The Lazy Life of Lipid-Linked Oligosaccharides in All Life Domains

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Abstract

Lipid-linked oligosaccharides (LLOs) plays an important role in the N-glycosylation pathway as the donor substrate of oligosaccharyltransferases (OSTs), which are responsible for the *en bloc* transfer of glycan chains onto a nascent polypeptide. The lipid component of LLO in both eukarya and archaea consists of a dolichol, and an undecaprenol in prokarya, whereas the number of isoprene units may change between species. Given the potential relevance of LLOs and their related enzymes to diverse biotechnological applications, obtaining reliable LLO models from distinct domains of life could support further studies on complex formation and their processing by OSTs, as well as protein engineering on such systems. In this work, molecular modeling, such as quantum mechanics calculations, molecular dynamics simulations, and metadynamics were employed to study eukaryotic (Glc₃-Man₉-GlcNAc₂-PP-Dolichol), bacterial (Glc₁-GalNAc₅-Bac₁-PP-Undecaprenol) and archaeal (Glc₁-Man₁-Gal₁-Man₁-Glc₁-Gal₁-Glc₁-P-Dolichol) LLO in membrane bilayers. Microsecond molecular dynamics simulations and metadynamics calculations of LLOs revealed that glycan chains are more prone to interact with the membrane lipid head groups, while the PP linkages are positioned at the lipid phosphate head groups level. Dynamics of isoprenoid chains embedded within the bilayer are described and membrane dynamics and its related properties are also investigated. Overall, there are similarities regarding the structural and dynamics of the eukaryotic, the bacterial and the archaeal LLOs in bilayers, which can support the comprehension of their association with OSTs. This data may support future studies on the transferring mechanism of the oligosaccharide chain to an acceptor protein.

Introduction

The glycosylation of asparagine residues is the predominant protein modification throughout all domains of life.¹ This post-translational modification is called N-glycosylation, which is important in many aspects of biology and affect various properties of the proteins, including folding, conformation and solubility.^{1,2} The N-glycosylation occurs in the consensus motif

(referred to as the sequon) represented by Asn-X-Ser/Thr, where Xaa can be any residue except Pro.^{3,4} Lipid-linked oligosaccharides (LLOs) are the substrates of oligosaccharyltransferase (OST), which catalyzes the transfer of the oligosaccharide to the acceptor asparagine residue of nascent proteins.⁵

The LLO molecule can be subdivided in two main parts: the isoprenoid moiety and the oligosaccharide chain. The link between these two portions is made by one or two pyrophosphate groups.⁶ The lipid component of LLOs varies for each species, as well as the number of isoprene units. On eukarya and archaea, the isoprenoid moiety is a dolichol, while in bacteria, the isoprenoid is an undecaprenol. In eukarya, the oligosaccharide portion is more conserved, composed by a Glc₃-Man₉-GlcNAc₂ chain, while on the other domains of life it becomes more diverse. Despite of all the differences in their structure, the oligosaccharide transfer mechanism seems to be conserved in all domains of life.⁷

In Bacteria, the N-glycosylation final step is performed by a single-subunit oligosaccharyltransferase (PglB).⁸ However, in most eukaryotes, the same is achieved by the action of a large, multi-subunit, membrane-embedded OST complex, usually containing eight subunits in yeast, but possibly showing even more in multicellular organisms. Recently, the structure of the yeast OST was characterized by employing single-particle cryo-EM,⁹ revealing a conserved subunit arrangement. In this structure, the catalytic STT3 subunit displays full access to its substrates, since its active site is not located in the inner area of the complex. The dolichol-pyrophosphate portion of the eukaryotic LLO binds to a hydrophobic groove of STT3, similarly to other OSTs,^{10,11} while the glycan chain binds to the pocket formed in the interface of both domains of the STT3 unit, also comprised by two noncatalytic subunits and an ordered N-glycan. This suggests that these LLO molecules might populate conformations in which the sugar moieties are parallel to the membrane. In this context, there is only one previous computational report¹² that assessed this information at an atomic level (only for bacterial and eukaryotic LLOs) which demonstrated the importance of the oligosaccharide chain behavior and its orientation with respect to the bilayer before its binding.

In our work, we employed a combination of quantum mechanics (QM) calculations, molecular modelling techniques, molecular dynamics (MD) and enhanced sampling simulations to study conformational properties, such as the orientation and the dynamics of LLOs from all domains of life: i) an eukaryotic LLO¹² (Glc₃-Man₉-GlcNAc₂-PP- Dolichol; G₃M₉Gn₂-PP-Dol; Fig. 1 A) embedded in 1-Palmitoyl-2-oleoyl-SN-glycero-3-phosphocholine (POPC) membrane; ii) a bacterial LLO¹² (Glc₁-GalNAc₅-Bac₁-PP-Undecaprenol; G₁Gn₅B₁- PP-Und; Fig. 1B) embedded in 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) membrane; iii) an archaeal LLO^{13,14} (Glc₁-Man₁-Gal₁-Man₁-Glc₁-Gal₁-Glc₁-P-Dolichol; G₁M₁Gal₁M₁G₁Gal₁G₁-P-Dol; Fig. 1C) embedded in a POPE membrane. The eukaryotic, bacterial and archaeal LLOs without its glycan chains were also analyzed. Improvements on the parameters of a torsional angle were achieved, an important correction for a better reproduction of some experimental properties of the isoprenoid unit. In addition, novel parameters for the sulfate groups in the archaeal LLO were also generated. Our microsecond molecular dynamics (MD) simulations analysis allowed us to identify clusters of conformations that were submitted to molecular docking calculations with the available crystallographic structures for all domains of life. We generated enzyme-ligand poses that were in agreement with expected crystal structure distances for all cases, achieving the first set of full complexes OSTs for the eukaryal and the archaeal enzymes.

