providing a break point in the turbidity plot; then it gradually increased. The critical molar value (ganglioside/phosphatidylcholine), at which both turbidity and $-NH_2$ outsidedness started changing was higher, the lower

the sialic acid content of ganglioside: in fact it was about 25% for G_{M1} (monosialoganglioside), 15% for G_{D1a} (disialoganglioside) and 10% for G_{T1b} (trisialoganglioside).

Therefore vesicular structures were surely present when the ganglioside content of the system was lesser than the above molar values. Over those values, smaller and more soluble metastable phases moving towards mixed micelles [26] were probably being formed, as demonstrated by the gradual decrease of turbidity and increase of amino groups available to TNBS.

Provided that the ganglioside content did not exceed the above critical values, preparations of unilamellar vesicles maintained unchanged values of turbidity and of outer-sided amino groups for at least a 12 h period of storage at room temperature. This is a proof for their stability under these conditions.

The behavior of ganglioside in the phosphatidylcholine vesicles was studied with two fatty acid spin-label probes: 5-nitroxystearic acid and 16-nitroxystearic acid, the former sounding near the surface region of the lipid bilayer, the latter near the deep one. The EPR spectra showing the mobility respectively of 5-nitroxystearic acid and of 16-nitroxystearic acid in the phosphatidylcholine vesicles and in the ganglioside-phosphatidylcholine vesicles are presented in Figs. 2 and 3. In the spectrum referring to 5-nitroxystearic acid (Fig. 2), the hyperfine splitting ($2T_{\parallel}$) between the extreme high- and low-field peaks was smaller in the phosphatidylcholine than in the ganglioside-phosphatidylcholine vesicles. This indicates a more restricted mobility of the hydrocarbon chains, or a more rigid environment around the spin label probe in the ganglioside-phosphatidylcholine bilayer if compared to that of phosphatidylcholine alone. With the 16-nitroxystearic acid probe (Fig. 3) slight but significant differences in the correlation time, τ_0, were recorded between the phosphatidylcholine and the ganglioside-phosphatidylcholine vesicles, meaning that the tendency to a more rigid disposition of the lipid moieties around the probe, caused by gangliosides, was present in the inner core of the bilayer, too.

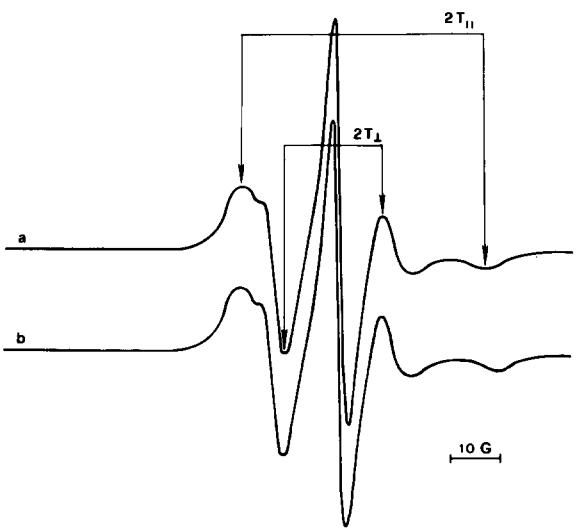


Fig. 2. EPR spectra of 5-nitroxystearic acid in (a) egg phosphatidylcholine unilamellar vesicles and in (b) egg phosphatidylcholine-ganglioside G_{M1} (22% in molar terms) unilamellar vesicles.

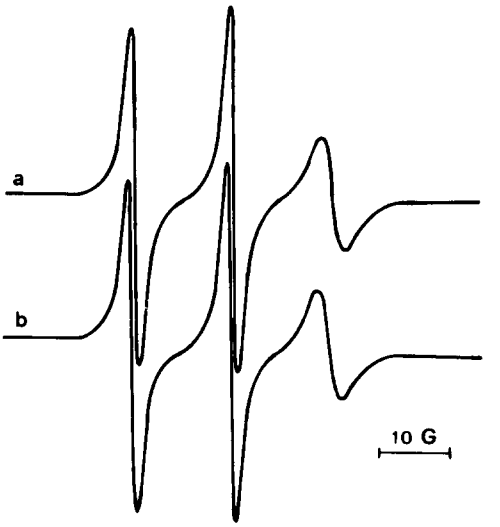


Fig. 3. EPR spectra of 16-nitroxystearic acid in (a) egg phosphatidylcholine unilamellar vesicles and in (b) egg phosphatidylcholine-ganglioside G_{M1} (22% in molar terms) unilamellar vesicles.

