Membrane restructuring following in situ sialidase digestion of gangliosides: complex model bilayers by synchrotron radiation reflectivity.

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Abstract

Synchrotron radiation reflectometry was used to access the transverse structure of model membranes under the action of the human sialidase NEU2, down to the Ångström length scale. Model membranes were designed to mimic the lipid composition of so-called Glycosphingolipids Enriched Microdomains (GEMs), which are membrane platforms specifically enriched in cholesterol and sphingolipids, where also typical signalling molecules are hosted. Gangliosides, glycosphingolipids containing one or more sialic acid residues, are asymmetrically embedded in GEMs, in the outer membrane leaflet. There, gangliosides are claimed to directly interact with growth-factor receptors, modulating their activation and then the downstream intracellular signalling pathways. Thus, membrane dynamics and signalling could be strongly influenced by the activity of enzymes regulating the membrane ganglioside composition, including sialidases. Our results, concerning the structure of single membranes undergoing in-situ enzymatic digestion, show that the outcome of the sialidase action is not limited to the emergence of lower-sialylated ganglioside species. In fact, membrane reshaping occurs, involving a novel arrangement of the headgroups on its surface. Thus, sialidase activity reveals to be a potential tool to dynamically control the structural properties of the membrane external leaflet of living cells, influencing both the morphology of the close environment and the extent of interaction among active molecules belonging to signalling platforms.

1. Introduction
Signal transduction, cell migration, endocytosis and exocytosis of proteins and lipids are largely influenced by the dynamic structure of the plasma membrane and by the local aggregation of specific lipids and proteins [1-4]. The assembly of receptors and associated transducing elements typically occur in the lipid bilayer matrix of membranes. Specific lipid classes, like sphingolipids, are driving agents for membrane partition into functional micro-domains [5,6]. Membrane micro-domains enriched in cholesterol and sphingolipids, including glycosphingolipids, leading to a liquid ordered phase, are usually identified by the term GEMs (Glycosphingolipid Enriched Membrane micro-domains) [7]. Gangliosides and cholesterol have been found to act synergistically, forcing a preferential redistribution of components across the membrane and contributing to the super-structuring of their environment [8-10].

Moreover, specific lipid mixtures have been found in association with particular types of signalling molecules. To this regard, the FAS/CD95 receptor is typically found in association with the ceramide/sphingomyelin/gangliosides complexes, while it has been reported that the epidermal growth factor receptor (EGFR) is mostly associated with the cholesterol/sphingomyelin/gangliosides ones [11]. Based on recent studies, the emerging concept is that the local environment of any protein within membrane microdomains may modulate its propensity to interact with other proteins [12]. Within GEMs, gangliosides appear to play a multifaceted role, entangling structural and functional properties. In particular, the interactions between gangliosides and receptors have been demonstrated to be crucial to modulate downstream signalling pathways. In fact, the interaction between ganglioside GM3 and multi N-acetylglucosamine (GlcNAc) residues of N-linked glycans belonging to EGFR down-regulates the activation of the receptor [13]. Moreover, the interaction with gangliosides GM3 and GM2 has shown to inhibit the activation of the hepatocyte growth factor receptor, cMet [14].

In this view, the remodelling of gangliosides in plasma membranes could deeply alter cell signalling, and enzymes regulating ganglioside metabolism could play a key role in this context.
Sialidases (EC 3.2.1.18), which catalytically remove sialic acid from different sialo-glycoconjugates, including gangliosides [15], have been demonstrated to be involved in these mechanisms.

In mammals, four sialidases (NEU1, NEU2, NEU3, and NEU4) are present. They are involved in several key physiological events [16] and their deregulation appears to be related to cancer transformation [17-23]. The typical plasma membrane-associated sialidase NEU3 shows strict substrate specificity for gangliosides [16] and is prevalently associated with GEMs [24]. Through the regulation of membrane ganglioside composition, NEU3 modulates the activation of membrane receptors including EGFR [19,25], androgen receptor [26], and β1 integrin trafficking [23]. Nonetheless, recently, it has been demonstrated that NEU3 is not the unique sialidase present in the plasma membrane but also other sialidases, typically located in other cellular districts, can move to the cell surface and may be involved in many events. Lysosomal sialidase, NEU1, can migrate to the plasma membrane in different cells, like T lymphocytes [27], macrophages [28], and erythrocytes [29], there accomplishing important physiological tasks. Sialidase NEU2 is cytosolic and occurred to be found associated to mouse thymus cells [30]. Thus, plasma membrane proteins, lipids and enzymes involved in their metabolism appear to be dynamic entities that can move, entering and exiting from dynamical structures. Alterations occurring in such dynamics could be related to the deregulation of signal transmission or protein trafficking.