Results and discussion

Simulation results are presented and discussed for the eukaryotic, bacterial and archaeal LLOs embedded in their respective membrane bilayers consisting of POPC, POPE and POPE lipids, respectively. The systems without glycan portion are presented as controls for further comparisons.

Figure 1: Schematic representations of the (A) eukaryotic, (B) bacterial and (C) Archaea LLOs used in this study. Blue circle = D-Glucose, Blue square = N-Acetyl-D-glucosamine, Yellow square = N-Acetyl-D-galactosamine, Yellow circle = D-Galactose, Green circle = D-Mannose and Bac = Bacillosamine.

Torsional potential and force field calibration

The functional form of the potential energy term, associated with the torsion around dihedral angle m , is given in GROMOS by:

$$V_{\phi,m} = k_{\phi,m} [1 + \cos \delta_m \cos(n_m \phi_m)] \quad (1)$$

where ϕ_m is the dihedral angle value, n_m the multiplicity of the term, δ_m the associated phase shift, and $k_{\phi,m}$, the corresponding force constant, which are applied. It is worth noting that a given dihedral angle may be described by more than one torsional potential energy term with different multiplicities and/or phase shifts.

While some of the torsional potentials were preserved from the GROMOS53A6 original parameter set when building LLOs topology, torsional preferences of the isoprenoid chain were re-evaluated based on QM data fitting. Torsional energy profiles obtained from QM calculations at B3LYP, MP2 and HF/6-31G(d) theory level were generated for 2-Methylbut-1-ene dihedral angle rotation and allowed the derivation of three new torsional dihedral potentials that were fitted to QM energy profiles (Figure 2). The newly obtained potentials (Table 1) adequately reproduce the QM-obtained energy profile related to such torsion. However, analyses of the dihedral profiles showed important divergences between QM-calculated and CHARMM36/GROMOS53A6 energy profiles for dihedral angle (Figure 2). Although accounting for two energy minima at -120° and 120° , conformational barriers obtained for the original GROMOS53A6 parameters are not sufficiently elevated to properly describe the bond rotation. Additionally, the 0° conformer is described as the energy global maximum, instead of a local minimum (Figure 2). The profile obtained for CHARMM36 force field parameters also show divergences, presenting only one energy global minimum at $180^\circ/-180^\circ$ and a global maximum of 22kJ/mol at 0° , while conformers at -120° and 120° are described as energy local maximum, instead of local minimum (Figure 2).

Organic liquid simulations were performed in order to validate the parameters used in topologies for LLOs building blocks. This validation strategy of comparing experimental and simulated condensed-phase (ρ and ΔH_{vap}) thermodynamic properties was employed in other works involving molecules parametrization in GROMOS force field^{15,16} and for the establishment of force field benchmarks.¹⁷ The simulation of geraniol in liquid phase yielded ρ and ΔH_{vap} values in good agreement with experimental data (Table 2), which indicates that the obtained set of torsional potentials is suitable to reproduce experimental properties.

Figure 2: Comparison of energy profiles calculated at classical (GROMOS53A6/CHARMM36) and QM (B3LYP, MP2 and HF/6-31G*) levels in the gas phase. The fitting has generated parameters that reproduce well the QM potential energy in the MM calculations.

Table 1: Torsional parameters obtained based on QM calculations of 2-Methylbut-1-ene dihedral angle

Compound	δ	$k_{\phi,m}$	n
	0	5.97	0
2-Methylbut-1-ene	0	-3.67	1
	0	-5.17	3

LLOs dynamics and spatial distribution within membrane

The density profiles calculated along the vector normal to the membrane (Z axis) indicated that, in general, the saccharidic residues remains above the lipid head group, while the pyrophosphate (PP) group mostly populate the same area as the lipid head groups. Moreover, LLOs hydrophobic chain (Dolichol-PP, Dolichol-P, and Undecaprenol-PP) fluctuates inside the membrane bilayer (Figure 3). We detected a general asymmetric behavior for the LLO lipid chain, with the highest density peaks being found in the middle of the two leaflets, along with a higher population of the upper leaflet area. The carbon at the end of the LLO tail demonstrates the flexibility of this moiety, as it populates distinct areas of the bilayer,

Table 2: Obtained values of thermodynamic properties obtained from geraniol simulation in liquid phase

Compound	Temp. [K]	Exp. ρ [g/cm^3]	Calc. ρ [g/cm^3]	Error	Exp. ΔH_{vap} [kJ/mol]	Calc. ΔH_{vap} [kJ/mol]	Error
Geraniol	298.15 ¹⁸	0.89 ¹⁹	0.89	0%	58.83 ¹⁸	64.24	9.33%

with a higher density between the center area of the membrane and the lower leaflet. This same pattern was observed for the non-glycosilated LLO chains (Figure S1), suggesting that the presence of oligosaccharide moieties in LLO structure does not influence the dynamics of its lipid tails within the membrane bilayer in terms of Z axis.