The effect of ganglioside concentration and species (G_{M1}, G_{D1a}, G_{T1b}) was also studied and the corresponding data reported in Table II. With 5-nitroxystearic acid the increase of the hyperfine splitting ($2T_{\parallel}$) and the corrected order parameter S was, at the same molar concentration, higher with G_{M1} than with G_{D1a} and G_{T1b}, and was proportionately dependent, at least to a certain extent, upon ganglioside

TABLE II

PARAMETERS EXPRESSING THE FREEDOM OF MOTION OF LIPID SPIN LABELS IN PHOSPHATIDYLCHOLINE AND IN PHOSPHATIDYLCHOLINE-GANGLIOSIDE UNILAMELLAR VESICLES

Vesicle composition	5-Nitroxystearic acid [b]		16-Nitroxystearic acid
	$2T_{\parallel}$ (G)	S	τ_0 (ns)
Phosphatidylcholine	49.8	0.587	1.06
Phosphatidylcholine [a]			
$+G_{M1}$			
4	50.4	0.605	–
12	51.3	0.623	–
22	51.8	0.629	1.34
$+G_{D1a}$			
4	50.1	0.593	–
8	51.2	0.610	–
12	51.3	0.610	1.16
$+G_{T1b}$			
4	50.3	0.594	–
10	51.0	0.603	1.20

[a] The molar percentage of ganglioside to total lipids is given. is given.

[b] Each value represents the mean of the closely agreeing determinations on at least two separate vesicle preparations. $2T_{\parallel}$ and S values agreed within 0.3 G and 0.01, respectively.

concentration. Using 16-nitroxystearic acid, the motion parameter, τ_0, underwent a slight enhancement which appeared to depend on ganglioside concentration while not, at least to any significant degree, on the ganglioside species.

On addition of Ca^{2+} to the ganglioside-containing vesicle suspension (see Table III), the 5-nitroxystearic acid spectrum, immediately recorded, showed a significant increase in the hyperfine splitting and in the order parameter S. The effect had about the same magnitude with all gangliosides (G_{M1}, G_{D1a} and G_{T1b}). On the other hand, the addition of Ca^{2+} to vesicles of phosphatidylcholine alone caused no measurable perturbations of the spectrum. The effect of Ca^{2+} was already present at 5 mM and remained unmodified by increasing ion concentration to 50 mM. It is worth noting that the effect of Ca^{2+} seemed to be somewhat time dependent. It tended to diminish, and in most cases it had actually disappeared 5 h after ion addition. No significant effect due to Ca^{2+} was recorded when using the 16-nitroxystearic acid probe.

TABLE III

EFFECT OF Ca^{2+} ON THE PARAMETERS EXPRESSING THE FREEDOM OF MOTION OF LIPID SPIN LABEL (5-NITROXYSTEARIC ACID) IN PHOSPHATIDYLCHOLINE AND IN PHOSPHATIDYLCHOLINE-GANGLIOSIDE UNILAMELLAR VESICLES

Each value represents the mean of the closely agreeing determinations on at least two separate vesicle preparations. $2T_{\parallel}$ and S values agreed with 0.3 G and 0.01, respectively.

Vesicle composition	Ca^{2+} added (mM, final concentration)	Time (h)	$2T_{\parallel}$ (G)	S
Phosphatidylcholine	0	–	49.8	0.587
	5	0	50.0	0.588
	50	0	50.1	0.588
Phosphatidylcholine +22% G_{M1}	0	–	51.8	0.629
	5	0	52.4	0.640
	5	5	52.0	0.632
	50	0	52.5	0.641
	50	5	52.3	0.638
+12% G_{D1a}	0	–	51.3	0.610
	5	0	51.7	0.624
	5	5	51.3	0.610
	50	0	52.0	0.630
	50	5	51.6	0.618
+10% G_{T1b}	0	–	51.0	0.603
	5	0	51.5	0.616
	5	5	51.2	0.605
	50	0	51.6	0.617
	50	5	51.2	0.604

Discussion

Gangliosides are membrane-bound glycosphyngolipids containing oligosaccharide chains of different composition and one or more residues of sialic acid. The oligosaccharide chains of gangliosides, protruding from the membrane surface, are available for a variety of interactions with other membrane components and with different external ligands [4–11]. The large oxygen-rich carbohydrate surfaces provide an extended lattice of hydrogen bonds and the acidic groups supply potential negative charges for these

interactions. The stereochemical orientation of the different carbohydrate chain sequences may constitute the code system for specific interactions.

