

Membrane Deformation by Neoelectins with Engineered Glycolipid Binding Sites**

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Abstract: Lectins are glycan-binding proteins that are involved in the recognition of glycoconjugates at the cell surface. When binding to glycolipids, multivalent lectins can affect their distribution and alter membrane shapes. Neoelectins have now been designed with controlled number and position of binding sites to decipher the role of multivalency on avidity to a glycosylated surface and on membrane dynamics of glycolipids. A monomeric hexavalent neoelectin has been first engineered from a trimeric hexavalent bacterial lectin, From this neoelectin template, 13 different neoelectins with a valency ranging from 0 to 6 were designed, produced, and analyzed for their ability to bind fucose in solution, to attach to a glycosylated surface and to invaginate glycolipid-containing giant liposomes. Whereas the avidity only depends on the presence of at least two binding sites, the ability to bend and invaginate membranes critically depends on the distance between two adjacent binding sites.

By deciphering the glycode, lectins play crucial roles in many biological or pathological processes.^[1] Whereas individual lectin binding sites have rather low affinity for glycans, multivalency results in high avidity for carbohydrates presented in several copies on glycoconjugates at the cell

surface.^[2] Moreover, multivalency also triggers the clustering of glycoproteins or glycolipids on cell surfaces.^[3] Shiga and cholera toxin B-subunits as well as capsid lectins from SV40 and norovirus have been demonstrated to cluster glycosphingolipids, resulting in negative membrane curvature and formation of membrane invaginations.^[4]

Multivalency of carbohydrates has been extensively studied, but not that of lectins, mainly because of the difficulty to control the number and location of binding sites in oligomeric proteins. An alternative approach is to use lectins presenting internal repeats such as β -proteins that are built on the cyclic repetition of 5 to 7 β -sheets.^[5] The bacterial lectin, RSL from *Ralstonia solanacearum*, is an ancestor of this fold, as it consists of a tandem repeat of two β -sheets that trimerizes as a six-bladed β -propeller (Figure 1 a,b).^[6] The resulting hexavalent trimer has the capacity to invaginate lipid membranes containing fucosylated glycolipids.^[7] Reducing the valency from six to three resulted in a trivalent lectin that lost the capacity to induce membrane invaginations in giant liposomes.^[7] However, the influence of topology, that is, repartition of binding sites in space and the distance between them, has not been investigated.

We therefore propose herein a novel approach for controlling valency. The “neoelectin” concept consists in the

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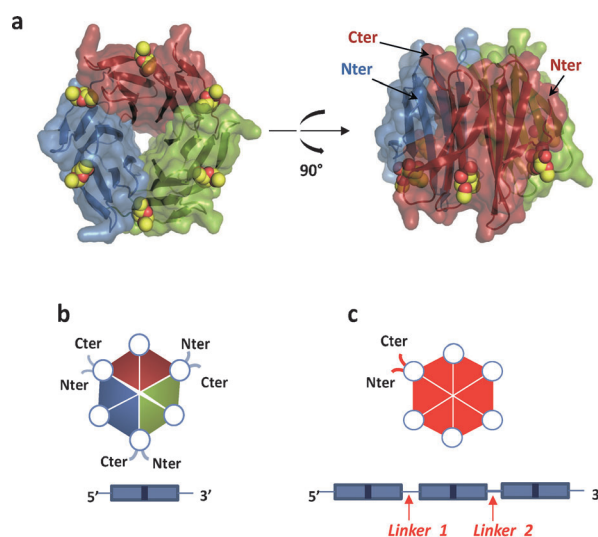


Figure 1. a) Overall structure of wt-RSL complexed with α MeFuc displayed as spheres (PDB code: 2BT9). Each monomer is represented by a different color. b) Representation of protein RSL and gene encoding RSL. The fucose binding sites are represented by white circle. c) Representation of protein neoRSL_VI and gene encoding neoRSL_VI.