Figure 3: Density profiles for the subdivided parts of the studied LLOs considering the vector normal to the membrane bilayer (Z axis): membrane head groups (yellow), lipid acyl chains (black), isoprenoid chain (blue), oligosaccharide (purple), pyrophosphate linkage (red), and the last carbon atom of isoprenoid chain (green). We enhanced the oligosaccharide distribution scaling it by a factor of five to facilitate the observation of data. The same procedure was performed for the pyrophosphate linkage, dolichol chain, and dolichol's last carbon distributions, but using a scale factor of ten.

To better understand the conformational space of LLOs lipid tails among the membrane XY axis, we measured the distance distributions between the first carbon (C1A - all simulations) and the middle carbon (C9E - Eukarya, C6E - Bacteria, and C8E - Archaea), and between the first carbon (C1A - all simulations) and the last carbon (C19E - Eukarya, C11E - Bacteria, and C12E - Archaea) of the lipid chain (Figure 4). The distributions found for bacterial LLOs revealed a more restricted area of occupation for the first half of the chain (Figure 4A - red line), specially on the bacterial molecule, mainly due to its reduced length, while the full length distance distribution (Figure 4A - blue line) displays a pronounced

flexibility, with a 10 Å difference in the stretch of the eukaryotic LLO. The same carbon-carbon distances were calculated as a function of trajectory time blocks of 200ns to confirm convergence (Table S1). Interestingly, the archaeal LLO first half chain presents a distribution pattern comparable to the eukaryotic LLO, despite having a difference of almost half of the isoprenoid units. When comparing the lipid dynamics of the complete LLO structures (Figure 4B) with the dynamics of LLOs without their glycan chains (Figure S2B), eukaryotic LLO presented a greater lipid motion in the absence of its glycan chain, while the archaeal LLO presented a more limited motion in the presence of its glycan chain. Together with the densities of LLO portion along the membrane, it is possible to infer that LLOs lipid tail motion is mostly confined to the XY axis at the region between the membrane leaflets. This behavior demonstrates the influence of the sugar portion on the dynamics of these glycoconjugates.

Membrane bilayer dynamics in the presence of LLOs

In order to detect possible effects of LLOs presence on the surrounding biochemical environment, we evaluated membrane properties, such as area per lipid and thickness, throughout the 1 μ s simulations (Table 3). For Eukarya and Bacteria LLOs, the presence of glycan chains did not influenced the area per lipid, even though they present drastic differences on the number of saccharides ramifications or sugar length. However, the presence of glycan chain on Archaea LLO yielded a higher area per lipid than its LLO in absence of the sugar moiety, suggesting that its composing monossacharides are capable to recruit lipids. In fact, the presence of a -NH_3^+ group at the POPE lipids head group make it is suitable for strong electrostatic interactions with the sulfate groups at the base of LLOs glycan chain, which was monitored throughout simulations and confirmed (data not shown). Moreover, the presence of glycan chains did not altered membrane thickness for any of the simulated systems. However, despite being composed by the same POPE membrane lipids, both Bacteria and Archaea systems presented different membrane thickness values, suggesting that the length

Figure 4: Distributions of the LLOs hydrophobic tail in lipid bilayer. A) histograms of the distances between C1A and C9E - Eukarya, C6E - Bacteria, C8E - Archaea (red line) and carbons C1A and C19E - Eukarya, C11E - Bacteria, and C12E - Archaea (blue line) for every LLO. B) 3D heatmap of C9E - Eukarya, C6E - Bacteria, C8E - Archaea occupancy and C) of C19E - Eukarya, C11E - Bacteria, and C12E - Archaea occupancy in membrane, taking C1A as [0,0] cartesian coordinates. In B and C, each value corresponds to the coordinates populated in X (horizontal) and Y (vertical) by the selected carbons. Highly occupied regions are colored in red, while poorly occupied region are colored in blue.

of the lipid tail may influence on local lipid structure and dynamics. These findings suggest that, depending on LLO chemical composition, these glycoconjugates may rearrange local membrane structure properties, which might be related to its biological function.

Table 3: Average Area per lipid and thickness of membranes throughout MD trajectory.

LLO	Area per lipid ($\text{\AA}^2$)	Thickness ($\text{\AA}$)
<i>Eukarya</i>	62.69 ± 1.02	38.07 ± 0.57
<i>Bacteria</i>	59.02 ± 0.83	40.15 ± 0.52
<i>Archaea</i>	61.83 ± 1.28	37.75 ± 0.67
<i>Eukarya</i> (no glycans)	61.41 ± 1.14	38.63 ± 0.62
<i>Bacteria</i> (no glycans)	58.49 ± 0.92	40.16 ± 0.56
<i>Archaea</i> (no glycans)	57.44 ± 1.13	39.22 ± 0.62

LLOs’ oligosaccharidic moiety orientation in membranes

Aiming to properly describe the motion and orientation of the oligosaccharidic portion from LLOs, we analyzed the tilt angle of the glycan chain relative to the vector normal to the membrane. For this, we defined a vector connecting distant monosaccharides, as follows: for the bacterial LLO, the monosaccharide bacilosamine (Bac) nearest to the reducing end and the one in the oxidizing end (NAc-Galactosamine) were selected; for the archaeal LLO, two measurements were performed (due to the existing branch ramifications in the glycan), one using 1 and 4’ (red line), and the other one using 1 and 4 (blue line); for the eukaryotic LLO, also two measurements were taken, using 1 and 3’ (red line), and 1 and 3 (blue line). As shown in Figure 5A, the main vector indicates that the preferred position for all glycan chains is in parallel with the bilayer (~ 90 degrees). This observations are further confirmed by the average positions of the saccharadic residues relative to the Z axis (Figure 5B), showing that the majority of the monosaccharide residues are close to the membrane.