A simple study model for membranes is the lipid bilayer of unilamellar vesicles. With this model we tried to investigate the ganglioside influence on the membrane properties by EPR spectroscopy. As spin-label probes we used 5- and 16-nitroxystearic acid, which penetrate the lipid layer and expose their spin-label groups to the inner core (16-nitroxystearic acid) or more superficially (5-nitroxystearic acid), respectively, at approx. 18 Å and approx. 6 Å from the carboxyl group [14]. Therefore they can monitor membrane events occurring in the deep hydrophobic region, or in the vicinity of the polar head group of the bilayer.

The signals released by 5-nitroxystearic acid clearly showed that the presence of gangliosides reduced the mobility of the hydrocarbon chains around the probe. Previous papers [11,27], in which EPR probes linked to the oligosaccharide portion of gangliosides were employed, provided evidence for strong side-side interactions among the head groups of gangliosides. Therefore the effect we observed can be the consequence of two kinds of interaction: one among the oligosaccharide chains of gangliosides, mediated mainly by hydrogen bonds; the second between gangliosides and phosphatidylcholine.

The chemical basis for these latter interactions could be: (a) the amido and the hydroxyl groups of long-chain bases and the hydroxyl groups carried by ganglioside saccharide chains: these can lead to the formation of hydrogen bonds with the phosphate groups of phosphatidylcholine. The occurrence of similar bonds was reported for the sphingomyelin/phosphatidylcholine system [28]; (b) the sialic acid residues of gangliosides which can establish electrostatic or dipole-dipole interactions with phosphatidylcholine headgroup [29]; (c) the hydrophilic portion of gangliosides which can remove hydration water from phosphatidylcholine headgroups, causing a decrease of mobility in the neighboring lipid portions. These interactions, individually or altogether, necessary lead the lipid chains to assembly themselves in a more ordered (rigid) fashion with concurrent decrease of membrane fluidity.

It is worth noting that this effect increased, up to a certain level, by increasing ganglioside concentration, while at a fixed ganglioside concentration it diminished from monosialoganglioside G_{M1} to disialoganglioside G_{D1a} and trisialoganglioside G_{T1b}. We can expect that by increasing the proportion of ganglioside in the bilayer a lattice of hydrogen bonds among the saccharide chains would form until the whole vesicle surface is covered. In addition, the superficial repulsive effect due to sialic acid charges would increase. By consequence, the interactions within the saccharide chains will compete with and balance the interactions between the same carbohydrate chains and the polar head groups of phosphatidylcholine. The critical concentration for a steady state will be attained at ganglioside concentrations which are lower, the higher the sialic acid content (or charge density) per mol of ganglioside. The decrease of hydrocarbon chain mobility around the probe was present, even if to a lesser extent, also in the hydrophobic core of the bilayer, as monitored by the 16-nitroxystearic acid probe. This means that the primary interaction between the polar groups of ganglioside and phosphatidylcholine are followed by a change of mutual interactions along the full length of the hydrocarbon chains, resulting in a decreased bilayer fluidity. Of course nearer the hydrophobic core this effect is balanced by the decrease of the order state and geometry of the hydrocarbon chains caused by the insertion of gangliosides.

The addition of Ca^{2+} appears to cause remarkable changes in the surface dynamics of ganglioside/phosphatidylcholine bilayer. In fact, the 5-nitroxystearic acid spectra indicated a net, but temporary, rigidification of the bilayer. With time the bilayer tended to return to previous fluidity, especially in the case of G_{D1a} and G_{T1b}. This behavior probably corresponds to the formation in the bilayer of two phases, one richer of gangliosides (and more rigid) and one poorer (and more fluid). This phenomenon, already observed by other authors [11,27], can be explained as due to 'clustering' or 'cluster stabilization' of gangliosides induced by calcium. Ca^{2+}, by preferentially bonding sialic acid (or phosphate groups), may remove gangliosides from their crosslinkages with phosphatidylcholine; therefore side-side hydrogen bonds among saccharide chains would prevail, leading gangliosides to cluster in a stable way. Therefore part of the probe would initially be trapped in the ganglioside richer phase, the remainder being left in the phosphatidyl-

choline phase. Later on, the probe would laterally diffuse between phases till reaching an even distribution. Concomitantly, the EPR spectra will show an increase of fluidity toward the level present prior to the addition of Ca^{2+} (in fact S, after 5 h from calcium addition, tended to return to its initial value).

In conclusion, our data suggest that the interactions among ganglioside oligosaccharide chains and between ganglioside and phosphorylcholine polar head groups can markedly influence the dynamics and fluidity of the membrane and mediate the response of membrane architecture to evironmental factors of physiological importance such as Ca^{2+}. The flexibility and reversibility of these interactions may also contribute to give the oligosaccharide components of membrane glycocalix precise functional orientation.

Acknowledgements

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