Deciphering multivalent glycocluster–lectin interactions through AFM characterization of the self-assembled nanostructures
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Deciphering multivalent glycocluster-lectin interactions through AFM characterization of the self-assembled nanostructures


Pseudomonas aeruginosa is a human opportunistic pathogen responsible for lung infections in cystic fibrosis patients. The emergence of resistant strains and its ability to form a biofilm seems to give a selective advantage to the bacterium and thus new therapeutic approaches are needed. To infect lung, the bacterium uses several virulence factors, like LecA lectins. These proteins are involved in bacterial adhesion thanks to their specific interaction with carbohydrates of the host epithelial cells. The tetrameric LecA lectin specifically binds galactose residues. A new therapeutic approach is based on the development of highly affine synthetic glycoclusters able to selectively link with LecA to interfere the natural carbohydrate-LecA interaction. In this study, we combined Atomic Force Microscopy imaging and Molecular Dynamics simulations to visualize and understand the arrangements formed by LecA and five different glycoclusters. Our glycoclusters are small scaffolds characterized by a core and four branches, which terminate by a galactose residue. Depending on the nature of the core and the branches, the glycocluster-lectin interaction can be modulated and the affinity increased. We show that glycocluster-LecA arrangements highly depend on the glycocluster architecture: the core influences the rigidity of the geometry and the directionality of the branches, whereas the nature of the branch determines the compactness of the structure and the ease of the binding.

Introduction

The human opportunistic pathogen Pseudomonas aeruginosa (PA) is a Gram-negative bacterium which causes chronic infections and degradation of the respiratory tract of cystic fibrosis and immune-compromised patients¹. This bacterium is resistant against conventional antibiotic therapies² due to the emergence of highly resistant strains³ and its ability to develop biofilms. The bacteria take advantage of the malfunctioning of the host defenses to use an arsenal of virulence factors which are involved in the adhesion, colonization and infection processes. As a consequence, great efforts have been devoted towards the development of new therapeutic approaches targeting PA virulence factors, including those involved in biofilm formation⁴. Among them, two lectins, LecA and LecB, were identified as potential therapeutic targets due to their role in cell recognition, biofilm formation and cohesion⁵. In particular, LecA (Fig. 1a) is a tetrameric lectin characterized by four identical units. Each of them presents a Carbohydrate Recognition Domain (CRDs) that allows the specific binding to one D-galactose residue (Gal). The binding between the CRD of the lectin and the residue is mediated by hydrogen bonds, hydrophobic contacts, and coordination with a calcium ion (Fig. 1a, purple spheres). The natural ligand of LecA has been identified as the globotriaosylceramide Gb3, which is highly present in human epithelial cells⁶. In order to inhibit the natural interaction between the lectin and the ligand of host cells⁶, it has been shown that certain galactosylated glycoclusters have a high selective affinity with LecA⁷,⁸ due to their multivalency⁹ and the so called “glycoside cluster effect”⁸,¹⁰. Their inhibition effect on the activity of lectin-induced PA lung infections has been demonstrated in animal model and represents a promising therapeutic strategy against such bacterial infections¹¹. To follow this therapeutic approach, our collaborative group has synthesized more than 150 different glycoclusters⁶, ⁸, ¹²-¹⁸. Different strategies to build these scaffolds were explored but most of them present a core with multiple branches terminated by a Gal residue capable of interacting with LecA’s CRD. The design of the core and the branches are key parameters for achieving optimal topologies toward a maximal cluster effect¹³,¹⁴,¹⁹.
In the present study, Atomic Force Microscopy (AFM) was chosen to observe at the nanoscale the arrangement formed by five different glycoclusters when bound to LecA. In addition, Nanosecond Molecular Dynamics (MD) Simulations were performed to better understand these arrangements. The data were analyzed taking into account the values of the dissociation constants ($K_d$) and the stoichiometry numbers ($n$) obtained by Isothermal Titration Calorimetry (ITC), in order to find a link between the morphology of the structures, the nature of the glycoclusters (core and branch) and the affinity to LecA.