Correspondingly, the same behavior is depicted by the residence time of all saccharidic residues at the membrane (Figure 5C). Most residues interact with the membrane during the whole simulations time, with few exceptions, such as Archaea residues 4, 5, and 3’ with a small decrease and Eukarya residues 3, 4, and D3, which possess lower residence time interacting with the membrane. Possibly, an higher number of ramifications and the presence of flexible glycosidic bonds (β -1,6 bond type) could yield a decrease on the residence time for some monosaccharide residues.

Furthermore, in order to confirm the orientation behavior we found in the unbiased MD simulations, we employed metadynamics calculations to describe the free-energy surfaces (FES) of the oligosaccharidic motion relative to the membrane. To obtain this, we employed a collective variable (CV - See Experimental Section) which could differentiate between the possible orientation states of the glycan chain. As shown in Figure 6, there are two low energy states depicted by the FES of the tilt angle: one is at 0 degrees, where the oligosaccharide chain is perpendicular to the membrane, and one comprehending values between 60 and 100 degrees (depending on the LLO type), where the glycan is in parallel with the membrane. For all systems, the lowest energy state detected is the one in parallel with the bilayer, with some of the structures located in this minimum possessing a slightly curved shape (Figure 6). The ΔG between states ranges from 35 to 70 kJ, reinforcing a clear preference for this conformational state.

Looking at the carbohydrate residues coloured by root mean square fluctuation (Figure 5D), it is possible to identify that, in general, the central core of the glycan chains is mainly rigid, while flexibility is higher at the ramifications and at the terminal residues. The bacterial LLO is indeed the most rigid among them, probably due to the absence of branching in its structure. The archaeal and eukaryotic LLOs show increased mobility in their chains, especially the latter with its multiple branched residues, which can sometimes prevent a full interaction in a parallel pattern with the membrane. As seen in the FES, the archaeal oligosaccharide chain has a shifted minimum, but this does not correlate with an increased flexibility in its structure. A possible explanation to this is the presence of the three sulphate groups that can interact firmly with the polar headgroups, recruiting more phospholipids to its surrounding, a feature that might block a further increase in the tilt angle.

Finally, we performed conformational analysis on LLOs glycan chains based on the glycosidic dihedral linkages using the same methodology proposed by Arantes et al.²⁰ (Table 4). For better understanding, in the Supporting Information, Figure S3 shows each glycosidic linkage on the LLOs structure with their dihedral angles ϕ , ψ and ω highlighted.

Figure 5: Tilt angles calculated for the vectors describing the motion from LLOs' glycan chains. A) Probability density histograms describing tilt angle values found during unbiased MD simulations. The blue line describes the vector connecting 1 and 4 in Eukarya, 1 and 6 in Bacteria, and 1 and 3 in Archaea. The red line describes the vector connecting residues 1 and 4' in Eukarya, and 1 and 3' in Archaea. B) Positions of each sugar residue relative to the Z-axis are represented by blue dots (average position in blue, error bars in red); C) Residence time of each sugar residue in the membrane interface, calculated using a cutoff of 4.5 Å. D) Root mean square fluctuation B-factor of LLOs. The color scale ranges from blue (hard) to yellow/orange (flexible).

As previously demonstrated for bacterial LLO,²¹ the average conformational profile of the oligosaccharide chain attached to the lipid remains mostly rigid during the simulations of all LLOs, with minor fluctuations to less frequent conformational states. These results may be related to the interaction of oligosaccharide chains with the phosphate head groups of the

Figure 6: Metadynamics simulations results: A) Free-energy surface (FES) in function of tilt angle for LLOs' glycan chains. B) Representative conformations of the eukaryotic, the bacterial and the archaeal LLOs, in their respective main minimum energy region. Average Error in tilt angles of LLOs: Eukarya = 1.72 ± 2.84 KJ/mol, Bacteria = 2.23 ± 2.73 KJ/mol and Archaea = 2.63 ± 0.88 KJ/mol.

membranes during the MD simulations of all LLOs. On the other hand, a higher flexibility is observed for the dihedral angles of monosaccharides linked to phosphate (ϕ_1 and ψ_1), when compared to the remaining glycosidic linkages. This behavior may be related to the lipid chain movement within the membrane. These data were used for identification of the most abundant conformations of LLOs molecules and subsequent docking calculations.

Molecular Docking of LLOs in each OST model

By exploring the conformational space of glycan moieties sampled of each simulation using the same approach as described in Arantes et al., we were able to identify conformational

Table 4: Average dihedral angles for the glycosidic linkages composing LLOs and their relative abundance throughout MD trajectory.