The study of portions of plasma membrane undergoing enzymatic action is then a very interesting topic. In particular, nanoscale modifications of the membrane structural properties induced by the punctual action of an enzyme on one of its components are now made possible by innovative experimental techniques on model systems. Among them, the synchrotron radiation reflectometry technique [31] is a powerful tool to access the transverse structure of a membrane down to the Ångström length scale.
In the work presented here, we applied synchrotron radiation reflectometry to perform a structural study of single ganglioside-containing model membranes under the action of sialidase NEU2. In particular, our aim was to observe the changes induced in the cross structure of a biomimetic membrane by the in situ chemical conversion of one component, namely GD1a ganglioside, in its metabolic product. It follows previous works we performed concerning the sialidase digestion of GD1a substrate in the form of colloidal aggregates in solution, either as pure GD1a micelles, or as mixed micelles or vesicles containing GD1a. [32]. The chemical formula of GD1a ganglioside is reported in Figure 1 (MW=1849 Da). Among others, the human sialidase NEU2 (HsNEU2) was chosen because it is water soluble, and therefore it could be added directly in the measuring cell to the buffer in contact with a pre-existing and characterized target membrane, without the help of any solubilizing agent. In addition, HsNEU2 is well characterized as a globular protein of ~42 kDa, it has been successfully purified and its structure and kinetic properties toward many substrates (gangliosides, oligosaccharides, and sialoglycoproteins) have been determined [33,34]. Within the family of neuraminidases, HsNEU2 has been found to be the most specific in substrate [35], being very efficient in removing the α2-3 linked-external sialic acid residues, as in GD1a, GD1b, GT1b, GM3, sialyllactose, and sialoglycoproteins [34], while showing no activity on internal sialic acid residues, like in GM1 or in GM2. Some residual activity has been detected on monomeric dispersions of GM1 [34]. Moreover, we observed [32] that, while chemically undergoing the enzymatic action, biomimetic mixed GD1a-phospholipid vesicles in solution display a transient response to sialidase, followed by full recovery.

Structural studies often require simplified model membranes. Still, some important features of biological membranes should be maintained. When mimicking GEMs, the presence of cholesterol and the asymmetric disposition of gangliosides are of primary importance. By a combination of Langmuir-Blodgett and Langmuir-Schaefer techniques [36], we deposited single asymmetric membranes composed by DPPC, cholesterol and gangliosides (only in the outer layer), on macroscopic silicon supports, with biomimetic molar ratio and disposition [37].
The reflectometry experiment is illustrated in Figure 1. Two replicas of a GD1a containing membrane have been built and accurately characterized. Then the target biomimetic membranes were submitted to the enzymatic treatment, and, finally, the structural properties of the digested membrane were assessed. Moreover, a much larger experimental landscape has been designed, as reported in the scheme of Figure 1. On this wider basis, we could finally gain novel and important additional suggestions on membrane reshaping following sialidase action.

![Scheme of the experimental design. The GD1a-containing membranes mA and mB are compared to their own in situ digested membrane and with other putative-product membranes. Membrane C and Membrane D have GM1 and AsialoGM1 gangliosides respectively embedded, instead of GD1a ganglioside. The lightning symbol stands for enzyme intervention. Nomenclature is according to Chester [38]. At the bottom: structure of ganglioside GD1a.](image)

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### 2. Materials and Methods

#### 2.1. Membrane preparation
DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine) was purchased from Avanti Polar Lipids. Cholesterol was from Sigma-Aldrich Co. GD1α (α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer), GM1 (β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer) and AsialoGM1 (β-Gal-(1-3)-β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer) were extracted and purified as described by Tettamanti et al. [39] and obtained as sodium salt powder. HsNEU2 sialidase expression and purification were conducted following the procedure described by Tringali et al. [33]. In order to obtain stable, single asymmetric membranes deposition, we used the Langmuir-Blodgett/Langmuir-Schaefer techniques [40]. A brief description of the technique is reported in SI Materials and Methods.