<table>
<thead>
<tr>
<th>Name</th>
<th>Core</th>
<th>Branches</th>
<th>Termination</th>
<th>$n$</th>
<th>$K_d$ (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Calix[4]-arene</td>
<td>OMTz</td>
<td>EG$_3$</td>
<td>Galactose</td>
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<tr>
<td>P1</td>
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<td>EG$_3$</td>
<td>Galactose</td>
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<td>EG$_3$</td>
<td>Galactose</td>
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<tr>
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<td>POProTz</td>
<td>AcNPh</td>
<td>Galactose</td>
<td>0.46</td>
</tr>
<tr>
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<td>Mannose</td>
<td>POEG2MTz</td>
<td>AcNPh</td>
<td>Galactose</td>
<td>0.52</td>
</tr>
<tr>
<td>M4</td>
<td>Mannose</td>
<td>POEG2MTz</td>
<td>AcNPh</td>
<td>Glucose</td>
<td>No Interaction</td>
</tr>
</tbody>
</table>

**Table 1: Structural composition of glycoclusters under study. Stoichiometry values $n$ and dissociation constants $K_d$ were determined by ITC to estimate the affinity with LecA. Pro = CH$_2$CH$_2$CH$_2$; Tz = triazole C$_3$H$_7$N$_3$; EG$_3$ = (CH$_2$CH$_2$O)$_3$; Ac = COCH$_3$; M = CH$_2$; Ph = phenyl, PO = phosphodiester P(=O)(O-)O.**

**Experimental**

**Glycoclusters**

The six glycoclusters used in this study are presented in Table 1 and their final structures are shown in the Supporting Information. Glycoclusters synthesis was extensively described in Reference, as well as the anti-adhesive properties of C1 and M3 on in vitro biofilm assays. They are characterized by a core, four branches formed with two different linkers. The branches are ended by one monosaccharide residue.

**AFM Imaging**

As previously described, the LecA-glycocluster complexes were prepared in vitro by mixing 20 µL of CaCl$_2$ (final concentration 0.3 µmol/L), 10 µL of LecA (final concentration 25 pmol/L) and 10 µL of glycocluster (final concentration 25 pmol/L). The complex was prepared with the same proportion of lectin and glycocluster (1:1) to favor the binding of one galactose residue to each CRD of the lectins. It was shown by ITC measurements that the complex forms in solution in the early stage of the titration process and this low concentration of 25 pmol/L was chosen to avoid the creation of large aggregates. Then, the solution was incubated 1 hour at room temperature. Finally, 20 µL of the final solution were deposited on freshly cleaved mica surface and dried overnight in a desiccator at room temperature and ambient pressure. Topographic images were acquired in air and at room temperature, using a SMENA B (NT-MDT, Russia) AFM microscope in Amplitude Modulation (AM) AFM mode with triangular cantilevers (NSC 21 from MikroMasch, Bulgaria) having a tip radius of 10 nm. The data analysis was performed with Gwyddion Software. By adjusting amplitude ratio, it is possible to image these soft molecules without damaging them. Measurements were performed in air for two main reasons: (i) the adsorption of the lectin on mica may not be strong enough to allow to image in liquid, and (ii) to ensure the highest resolution conditions to correlate the morphology as observed by AFM and the simulation results. The only artefact derived from this choice is the detection of a smaller height of aggregates, as previously described by Sicard et al.

**Computer Simulations**

Simulations were carried out by combining Monte Carlo method with SPASIBA (Spectroscopic Potential Algorithm for Simulating Bimolecular conformational Adaptability) force field that is characterized by the combination of AMBER Van der Waals, electrostatic interactions and Urey-Bradley-Shimanouchi terms for bond stretching, valence angle bending and torsional parameters. Monte Carlo simulations were run to minimize the energy of the complex and find the most probable conformations. The protein structures were considered as aggregates and the final assembly was optimized with all atoms released by applying the SPASIBA force field by means of molecular docking. The interaction potential energies ($\Delta E$) of the possible arrangements were calculated by the difference between the energy of the complex ($E_{\text{complex}}$) and the energies of both the protein ($E_{\text{protein}}$) and the ligand ($E_{\text{ligand}}$) independently, as shown in equation (1):

$$\Delta E = E_{\text{complex}} - (E_{\text{protein}} + E_{\text{ligand}})$$

$\Delta E$ values were used to evaluate the more energetically stable structures. The LecA lectin structure file was retrieved from the RCSB Protein Data Bank website (PDB code 4LJH, since it shows...
the Gal molecules in the CDRs of the lectin). Molecular graphics and analysis were performed using the Discovery Studio Visualizer 4.0 Software (Accelrys, San Diego, CA, USA).