	Frequency		Average($^{\circ}$)		Frequency		Average($^{\circ}$)
<i>Archea</i>	ϕ_1	1.00	99.77 ± 17.80	<i>Eukarya</i>	ϕ_1	1.00	113.21 ± 13.63
	ψ_1	0.88	172.43 ± 25.67			0.23	90.43 ± 9.85
		0.11	-84.07 ± 14.33		ψ_1	0.70	139.88 ± 12.41
	ϕ_2	0.91	-34.96 ± 19.99			0.07	-86.32 ± 13.57
		0.09	28.38 ± 9.93		ϕ_2	1.00	-37.80 ± 17.85
	ψ_2	1.00	119.64 ± 12.77		ψ_2	1.00	105.13 ± 10.16
	ϕ_3	1.00	-66.91 ± 14.20		ϕ_3	1.00	-85.92 ± 9.93
	ψ_3	1.00	-97.08 ± 13.73		ψ_3	1.00	94.30 ± 9.57
	ϕ_4	1.00	79.45 ± 15.26		ϕ_4	1.00	142.94 ± 12.57
	ψ_4	1.00	-131.40 ± 18.43		ψ_4	1.00	-178.73 ± 11.27
	ϕ_5	1.00	91.84 ± 14.10		ω_4	1.00	169.45 ± 9.83
	ψ_5	1.00	-98.68 ± 20.19		ϕ_5	1.00	138.98 ± 18.13
	ϕ_6	0.99	75.68 ± 12.57			0.92	-171.39 ± 19.19
	ψ_6	1.00	-149.85 ± 19.64		ψ_5	0.08	-102.05 ± 9.54
	ϕ_7	0.99	79.58 ± 12.76		ω_5	0.97	169.78 ± 11.42
	ψ_7	0.25	81.38 ± 13.72		ϕ_6	0.99	84.19 ± 18.58
		0.75	178.34 ± 19.11		ψ_6	1.00	-102.21 ± 19.96
	ω_7	1.00	88.28 ± 10.36		ϕ_7	1.00	92.11 ± 10.78
<i>Bacteria</i>	Frequency		Average($^{\circ}$)	<i>Eukarya</i>	ψ_7	1.00	-93.32 ± 10.98
	ϕ_1	0.58	109.78 ± 10.98		ϕ_8	0.98	71.05 ± 15.37
		0.42	143.13 ± 10.11			0.87	-139.40 ± 11.19
	ψ_1	0.68	-156.49 ± 11.02		ψ_8	0.13	-86.10 ± 10.83
		0.32	-111.59 ± 6.62		ϕ_9	1.00	97.31 ± 14.10
	ϕ_2	1.00	71.32 ± 7.51		ψ_9	1.00	-90.93 ± 17.47
	ψ_2	1.00	-141.53 ± 7.95		ϕ_{10}	0.52	76.63 ± 12.72
	ϕ_3	0.99	92.46 ± 8.37			0.48	123.15 ± 13.66
	ψ_3	0.99	-88.41 ± 10.04		ψ_{10}	0.40	-125.12 ± 13.73
	ϕ_4	1.00	91.91 ± 17.20			0.60	-73.58 ± 13.51
		0.26	-124.25 ± 7.45		ϕ_{11}	1.00	96.60 ± 14.56
	ψ_4	0.74	-90.60 ± 10.10		ψ_{11}	1.00	-86.29 ± 16.47
	ϕ_5	0.99	91.98 ± 9.40		ϕ_{12}	1.00	88.98 ± 12.42
	ψ_5	1.00	-92.17 ± 14.83			0.32	-129.95 ± 9.06
	ϕ_6	1.00	97.06 ± 17.30		ψ_{12}	0.68	-92.60 ± 13.29
		0.14	-129.59 ± 8.69		ϕ_{13}	1.00	76.13 ± 10.96
	ψ_6	0.85	-87.16 ± 11.31			0.91	-137.17 ± 10.47
					ψ_{13}	0.09	-95.70 ± 7.42
					ϕ_{14}	1.00	77.51 ± 8.88
					ψ_{14}	0.99	83.97 ± 11.24

populations for each LLO ensemble and submit them to molecular docking calculations (Experimental Section) in order to test if the obtained models were capable of binding to its corresponding OST counterpart (Figure 7).

For the bacterial LLO, the molecule mostly displayed a similar orientation as seen in the recent crystal structure (PDB ID 6GXC)²² of the full complex with an acceptor peptide and an inactive LLO, but with closer distances between the peptide acceptor and the LLO donor (3.5 Å vs 4.0 Å in the recent crystallographic structure). Main relevant distances reproduced on our model (apart from the restricted ones) includes: i) atom O7 from the N-acetylation in C2 and the OH group of Tyr468; ii) OH group from Tyr196 and the phosphate groups in LLO; iii) OH group from Ser198 and the phosphate groups. The undecaprenyl portion fitted into the previously identified hydrophobic groove, while the saccharidic moiety interacted along the cavity formed in the interface between TM domain and PP domain.

The archaeal LLO docking revealed an interesting feature for the WWDXGX motif, which in *A. fulgidus* AglB (PDB ID 5GMY)²³ is composed of WWDYGH. His555 plays the role of Tyr468 in the WWDYGY motif of *C. jejuni* PglB, performing electrostatic interactions between its imidazole side-chain ring and the sulfate group connected to the C2 of the reducing end monosaccharide from archaeal LLO (Glc1). This observations indicate that the WWDXGX motif is directly involved in the selectivity of the donor substrate of OSTs and the enzyme possibly evolved to better accommodate distinct glycan ligands. Another residue substitution occurs in the archaeal enzyme, where residue Tyr79 in *C. lari* becomes His81 in *A. fulgidus*. The position occupied by this amino acid, in close proximity to the sulfate group connected to C2 of the first monosaccharide, suggests that His81 (and Tyr79 in *C. lari*) acts as an additional interaction that ensures correct positioning of the LLO inside the enzyme cavity. Besides that, another residue was observed to interact with LLO phosphate groups, namely Trp215, performing a similar role seen in the enzymes from the other domains of life (Trp208 in eukarya and Tyr196 in Bacteria),^{24,25} explaining the decrease in the enzyme activity when it was mutated to alanine.¹¹ The remaining carbohydrate residues of the