After the Langmuir-Schaefer deposition of the planned layers on a UV-Ozone treated [41] single crystal of silicon (5 x 5 x 1.5 cm³), the sample were closed on a homemade Teflon holder [42], directly mounted on the measuring station of ID10B beamline. The sample was mounted horizontally, face down, as schematically reported in Figure 2.

Figure 2 Schematic representation of the experimental set-up mounted on ID10B beamline.

2.2. Synchrotron Radiation Reflectivity

A reflectivity experiment allows to get information about the internal structure of stratified samples
Details of the technique are given in SI Materials and Methods.

Reflectivity measurements were performed on the horizontal reflectometer ID10B at the European Synchrotron Radiation Facility in Grenoble, France. Data were analyzed using the software Motofit [47] with a 7 layers model (hydrophilic out, 3 hydrophobic, hydrophilic in, a water layer and the silicon oxide). Electron densities were calculated starting from Nagle and Tristram-Nagle [48], Boretta et al. [49] and Greenwood et al. [50].

3. Results

The scheme of the performed reflectivity experiment is reported in Figure 1. We investigated the changes induced in model membranes by in situ enzymatic removal of sialic acid from parent gangliosides. Afterwards, we compared the structure of the so-obtained final membrane with that of a membrane originally built up with the lower-sialylated ganglioside. Moreover, we submitted also these last membranes to sialidase, in order to test for residual activity on internal sialic acid residues and for possible non specific interaction with the membrane itself.

The contrast of a material for synchrotron radiation is related to its electron density, which is higher for the glycolipids than for other lipids (see SI, Table S2) in the region of the sugar heads. Then, reflectometry allows detecting structural changes of the saccharidic substrates of HsNEU2, within the membrane, with high sensitivity. To our scope, we prepared and studied four supported membranes, containing each a different ganglioside species: GD1a (two replicas), GM1 and AsialoGM1, differing in the number of sialic acid residues in their polar region (see Figure 1). The lipid matrix consisted of di-palmitoyl-phosphatidyl-choline (DPPC), with a distinct asymmetric disposition of cholesterol and gangliosides in the two leaflets. The overall components molar ratio was DPPC: cholesterol: ganglioside = 10: 1.25: 0.5. Gangliosides were deposited in the outer leaflet of the membranes, exposed to the bulk water, together with 30% of cholesterol. The inner leaflet composition was therefore DPPC: cholesterol = 10: 1.75 mol, whereas the outer leaflet composition was DPPC: cholesterol: ganglioside = 10: 0.75: 1 mol. Saturated lipids were used, as they are best
suited to prepare full-coverage bilayers. Samples were submitted to annealing (see SI) and then reflectivity spectra were collected at 22°C.

The GD1a-containing membrane (sample $mA$) was measured both in pure water and in 150 mM NaCl aqueous solution as bulk solution. The obtained results indicated that the membrane was stable and resistant against changes in the ionic strength of the solvent. This feature is highly desirable for a model membrane, suitable for the study of the interactions between the membrane and approaching macromolecules, usually dispersed in buffer solutions, or for multistep processes, requiring buffer replacing. Upon salt addition, a slight increase in membrane thickness was detected (5.7 nm instead of 5.4 nm in pure water). This is consistent with the screening of electrostatic repulsive interactions among charged ganglioside headgroups, allowing tighter packing of lipids within the membrane. Then, for all other membranes, experiments were made in the presence of 150 mM NaCl solution, safely flushed before reflectivity measurement.

After characterization of the target membrane $mA$, the solution containing HsNEU2 sialidase was injected directly in the measuring cell and, after 0.5 h, reflectivity was measured again. Based on the known kinetics of HsNEU2 [34], such a delay is suitable for the enzymatic reaction to be fully completed. In Figure 3A, the reflectivity spectra of the membrane $mA$ before and after the action of HsNEU2 are shown, together with the best fitting curve. The density profiles, corresponding to the best fit of the reflectivity curves and describing the cross structure of the membrane, are reported in Figure 3B. Vertical dashed lines are drawn to guide the eye and approximately identify different regions of the deposited raft-mime membrane. Profiles differ mainly in the outer hydrophilic layer (region $b$), where ganglioside headgroups are exposed to the enzyme. In particular, the action of HsNEU2 resulted in a significant increase of the contrast, and therefore of the electron density, of this region.