de novo creation of a monomeric hexavalent lectin adopting the β -propeller architecture. Engineering was performed at the genomic level by linking three divalent RSL monomers with a SSTVPGD peptide linker (Figure 1c). Differences introduced in gene design insure that all sites can be individually mutated in order to inactivate them, and allowed thereafter to control the number and position of the active binding sites. First, the monomeric neolectin with six active binding sites (referred to as neoRSL_VI) was designed and led to a 273 amino acids protein, with six blades and two engineered linkers (Figure 1; Supporting Information, Figure S1 and Table S1). The resulting recombinant protein has the expected MW of 30 kDa and a denaturation temperature of 66° (Table 1; Supporting Information, and Figure S2) that is stabilized in the presence of fucose (T_m 88°C), which is indicative of correct refolding with conserved ligand binding properties.

Table 1: Comparison of wild-type RSL and neoRSL_VI.^[a]

	wt-RSL	NeoRSL_VI
molecular weight	9.86	29.45
oligomerization state	trimer	monomer
thermal stability (TSA):		
T_m apo protein (°C)	86 ^[a]	66
T_m with fucose (°C)	96 ^[a]	88
affinity for fucose-chip (SPR):		
$K_{d,affinity}$ (μ M)	0.7	0.9
$K_{d,avidity}$ (nM)	9	5
affinity for α MeFuc (ITC):		
n (stoichiometry)	1.84 $\pm$ 0.01	5.65 $\pm$ 0.02
K_d (μ M)	1.23 $\pm$ 0.06	1.04 $\pm$ 0.06
ΔH (kJ mol ⁻¹)	-47.9 $\pm$ 0.2	-45.5 $\pm$ 0.2

[a] From Ref. [7].

NeoRSL_VI was tested for its ability to bind to carbohydrates in solution and to glycosylated surfaces. Using ITC, titrating neoRSL_VI with the monosaccharide ligand α -methyl fucoside (α MeFuc) (Figure 2b; Supporting Information, Figure S3 and Table S2) confirmed that all six binding sites of the neolectin were active ($n=5.6$). The affinity for α MeFuc was in the micromolar range ($K_d=1.04 \mu$ M), with no significant difference to wt-RSL ($K_d=1.23 \mu$ M). Surface plasmon resonance data were measured using a surface of polyacrylamide (PAA)-decorated with α -fucose residues (Figure 2c; Supporting Information, Figure S4 and Table S3) with different concentrations of lectins. Because of multivalent attachment, the binding curves were analyzed as a function of concentration (Supporting Information, Figure S4) using a two sites model that separates the affinity value at each site ($K_{d,aff}$) and the bulk avidity for the surface ($K_{d,av}$).^[8] NeoRSL_VI has single site affinity in the micromolar range ($K_{d,aff}=0.9 \mu$ M), which is in excellent agreement with ITC data above, and a strong avidity for the glycosylated surface in nanomolar range ($K_{d,av}=5$ nM). This 180-fold increase between the affinity for a single fucose and the avidity for multivalent fucose surface is the result of the clustering effect and is in the same range than previously