A) PglB + Bacterial LLO

B) AglB + Archaeal LLO

C) Stt3 + Eukaryotic LLO

Figure 7: Docking poses obtained for each studied LLO inside the corresponding OSTs. A) Bacterial LLO complexed with PglB depicting interactions Bac-O7 and Tyr468-OH, Asn-ND2 and Bac-C1; B) Archaeal LLO complexed with AglB depicting interactions Glc1-S2 and His555-ND1, Glc1-S3 and Trp215-NE1, Asn-ND2 and Glc1-C1; C) Eukaryotic LLO complexed with STT3 subunit depicting interactions NAcGlc1-O7 and Tyr530-OH, Asn-ND2 and NAcGlc-C1. All phosphate groups interacted with the respective ion, when present in the crystallographic structure.

molecule are in contact with AglB domains interface, where the last two monosaccharide are located near the N-terminal region of EL5.

The eukaryotic LLO docking was performed in the single catalytic subunit (PDB ID 6FTI),⁹ achieving a pose that fitted adequately into the donor cavity, despite this structure being obtained without the coordinating ion at the catalytic site, which organizes the structure around this area. When comparing our final docking complex to the small LLO found in the cryo-EM structure of the mammalian OST complex, it is possible to observe that the full LLO obtained from our data was not able to reproduce the exact conformation found in the experimental data. This is probably due to the larger number of monosaccharide units and the distinct conformations sampled in our simulations. Despite that, alignment of the currently formed complex to the full OST complex did not generate any clashes with the other subunits. Based on our obtained model and on the three reports of the recently released structures of the full OST complex, we verified that the glycan portion of the LLO molecule must interact with EL5, while inducing the ordering of TMH9, and that EL5 must be in a flexible state, not defined by a specific secondary structure. We could not observe a predicted entry route, since the high fluctuations of the hydrophobic tail generated conformations that were not compatible for fitting of its whole structure within the OST transmembrane domain.

Conclusion

Understanding the conformations and dynamics from distinct LLOs could support a better comprehension of the complex formation with OSTs, and, consequently, provide insights to enhance the efficiency of OSTs glycosylation turnover, as well as allow the engineering of these enzymes aiming biotechnological applications. In this report, we characterized three LLOs, representatives for each domain of life, employing a series of molecular modelling techniques.

By properly parameterizing the isoprenoid unit (one of the building blocks of the LLOs

hydrophobic tail) and achieving good agreement with experimental data (error less than 10%), we were able to describe the highly flexible behavior of this portion of the molecule, demonstrating its distribution inside the membranes. Additionally, the glycan chains were also evaluated in respect of their motions and interactions, demonstrating the clear preference for a parallel position relative to the membrane bilayer, mostly in close contact to the phospholipids head groups, similarly as previous reports.¹²

Molecular docking trials accessed the complexes for each LLO inside the respective OSTs cavities, which respected cutoff distances expected from previously determined OSTs structures. By correlating recent crystallographic data from the bacterial OST (PglB)²⁵ with our results from the archaeal LLO docking model, we were able to elaborate an explanation for the change in amino acids in the WWDXXG motif. This observations highlights the substrate selectivity role of this motif, allowing the prediction of interactions between glycans and this region. Furthermore, we described a rationale for the amino acid substitutions seen in Archaea, and the sulfate group in C2 observed on this LLO. Besides that, all of our models sampled conformations that could properly fit inside the donor substrate binding site, further validating our findings. We hope this work contributes for future investigations regarding the glycosylation activity and the distinct carbohydrate moieties distributed among other species.

Experimental

Nomenclature and software

The IUPAC proposed recommendations for nomenclature and symbols were used. Regarding MD simulations, the GROMACS 5.0.7 simulation suite²⁶ was employed, along with the GROMOS53A6 force field²⁷ for the systems without the glycan chain and the GROMOS53A6GLYC force field^{28,29} for glycan chain, in which X is "2", "3", "4" or "6" and its ϕ and ψ angles are defined as shown below:

$$\phi_{(1 \rightarrow X)} = O5 - C1 - OX - CX \quad (2)$$

$$\psi_{(1 \rightarrow X)} = C1 - OX - CX - C(X - 1) \quad (3)$$

Finally, for (1→6) linkage, the ω is defined as shown below:

$$\omega = O6 - C6 - C5 - C4 \quad (4)$$

Generation of new torsional parameter for isoprenoid chain

The QM torsional profile for the dihedrals within the isoprenoid structure were obtained using Gaussian09.[?] The 2-Methylbut-1-ene molecule was used due to its similarity to the isoprenoid portion. QM calculations were carried out using the *scan* routine combined with a *tight* convergence criterion, at HF level with the 6-31G* basis set, obtaining the relative energy associated with the rotation of dihedral by increments of 30°. Analogue MM calculations were performed in GROMACS 5.0.7, using the force field parameter set 53A6, as described in Pol-Fachin *et al.* and Arantes *et al.*,^{20,28,29} and CHARMM36³⁰, for comparison purposes. The MM torsional profile was fitted to the QM profile in Rotational Profiler server,³¹ providing proper torsional parameters for MM calculations to yield a torsional profile similar to the QM. These new parameters were then implemented in the LLOs topologies for MD simulations.