The reproducibility of this result was tested on a second replica of GD1a-containing membrane $mA$, namely membrane $mB$. The two membranes ($mA$ and $mB$) have been independently prepared in different days, following the same protocol, and are then independent replicas of the same
membrane system. The measured reflectivity curves are very nicely superimposed, as can be seen in *SI*, Figure S1. After exposition to sialidase, results obtained on the membrane transverse structure of the replica membrane *mB* confirmed that the enzymatic digestion produced a pronounced increase of the electron density of the external polar region, from 416 e\(^-\)/nm\(^3\) to 480 e\(^-\)/nm\(^3\) (as reported in *SI*, Table S1), the hydrophobic core being nearly unaffected.

**Figure 3** Reflectivity curves (A) and density profiles (B) of membrane *mA* before (blue dots) and after (red triangles) the action of HsNEU2 sialidase. The solvent is a 150 mM NaCl solution. T= 22°C. In A) symbols correspond to the experimental data, lines to the best fits, corresponding to the profiles in B). In B) vertical dashed lines are drawn to guide the eye to approximately identify 9 regions, referring to different portions of the interfacial system: bulk solution (*a*), outer hydrophilic layer (*b*), outer CH\(_2\) groups layer (*c*), CH\(_3\) groups layer (*d*), inner CH\(_2\) groups layer (*e*), inner hydrophilic layer (*f*), water layer (*g*), silicon oxide (*h*), silicon (*i*).

Then, we wondered whether the “digested” membrane, resulting from the *in situ* action of the sialidase on the matrix membrane containing GD1a, had the same structure of a membrane prepared by mixing the matrix with the digestion product, *i.e.* GM1. We therefore prepared an asymmetric membrane, *mC*, containing GM1 instead of GD1a and measured its reflectivity spectrum. The density profile is reported in Figure 4, in comparison with that of the digested-*mB* membrane.
Remarkably, *mB* membrane (and replica *mA*) obtained after HsNEU2 digestion of GD1a was different from *mC* membrane, prepared by directly mixing GM1 to the matrix. In particular, the electron density of the hydrophilic layer was higher in the digested-*mA* and digested-*mB* membranes (480 e⁻/nm³) than in the *mC* membrane (419 e⁻/nm³).

**Figure 4** Contrast profiles of the *in situ* digested-*mB* membrane (after the action of HsNEU2 sialidase, red) and of membrane *mC* (green). The solvent is 150 mM aqueous solution. T= 22°C. Vertical dashed lines are drawn to guide the eye, as in Figure 3.

Why digested-GD1a-containing membranes could be different from *ab initio* GM1-containing ones is not trivial. In principle, because of the reported activity of HsNEU2 on monomeric GM1 [34], a weak activity of sialidase on GM1 produced by GD1a hydrolysis in model membranes could not be excluded. Thus, to address this point, we exposed the GM1-containing model membrane, *mC*, to HsNEU2.

The reflectivity profiles of the *mC* membrane before and after the action of sialidase were almost superimposable, as seen in Figure 5. This finding also excludes that non specific interaction occurs between the enzyme and the membrane. A minor effect could be related to the mentioned weak action of the sialidase on dispersed GM1 molecules.
Figure 5 Reflectivity curves from membrane $mC$ before (green dots) and after (violet triangles) the action of HsNEU2 sialidase. The solvent is 150 mM NaCl aqueous solution. T= 22°C. Symbols correspond to the experimental data, line to the best fit, corresponding to the profile in Figure 4, green curve.

We performed reflectivity experiments on a membrane containing Asialo-GM1, $mD$. The corresponding spectrum, together with the best fit line, and the fitted cross profile are reported in Figure 6. The transverse structure of membrane $mD$ was different from membrane $mA$ both before and after HsNEU2 digestion.

Figure 6 Reflectivity curve from membrane $mD$ (A) and contrast profile (B). The solvent is 150 mM NaCl aqueous solution. T= 22°C. Symbols correspond to the experimental data, line to the best
fit, corresponding to the profiles in B). In B) vertical dashed lines are drawn to guide the eye, as in Figure 3.

Finally, in Figure 7, we compare all the recovered electron density profiles, restricted to the external headgroup region. It is easily seen that all of them appear very similar, except for the one corresponding to the digested-GD1a model membrane, remarkably different from the others. In fact, its external polar region has a much higher contrast than all of the other model membranes, including the chemically-identical GM1-containing membrane, mC.