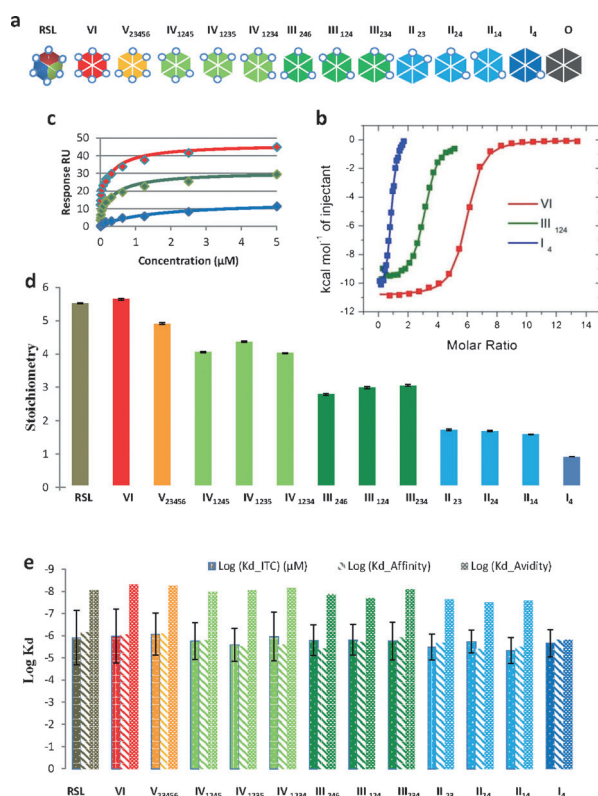


Figure 2. a) Representation of different neoRSLs with variation of the number of binding sites and position. Each active binding site is represented by a white circle. The proteins with the same valency share the same color. Naming of lectins is based on a roman number for the valency, and arabic numbers in subscript for the numbering of the active sites. b) ITC measurement of integrated heat of binding when titrating neoRSL_VI, III₁₂₄, and I₄ (0.03 mM) with α MeFuc (1.5 mM, 0.75 mM, and 0.25 mM, respectively) at 25°C in 20 mM Tris pH 7.5 with 0.1 M NaCl. c) SPR measurement with steady state analysis of responses at the end of association phase for the binding of three neoRSLs (VI: red; III₁₂₄: green and I₄: blue) to a fucosylated-PAA chips. d) Stoichiometry of neoRSLs determined by ITC. e) Affinity determined by ITC and SPR and avidity determined by SPR with the two-site binding model.

obtained for wt-RSL ($K_{d,aff}=0.7 \mu$ M and $K_{d,av}=9$ nM)^[7] confirming the integrity of neoRSL.

NeoRSL_VI has been cocrystallized with α MeFuc and the resulting crystal diffracted to 1.35 Å resolution (Supporting Information, Table S4). NeoRSL_VI folded as the expected six-bladed β -propeller with six occupied fucose binding sites, indicating that the engineering approach has been successful (Figure 3a). Electron density of one of the linker engineered between blades 4 and 5 could be clearly identified (Figure 3b) and it adopted roughly the same shape as the natural linker (Supporting Information, Figure S5). The second linker is more flexible, but superimposition demonstrates that the engineered linker connects two β -strands while maintaining the position they adopt in the wild-type protein.

Using the differences in gene sequence introduced in the binding site, it was possible to mutate each of them independently. Taking into account the circular symmetry of the protein, 13 different architectures can be produced,

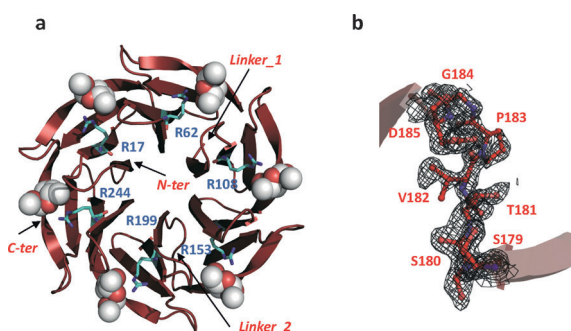


Figure 3. a) Overall structure of the neoRSL VI complexed with α MeFuc displayed as spheres. The arginine residues that are mutated in the neoRSL valency mutants are displayed by sticks. b) 2mFo-DFc electron density map for the engineered linker between blades 4 and 5 contoured at 1σ .

varying from valency zero to six, and presenting all possible arrangements of binding sites in space. Individual sites were inactivated by mutating the Arg residue previously demonstrated as crucial:^[6,7] R17A, R62A, R108A, R153A, R199A, and R244A, for site 1 to 6, respectively. Double to sextuple Arg to Ala mutants were also prepared to obtain the 13 neolectins (Figure 2a). For nomenclature, NeoRSL_II₂₄ is a divalent lectin with site 2 and 4 active, and site 1, 3, 5 and 6 inactivated by Arg to Ala mutation (Supporting Information, Figure S2). Denaturation temperature of neolectins varied from 66 °C for neoRSL_VI to 50.5 °C for neoRSL_II₂₄, and stabilization by bound fucose ligand has been always observed.