Parametrization strategy and topology construction

In order to describe LLOs through molecular mechanics simulations, a set of lipids commonly found in membranes was selected to act as building blocks in this work. Our parametrization strategy was based on accurately reproducing experimental values for physical-chemical properties of liquids. Topologies were constructed for LLOs using the potentials for bond

stretching, bond-angle bending, improper dihedral deformation and proper dihedral, as well as van der Waals interactions terms retrieved directly from GROMOS53A6²⁷ set, while potentials for diphosphate linkages were retrieved from previous works.²⁹ Moreover, in order to maintain compatibility with the general GROMOS force field, partial charges for the sulfate group were selected from those already distributed within GROMOS53A6 for the phosphate group (Table S2). Glycan chains topologies were built using the potentials for bond stretching, bond-angle bending, improper dihedral deformation and van der Waals interactions terms were retrieved directly from GROMOS53A6GLYC force field.^{28,29} Such models had their glycosidic linkage geometries adjusted to the main conformational states for each linkage, based on their relative abundance in the isolated disaccharides in water, as previously described.^{21,32,33}

Liquid and gas-phase simulations for assessment of thermodynamic properties

MD simulations were carried out by using GROMACS 5.0.7 package and physical-chemical properties of organic liquids (density and enthalpy of vaporization) were used as target to validate topology parameters, as previous works of parametrization of small biomolecules^{15,16,20} and benchmark of force fields.¹⁷ The geraniol fragment was chosen due to the availability of experimental values of density and enthalpy of vaporization. In order to calculate such thermodynamic properties, liquid phase was induced by simulating 125 randomly placed molecules in a cubic box under 100 bar and, after, scaling the simulation box in X, Y and Z axis in order to obtain a simulation box of 1000 molecules in liquid phase. All simulations were carried out with the Berendsen pressure and temperature coupling algorithms³⁴, using $\tau_T = 0.2$ ps and $\tau_P = 0.5$ ps, following previous works of GROMOS parametrization strategies²⁷. Non-bonded interactions settings followed the recommendations of Gonçalves et al.³⁵ by using particle mesh Ewald (PME) method³⁶ and a cutoff of 1.2 Å to treat electrostatic interactions, while using a cutoff scheme to treat van der Waals interactions within 1.2 Å.

Additionally to the liquid-phase simulation of 10ns, a gas-phase simulation was carried out for 100 ns using *leap-frog* and langevin algorithm³⁷, respectively. The potential energies associated with these systems ($E_{pot}(g)$ for gas-phase and $E_{pot}(l)$ for liquid-phase) were extracted and used to calculate (Eq. 5) the enthalpy of vaporization (ΔH_{vap}) of the fragments.

$$\Delta H_{vap} = (E_{pot}(g) + k_B T) - E_{pot}(l) \quad (5)$$

Organic liquid densities (ρ) were calculated from liquid-phase simulations using block averages of 5 blocks, as for ΔH_{vap} .

Membrane insertion of LLOs

Initial LLOs structures were oriented along the Z axis, perpendicular to the membrane patch. A bilayer model constituted of 120 POPE lipids for bacterial and archaea systems and a bilayer constituted of 120 POPC lipids for eukaryotic, were obtained at the end of the protocol (Figure 8). In order to insert LLOs into their respective membrane patch, The InflateGRO methodology³⁸ was employed. Briefly, this consists in inserting LLOs into a pre-equilibrated bilayer patch, with lipids overlapping the LLOs, and then expanding the dimensions of the box, as well as, translating all lipids laterally, so that no more overlap is found. After that, a series of minimization steps are applied, along with the box dimensions being compressed and the lipids translated back to the center of the system, until the system reaches the desired density. During all this process, the LLOs are under strong position restraints, so that the structures are not affected.

Molecular Dynamics Simulations

Following the membrane insertion steps, the rectangular box was then solvated with SPC water model³⁹ and periodic boundary conditions. Before this process, we did the common procedure of raising the van der Waals radius of the C atom from 0.15 to 0.375, aiming to

Figure 8: Initial models of the eukaryotic, bacterial, and archaeal LLOs embedded in membrane bilayers. Water molecules are omitted in this representation.

avoid water molecules filling any left space between the lipids. After solvation, this parameter was returned back to its original value. Counter ions were added to neutralize the systems, when needed. Steepest Descent algorithm was used in the energy minimizations performed. First, two simulations of equilibration were performed with position restraints: an NVT and an NPT of 2 ns and 50 ns, respectively. Subsequently, one microsecond unrestrained NPT MD simulations were performed for each of the six systems, generating the production run where data were collected for the systems analysis. The LINCS algorithm⁴⁰ was chosen to constrain covalent bond lengths and an integration step of 2 fs was applied. Calculations of electrostatic interactions were performed by using the particle mesh Ewald (PME) method³⁶ and a cutoff of 1.2 Å, while a cutoff scheme and a cutoff of 1.2 Å was used for Lennard-Jones contributions³⁵. The pressure barostat chosen was Parrinello–Rahman,^{41,42} with a 2.0 ps coupling constant, while the temperature thermostats chosen were V-rescale (NVT step)⁴³ and Nosé–Hoover (NPT equilibration and production MD),^{44,45} with a coupling constant of $\tau = 0.5$. Additionally, semiisotropic pressure coupling was applied, accounting for the presence of the membrane. Constant temperature of 310 K (Eukarya and Bacteria), 353 K (Archaea) and constant pressure of 1 atm were also implemented.