**Figure 7** Contrast profiles of the external head-group region of the different membranes in 150 mM NaCl solution: mA before (blue) and after (red) HsNEU2 digestion, mC before (green) and after (violet) HsNEU2 digestion and mD (black). T=22°C. The external polar region GD1a-digested membrane mA (red) has a remarkably higher contrast. The horizontal axis refers to distances from the mid-plane (z = 0) of the external head-group regions.

4. Discussion

In biological membranes, gangliosides are transformed into lower-sialylated species by sialidases, while being already packed in the membrane aggregate. From this start point, interesting issues emerge. Gangliosides disposition and relative concentration within the lipid structure could affect enzymatic activity and kinetics. Also, and more intriguing, the enzymatic action could not only
produce a new molecule in the membrane, but also induce changes in the membrane organization, such as lipid lateral segregation, membrane core fluidity and roughness, maybe reminiscent of its chemical and structural history.

In the experiment presented here, our aim was to study the transverse structure of ganglioside containing membranes submitted to sialidase digestion. So we applied synchrotron radiation reflectometry to four supported model membranes containing three different ganglioside species: GD1a, GM1 and AsialoGM1. We investigated the changes induced by sialic acid removal by in-situ enzymatic digestion, and we compared the final membrane with a membrane originally built up with the lower sialylated ganglioside. The successful deposition of single macroscopic membranes containing GD1a and AsialoGM1 was itself a first remarkable result, following the previous successful preparations with GM1 and cholesterol [8,51] and opens the way to the construction of model membranes with increasing bio-similarity.

The occurrence of a change in the cross structure of the GD1a-containing membrane, mA, upon enzymatic action is clearly visible in Figure 3. Then, the technique proves to be extremely sensitive in following this kind of membrane-enzyme interaction and constitutes an experimental and technical achievement. In fact, the technique is able to reveal structural changes in the sugar-containing hydrophilic layer of the membrane, where sialidase is acting. However, before the experiment had been performed, it was difficult to foresee the extent of experimental sensitivity to these structural changes. In fact, the mole fraction of GD1a with respect to the hosting phospholipids of the outer layer is as low as 1:10. In addition, the enzyme removes only the external sialic acid residue of GD1a (one sugar unit out of 6), likely located quite far from the membrane surface (about 1.5 nm distance), while the phospholipid headgroups of the matrix are confined in a closer layer (some 1 nm thick). Remarkably, despite the visibility of a clear effect was not obvious to anticipate, an unambiguous variation of the average electron density of the hydrophilic layer as a whole was observed. We note that the outer hydrophilic layer (region b) accounts for the dense hydrophilic region of the membrane, where DPPC headgroups (the majority compound) are
confined, contributing to space filling with the bound hydration water. Reversely, the protruding portions of the ganglioside headgroups, containing the external, terminal, sialic acid unit to be removed by the enzyme (see Figure 1), sparingly populate a far-layer that is not dense enough to effectively contrast against bulk water. In this far layer one can estimate that the headgroup surface occupancy is in the range 10-20% of the total available area.

The density profiles reported in Figure 3B differ mainly in the outer hydrophilic layer (region b), where ganglioside headgroups are exposed to the enzyme. Upon HsNEU2 digestion, a significant increase of the contrast of this region occurred, i.e., its electron density increased. We remark that the same result was obtained on the replica GD1a-containing membrane, mB; thus, the reproducibility stands as a strong proof of the reliability of the outcome.

This result is of great importance and adds interesting information on the cross local structure of the ganglioside-containing membrane, integrating the compositional result of GD1a turned to GM1 by biochemical catalysis. In fact, a decrease in the average specific volume of the layer, corresponding to increased lipid layer compactness, cannot be invoked to explain the observed large increase in the average electron density of the hydrophilic close-layer, about 1 nm thick. In fact, no corresponding change is seen in the electron density of the hydrophobic region of the outer layer (see SI, Table S1). A more tricky effect should occur.

We observe that an increase in the electron density profile of the hydrophilic close-layer, without affecting the hydrophobic region, can be obtained by replacing a fraction of the hydrophilic close-layer with an equal volume with higher electron density. This suggests that the headgroups of the GM1 molecules emerging from in situ digestion are not only shortened by one charged external unit, but also retracted closer to the membrane surface with respect to the parent-GD1a headgroups. It seems that they lay down on the membrane surface. We recall that the lying conformation, requiring the ganglioside headgroups to tilt with respect to the axis of the hydrophobic chains embedded in the membrane core, has often been hypothesized in the literature. The interconversion between the standing and lying conformations of headgroups has been observed to occur in
ganglioside aggregates as a consequence of the action of external parameters like temperature, dehydration, or salinity [9,52].