The stoichiometry of all purified neolectins was verified by ITC (Figure 2; Supporting Information, Figure S3). Affinity for α MeFuc in solution displayed limited variations, with K_d values between 0.85 μ M for neoRSL_V₁₂₄₅₆ and 4.67 μ M for neoRSL_II₁₄ (Supporting Information, Table S2). All neolectins with valency ranging from 1 to 6 maintained the same high affinity towards fucose in solution and no correlation could be observed as a function of the number or the location of the active binding sites. As expected, the neolectin with all sites inactivated by the Arg to Ala mutation, neoRSL_0, does not bind fucose efficiently anymore. However, a weak residual binding activity ($K_d = 4$ mM) was observed, at the limit of the detection method. Since residual affinity is 2000 weaker than the normal one, it does not interfere with the characterization of the other mutants.

From SPR experiments (Figure 2; Supporting Information, Figure S3 and Table S3), the local affinity for fucose, $K_{d,aff}$, did not present significant variations as a function of valency with values ranging from 0.8 μ M for neoRSL_V₁₂₄₅₆ to 4 μ M for neoRSL_II₁₄, in excellent agreement with those determined by ITC (Figure 2e). The avidity constant, $K_{d,av}$, displayed more significant variations, with an almost linear decrease from 5 nM to 30 nM when the valency is varied from 6 to 2. Surprisingly, the variation was only rather limited. Only the monovalent receptor, that is, neoRSL_I, displayed a sharp drop in binding with a micromolar affinity ($K_d = 13.4$ μ M), corresponding to the loss of any avidity effect.

Neolectins with controlled valency and topology were then assayed in a synthetic membranous environment in order

to test their potential to induce membrane invaginations. For this, the fucosylated glycolipid DOPE-Lewis a (DOPE-Le^a) was reconstituted in giant unilamellar vesicles (GUVs) with a typical diameter between 10 and 30 μ m (Supporting Information, Figure S6). As demonstrated previously, wt-RSL bound to Lewis a epitope (100 %) on GUV surface and induced membrane invaginations in 93 % of the cases ($n = 280$).^[7] NeoRSL_VI displayed the same properties with formation of invaginations in 90 % of liposomes ($n = 100$) (Figure 4; Supporting Information, Figure S6). When the valency is decreased to 5 or 4 (Supporting Information,

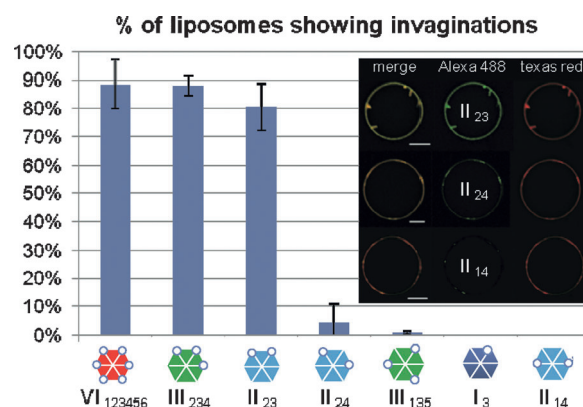


Figure 4. Percentage of liposomes showing membrane invaginations in presence of different neoRSLs. Confocal sections of the equatorial plane of GUVs containing glycolipids in presence of Alexa488-labeled proteins are displayed as insert. Proteins are shown in green and the membrane marker (DOPE-TR) in red. Scale bars: 5 μ m.