Results and discussion

LecA-glycocluster affinity

LecA lectin has a cobblestone shape of 7.0 x 3.2 x 1.9 nm³ with four identical CDRs which recognize specifically galactose residue, as shown in Fig. 1a. In order to understand the relationship between the glyocluster structural composition, the LecA-glycocluster affinity and the LecA-glycocluster complexes arrangement, five tetravalent glyoclusters, called as C1, P1, M1, M2 and M3 (Table 1), were selected for their affinity with the lectin20, 21. These synthesized molecules are composed by a core, two linkers, named here linker 1 and linker 2, forming the branches, and to finish a monosaccharide residue, in this case one galactose to interact with LecA (schematic drawing in Fig. 1b). On one side, three of them, C1, P1 and M1, present different cores but similar branches composed by two linkers: oxymethylenetriazole (OMTz) or phosphodiester propyl-triazole chain (PPOProTz) as linker 1 and a hydrophilic triethyleneglycol (EG3) as linker 2. The difference in length of the alkyl chain between PPOPro and OMTz compensates the difference in core size: a calix[4]arene for C1, a porphyrin for P1 and a mannose for M120, 24. On the other side, M1, M2 and M3 have the same mannose core but different branches. In M2, linker 1 is the same as in M1 (PPOProTz) and linker 2 is the same as in M3: a rigid aromatic acetamidophenyl (AcNPh), while, the linker 1 in M3 is a flexible phosphodiester diethyleneglycol methylene triazole (POEGMTz)21. Additionally, M4 presents the same core and branches than M3 glyocluster but exhibits glucose as terminal carbohydrate residue. It was used as a negative control since glucose does not interact with LecA.

Thermodynamic parameters including the stoichiometry value (n) and the dissociation constant (Kd) of the interaction with the lectin have been obtained by ITC 20,21,29 and presented in Table 1. The stoichiometry value indicates the number of binding between one glyocluster and the monomers of the lectin. Depending on the glyocluster structure, n values are close to 0.5 or 0.25. This indicates that one glyocluster can bind up to two lectin monomers for n = 0.5 (1:2), or to four monomers for n = 0.25 (1:4), without excluding the less probable intermediate case n = 0.33 (1:3, interaction with three monomers). For the five glyoclusters here studied, the n values were previously estimated at: n = 0.24 for C1, n = 0.28 for M1, n = 0.46 for P1 and M2 and n = 0.52 for M3, leading to different possible topologies when interacting with LecA. The dissociation constant Kd refers to the affinity of the lectin-glycocluster interaction: the lower the Kd the stronger the interaction. In this case, the dissociation constants were determined at: Kd = 170 nmol/L for C1-LecA, Kd = 194 nmol/L for M2-LecA and Kd = 157 nmol/L for M3-LecA. The Kd increases to Kd = 330 nmol/L for the P1-LecA and reaches the highest value of Kd = 11000 nmol/L for M1-LecA (Table 1 and 20, 21).

Effect of the core on nanostructures

Fig. 2 presents three AFM images of the complexes C1-LecA, P1-LecA and M1-LecA, respectively, created in solution and deposited on a mica surface. These three glyoclusters have roughly the same branch, a phosphodiester propyltriazole (PPOProTz) or a oxymethylenetriazole (OMTz) as linker 1 and the same flexible triethyleneglycol (EG3) arm as linker 2. As the images clearly show, the change in the nature of the core of the three glyoclusters leads to different arrangements formed with LecA:

- A mostly mono-dimensional arrangement with the calix[4]arene-centered glycocluster C1, where alternate lectins and C1 glyoclusters are observed in single filaments. In a previous work, we showed that lectins can be identified along the filament on high resolution images22.
- A smaller curved structure with larger holes is obtained with the porphyrin core glycocluster P1, in which the width of the sinusoidal branches vary from
40 nm to 125 nm suggesting that one to five lectins can be involved, taking into account the tip convolution. A large 2D organized lacy structure is found in presence of the mannose-centered glycocluster M1, where sometimes holes can be identified in the arrangement.

The average height of the structures is around $1.4 \pm 0.2$ nm on all the images. This value is slightly lower than the width of a single lectin measured by crystallography (see Fig. 1a), and previously evidenced by AFM. It seems that the lectins are lying flat on the mica surface, creating 1D or 2D arrangements. The lowering of the size can be attributed to the sample preparation protocol (dried overnight in dessicator) and the adsorption of the protein on the mica surface.