Well-tempered metadynamics simulations

For the structural assessment of glycan chains tilt angle in respect to the membrane, well-tempered metadynamics calculations were performed. Following the membrane insertion steps, a rectangular box was used and periodic boundary conditions applied. The systems were submitted to energy minimization by Steepest Descent algorithm, followed by an equilibration phase of 20 ns and subsequently to well-tempered (WT) metadynamics simulations. A CV was chosen to obtain discrimination between the orientation states of the glycan chain. This CV computed the angle between two fixed virtual atoms, positioned at the three-dimensional space of the box with XYZ coordinates corresponding to [3.0,3.0,8.57] and [3.0,3.0,5.55], and the center of geometry from the last monosaccharide from each LLO. These coordinates for the fixed virtual atoms were selected so that when the glycan chain is fully perpendicular this value would be equal to 0 and when the glycan chain is fully parallel it would equals to around 90 degrees. A bias factor of 15 was employed to rescale gaussian functions. The initial height of the Gaussian hills was set as $1.0 \text{ kcal}\cdot\text{mol}^{-1}$, while the hill width was chosen to be 0.1 degrees. An upper limit was defined for the CV around 115 degrees to avoid unwanted membrane insertion. The Parrinello–Rahman barostat^{41,42} was employed, with a 2.0 ps coupling constant, to keep pressure constant at 1 atm. Temperature values were coupled by a V-rescale thermostat (NVT step),⁴³ using a constant of $\tau = 0.1$. Covalent bond lengths were constrained by the Lincs method,^{40,46} allowing an integration step of 2 fs. PME method³⁶ was applied to treat electrostatic interactions. GROMACS 2018⁴⁷ interfaced with the PLUMED plugin package 2.5b⁴⁸ was chosen to perform the enhanced sampling simulations. To generate the free energy surfaces of the tilt angle, the sum hills tool from the PLUMED package was employed. Block-analysis technique was utilized for error calculation and the reweighting procedures were applied similarly to a previous report.⁴⁹ The eukaryotic and the archael LLOs were simulated during 500 ns, at 310 K and 353K respectively. The bacterial LLO was simulated during 250ns, at 310K. Systems were simulated until the FES were considered qualitatively converged (hence, the different simu-

lation run times): this was verified by evaluating the average deviation between free-energy surfaces generated at every 10ns. FES profiles were considered converged when reaching an average deviation of 5 kJ/mol.

Molecular Dockings

Clusters of conformations that represent the most abundant states on LLOs ensembles were identified based on a previously applied methodology.²⁰ After identifying these clusters, we submitted the most prevalent structures to PatchDock molecular docking⁵⁰ server against their corresponding OST counterparts. Patchdock molecular docking algorithm uses shape complementarity principles with two main steps: i) determining the molecular shape representation to find possible binding geometric patches, and ii) matching of the surface patches between the two molecules. Additionally, we employed distance restraints to reduce the search space, similarly to a previous report.¹² For the eukaryotic LLO docking, restraints were employed between OST residue Tyr530 at atom OH to monosaccharide Gn1 at atom O7, with boundaries limited between 1.0 to 5.0 Å. For the archaeal LLO docking, pairs of restraints were added for more atoms: i) acceptor peptide residue Asn-ND2 and atom C1 of the LLO G1 (min. 1.0 Å and max. 4.0 Å); ii) Aglb residue Arg426-NH1 and NH2 and atom P76 of LLO (min. 1.0 Å and max. 6.0 Å); iii) Zn^{2+} catalytic ion to atom P76 of LLO (min. 1.0 Å and max. 5.0 Å). The bacterial LLO docking was performed by setting restraints in the distances between: i) acceptor peptide residue Asn-ND2 and C1 of the LLO B1 (min. 1.0 Å and max. 4.0 Å); ii) Mg^{2+} catalytic ion and atom P2 of LLO (min. 1.0 Å and max. 6.0 Å); iii) Both NH1 and NH2 of Arg375 and atom P2 of LLO (min. 1.0 Å and max. 6.0 Å). From this methodology, we have obtained at least one complex that respected the expected distances and was located around the expected donor cavity. For the manipulation and visualization of structures, the softwares VMD⁵¹ and PyMOL⁵² were employed.

Acknowledgement

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), MCT, Brasília, DF, Brazil; the Coordenacao de Aperfeicoamento de Pessoal de Nivel Superior (CAPES) - CAPES/Drug Discovery grant number 23038.007777/2014-87, MEC, Brasília, DF, Brazil. The authors acknowledge the National Laboratory for Scientific Computing (LNCC/MCTI, Brazil) for providing HPC resources of the SDumont supercomputer, which have contributed to the research results reported within this paper. URL: <http://sdumont.lncc.br>

Supporting Information Available

The following files are available free of charge.

- Supplementary material: Density profiles for the subdivided parts of the studied LLOs, without glycan chains. Distributions of the LLOs, without glycan chains, hydrophobic tail in lipid bilayer and schematic representations of the oligosaccharide chains of LLOs.

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