Figure S6 and Table S5), the neolectins still bound efficiently to Le^a epitope and induced invaginations. For trivalent RSL, different results were observed. NeoRSL_III₂₄₆ only induced invaginations in 1 % of studied liposomes, as previously observed with trivalent RSL mutant R17A.^[7] However, other trivalent neolectins such as neoRSL_III₂₃₄ and neoRSL_III₁₂₄ with at least two neighboring binding sites were still able to induce invaginations in 88 % and 70 % of liposomes, respectively. The topology of binding sites therefore appears to have a crucial effect on glycolipid clustering and dynamics within membranes. Analyzing divalent neolectins with two binding sites confirmed this observation (Figure 4). Among the three possible topologies, only neoRSL_II₂₃ was able to induce membrane invaginations (80 %) whereas neoRSL_II₂₄ (4 %) and neoRSL_II₁₄ (0 %) were not at all efficient. To sum up, all Alexa488-labeled neolectins bound efficiently to GUVs, with the exception of neoRSL_0, which has only residual affinity for fucose, but only the ones that present adjacent binding sites were able to induce invaginations.

The process by which membrane glycosphingolipids trigger the internalization of bacterial and plant toxins and viruses through glycan binding has been the subject of few studies. Several pathways have been described and relevant biological functions were proposed to relate to cell internalization through vesicles formation.^[9] The origin of the energy required for this physical process is not clear but has been proposed to arise from line tension generated by the

formation of lipid domains.^[10] In the present study such mechanisms would imply that upon DOPE-Le^a binding, the neoelectins cluster on the membrane surface, triggering the formation of lipid domains. This would be in agreement with the patches observed on liposomes when lectin binds. We demonstrated herein that hexavalent to trivalent and even divalent lectins are able to cluster glycolipids and alter membrane shape, providing that the binding sites are close enough (Figure 5). The hypothesis that close proximity of

shed light on the evolution of these proteins, resulting from oligomerization or duplication as tandem repeat.^[11] Finally, these considerations would be of high importance for engineering cargo proteins designed for importing drugs through the binding to glycosphingolipids, as already pioneered for Shiga toxins.^[12]

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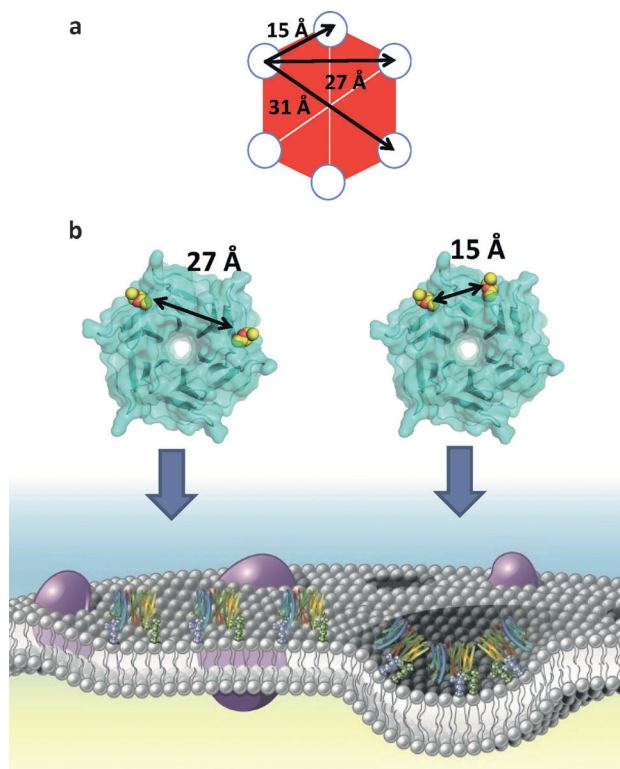


Figure 5. a) Representation of neoRSL_VI with distances between fucose binding sites. b) Representation of the effect of the distance between two binding sites on the plasma membrane. When the distance between two binding sites exceeds 27 Å, only lectin binding occurs. However, when only 15 Å separates two binding sites the neoelectins cluster glycolipids and bend the membrane.

carbohydrate binding sites is a prerequisite for membrane curvature by bringing lipids close to each other is in agreement with the topologies of RSL (distance of 15 Å). However the minimum distance for bending membrane may differ with the shape and curvature of the binding protein, as exemplified with viral capsids.^[4a,d]

Our work has demonstrated that the capacity of multivalent lectins to induce membrane invagination is not directly correlated with avidity to the glycosylated surface. This may

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