To understand the topological arrangement of the obtained complexes, MD simulations have been performed taking into account the stoichiometry $n$ values (Table 1), which differs from one glycocluster to another. The molecular models, corresponding to the four more energetically stable structures obtained with one glycocluster and LecA, are presented in Fig. 2 for the three complexes C1-LecA, P1-LecA and M1-LecA.

In particular, for the C1-LecA complex, the more energetically stable structures are obtained with one glycocluster bound to two lectins. This binding leads to two equally favorable possible configurations. The former presents an energy of $\Delta E = -328$ kcal/mol and the lectins are maintained in a $90^\circ$ orientation one to the other since the four branches of the glycocluster are alternated pointing upwards and downwards around the core structure, resulting in perpendicular planes. In the latter, with a potential energy $\Delta E = -318$ kcal/mol, the two lectins are maintained in a $0^\circ$ orientation by the glycocluster, leading to a filament structure where the two lectins are bound to the glycocluster from the same edge. These configurations are respectively 88 kcal/mol and 78 kcal/mol below the value of the third one ($\Delta E = -240$ kcal/mol), where the glycocluster binds three CRDs from three different lectins, leading to a “Y trimer” arrangement of the lectins on the plane. This organization and the one that involves the binding of one glycocluster with four different lectins (“X tetramer”, $\Delta E = -232$ kcal/mol) are less favorable. They allow the formation of defects in the structure, such as the bifurcations seen in the AFM images. Therefore, we can evidence that, for C1 glycocluster, the stiffness of the core reinforces the rigidity of the complex while the branches alignment leads to the creation of a 1D filament structure.

Figure 2: AFM topographic images and MD simulations of C1-LecA, P1-LecA and M1-LecA complexes. Image size: 2 x 2 μm².
For the P1-LecA complex, the stoichiometry is 0.46 close to a 1 glycocluster for 2 CRDs. The four more probable models are characterized by potential energies spanning from $\Delta E = -167$ kcal/mol to $\Delta E = -114$ kcal/mol. This leads to a general shape of the arrangements less constrained, with rounding filaments connected at some places in 2D structures. The porphyrin core is less rigid than the calix[4]arene and the branches present more degrees of freedom. We can consider that in the solution before deposition, the branches can orient themselves more freely with respect of the CRDs of the lectins. The spreading of the structure seems to be limited by the fourth most probable model (“Monomer”, $\Delta E = -114$ kcal/mol, Fig. 2), where two galactoses of the same glycocluster bind two CRDs of the same lectin.

For the M1-LecA complex, the models correspond to the interaction between four galactoses of the glycocluster and two, three or four lectins (including one or two CRDs per lectin), leading to dimer, trimer and tetramer dispositions, respectively. A mixture of these organizations seems to be responsible of the final architecture of the complex. The structures extend laterally over several hundreds of nanometers forming a compact arrangement with only some holes of different size, being the biggest arrangement of around $300 \times 300$ nm$^2$. This means that lectins are close together and might also interact with each other$^{23}$. The geometry is also favored by the flexibility of the mannose core, which authorizes a great degree of freedom to the branches in solution.

**Effect of the branches on nanostructures**

To study the influence of the branches on the complex arrangements, three glycoclusters with the same mannose core but different branches were imaged by AFM (Fig. 3). The branches differ from each other by the nature of the linker 1 and/or linker 2 (Table 1). Also in this case, different nanostructures were observed by AFM, as shown in Fig. 3. The first glycocluster M1 has been previously discussed. M2 and M3 have a higher affinity to LecA than M1 and similarly to C1- and P1-LecA affinity. Their interactions with LecA lead to the formation of smaller and more compact structures. For M2-LecA complex, the small structures are sometimes elongated with well-defined borders characterized by rectangular kinks that would remind the shape of the lectin. Instead, in the case
of M3-LecA complex, the structures are quite small with a size of 70 to 100 nm. These arrangements present also a huge heterogeneity about the structure height that ranges from 1.4 to 25 nm, meaning that the lectins are stacked one over the other at some places (data not shown).