By integration of the ρ(z) profiles in the region of the external polar heads of membrane mA before and after digestion (blue and red curves of Figure 7), the number density of additional electrons in the close-layer can be calculated. A 30% increase in the electron density excess with respect to water has occurred, corresponding to a 1.3% GM1 head retraction per unit volume. Very interestingly, this is consistent with a glycolipid head volume redistribution increasing its lateral hindrance by 4%, the same value estimated for the interfacial area increase upon GM1 tilting in micelles [53]. The corresponding variation in the low-contrast hydrophobic moiety, 4% each for the 10% ganglioside molecules in the layer, is not visible.

The comparison between the “enzyme digested” membrane and a membrane prepared by mixing the matrix with the digestion product, reported in Figure 4, is extremely interesting. In particular, the electron density of the hydrophilic layer is higher for the digested-mA (digested-mB) membrane than for mC.

This is an interesting point. Why should digested-GD1a-containing membranes be different from *ab initio* GM1-containing ones is not obvious. An incomplete digestion of GD1a into GM1 or further-proceeding digestion leading to AsialoGM1 can be excluded. In fact, despite direct biochemical assessment on the deposited membranes is unfeasible, enzymatic activity has been previously studied in different ganglioside-containing systems: incomplete digestion of GD1a is unlikely since HsNEU2, similarly to other sialidases, has been proved to go through full GD1a-to-GM1 conversion in different aggregated structures in solution, as micelles or mixed vesicles [32,54]. Moreover, the amount of added enzyme was intentionally in large excess with respect to the substrate. Moreover, being the electron density of the hydrophilic outer layer higher than both GD1a- and GM1-containing membranes, so out of any weighted average of the two, trivial incomplete digestion is unable to provide straightforward explanation of the experimental result. On the other hand, a weak activity of sialidase on GM1 inserted in model membranes, producing the
Asialo-GM1, could not be excluded [34] in principle. This was ruled out by testing that the reflectivity spectra of the GM1-containing mC membrane before and after exposure to sialidase were almost superimposable, as seen in Figure 5. Furthermore, the transverse structure of the Asialo-GM1-containing mD membrane is different from that of GD1a-containing membranes mA and mB, both before and after HsNEU2 digestion (Figures 6A and 6B).

From the comparison of the electron density profiles restricted to the external headgroups region, reported in Figure 7, we see that the lying conformation, corresponding to high close-shell contrast, is not the only one admitted for GM1, in the membrane. As well, GM1 headgroups can stand more perpendicular to the membrane (as in mC), displacing their center of mass farther from the surface. This arrangement appears to be similar to the one adopted by both GD1a and AsialoGM1 in membranes mA (and its replica mB) and mD. Notably, when the same protocol was followed for sample preparation, the cross profiles of the external close-layers at 40mN/m were nicely the same. Although the polar heads of the different gangliosides are different, their initial portion, included in the close-layer, is the same and, at the given surface dilution, behaves the same way. The obtained results (Figure 7) indicate that in this condition of surface concentration and pressure, the ganglioside headgroups assume the standing conformation with respect to the membrane plane. But, upon specific and proper stimulation, they can turned to lying, seemingly modulating the mechanical properties of their local environment.

5. Conclusion

The outcome of this work is that the action of the enzyme HsNEU2 has a deeper effect on biomimetic membranes than just turning GD1a to GM1 by external sialic acid removal. The in-situ produced GM1 glycosidic headgroup is retracted to the membrane surface, storing additional packing surface. Stored surface could eventually be used to promote or allow for mechanical deformations of the membrane, providing structural support to biological functions. In fact,
ganglioside-enriched domains are easily associated with membrane protrusion or caveolae. The present observations, then, support the hypothesis that the sialidase action drives the membrane to a structural turning point, where it can be readdressed to different final fates.

Finally, it is sometimes argued that the packing properties of aggregating molecules are likely to be washed out once in a large structure, like a membrane, where they happen to be mixed with other different species. In this experiment, as in many others, it comes out that, instead, molecular packing and membrane properties respond to each other, contributing to membrane structural and functional evolution.

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Graphical Abstract:

![Graphical Abstract Image]

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