The potential energies of stacked arrangements have been calculated for the three mannose-centered complexes. A value of $\Delta E = -229$ kcal/mol was obtained for M1-LecA, when two stacked lectins are linked to one glycocluster. Even if this energy value is not so far from the one obtained for plane-arrangements, AFM images and the height of M1-LecA complexes arrangement did not evidence such stacked structures. Only small brighter spots on the large 2D monolayer structures appear at some places. In the case of M2 glycocluster, the most energetically favorable arrangements are characterized by the interaction between two galactoses of the glycocluster and two CRDs of two or one lectins, leading to dimer and monomer dispositions respectively. The monomer arrangement can explain why the structures obtained with AFM are so small. We can assume that this monomer configuration blocks the extension of the structure in the plane, leading to small complexes. The stacked arrangement is at 97 kcal/mol from the monomer model, which does not really favor 3D structures formation compared to 2D compact arrangements. The M3 glycocluster leads roughly to the same behavior as observed with M2. Thus, the most energetically favorable arrangements are characterized by the interaction between two galactoses of the glycocluster and two CRDs of one or two different lectins. The monomer configuration is only at 15 kcal/mol from the most energetically favorable model, explaining the small lateral size of the structures. In addition, the stacked configuration is only at 27 kcal/mol far from the most energetically favorable arrangement, thus it can well explain why some 3D complexes were found. Moreover, these two glycoclusters present roughly the same stoichiometry value ($n = 0.52$ for M2 and $n = 0.46$ for M3) and similar low $K_d$, 194 nmol/L and 157 nmol/L for M2-LecA and M3-LecA, respectively. Once again, it appears that the structures are limited in lateral size for small $K_d$ with a high affinity between LecA and the glycoclusters.

**Influence of lectin/lectin interaction**

Due to the drying process, stacked structures appeared and therefore, we cannot exclude the presence of a lectin-lectin interaction. Consequently, one issue arises: how can the lectin-lectin interaction influence the complex formation? To answer this question, the complex formed between the lectin and M4 glycocluster (Table 1) was studied. The M4 glycocluster has the same structure than M3 but instead of having galactose residues, it presents glucose that does not interact with LecA. Cause of the lack of affinity with the lectin, the M4-LecA complexes should be mostly driven by the lectin-lectin interactions. Extended 2D monolayer complexes similar to the ones obtained with M1 were observed by AFM (Fig. 4). Thus, the interplay between the glycocluster-lectin and the lectin-lectin interactions must govern the formation of these complexes. MD simulations of lectin-lectin interactions shows that the scheme with a direct edge-contact between lectins leading to an $\Delta E = -98$ kcal/mol is not negligible and can explain the lateral extension of the structures.

This study contributes to new findings to understand the relation between lectin-glycocluster interaction and the structure of these formed complexes. Hence, five glycoclusters were selected to study the arrangement of the glycoclusters-mediated self-assembly with LecA, since they have different topologies and/or their interaction with LecA exhibited different stoichiometry as determined by ITC. Therefore, we do expect their interaction with LecA to be govern by different mechanisms that could be deciphered by characterizing the resulting nanostructures. In particular, the effects of the core and the branches on the nanostructures have been studied by comparing the arrangement formed with the different glycoclusters. Mainly, the comparison of the C1-LecA, P1-LecA and M1-LecA complexes can show the influence of the core on the complex arrangement, since C1, P1 and M1 have the same branches but different cores. Whereas, the comparison of M1-LecA, M2-LecA and M3-LecA nanostructures can elucidate the influence of the branch as M1, M2 and M3 are mannose-centered glycoclusters with different branches.
Conclusions

In conclusion, these AFM studies combined to MD simulations revealed that the core rigidity and the nature of the linker 2 influence drastically the morphology of the glyocluster-lectin complexes. A relation between the affinity of glyocluster-LecA interaction and the morphology at the nanoscale of the created complexes could be established. A high affinity (K_d around 100 to 200 nmol/L) leads to small structures, compact for M2 and M3, sometimes 3D or limited to the size of the lectin as for C1. When the affinity decreases, the structures extend laterally to large 2D monolayers, observed for M1 and M4. In that case, the lectin-lectin interaction should be taken into account in the process of complex formation. For M1, it competes with the glyocluster-lectin interaction. P1 glyocluster, with an affinity of K_d = 330 nM, presents very specific morphology in between the one obtained for C1 and M1. In ongoing projects, M3 glyocluster was chosen for AFM spectroscopic measurements at the molecular level to quantify the lectin-M3 interaction, and also at the cell level to evaluate its anti-adhesive effect on the adhesion of Pseudomonas aeruginosa bacteria to host cells. As a future perspective, to better understand the lectin-glyocluster arrangements, the correlation between nanometric structural description and local chemical information is foreseen by means of AFM-IR.

Conflicts of interest

There are no conflicts to declare.

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Notes and references


