

# A Conformationally Unambiguous Spin Label for Exploring the Binding Site Topology of Multivalent Systems

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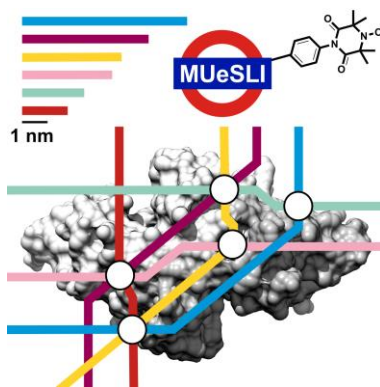
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**ABSTRACT.** Multivalent carbohydrate-lectin interactions are a key concept in biological processes mediating, e.g., signaling and adhesion. Binding affinities of multivalent ligands often increase by orders of magnitude compared to a monovalent binding situation. Thus, the design of multivalent ligands as potent inhibitors is a highly active field of research, where knowledge about the binding site topology is crucial. Here, we report a general strategy for precise distance measurements between the binding sites of multivalent target proteins using monovalent ligands. We designed and synthesized Monovalent, conformationally Unambiguously Spin-labeled Ligands (MUeSLI). Inter-binding site distances of the complex model lectin wheat germ agglutinin were determined using pulsed electron paramagnetic resonance spectroscopy. This approach is an efficient way for exploring multivalent systems with monovalent ligands and it is readily transferable to other target proteins allowing the targeted design of multivalent ligands without structural information available.

## TOC GRAPHICS



## KEYWORDS

EPR spectroscopy – DEER – distance measurements – spin labeling – multivalency – ligand design

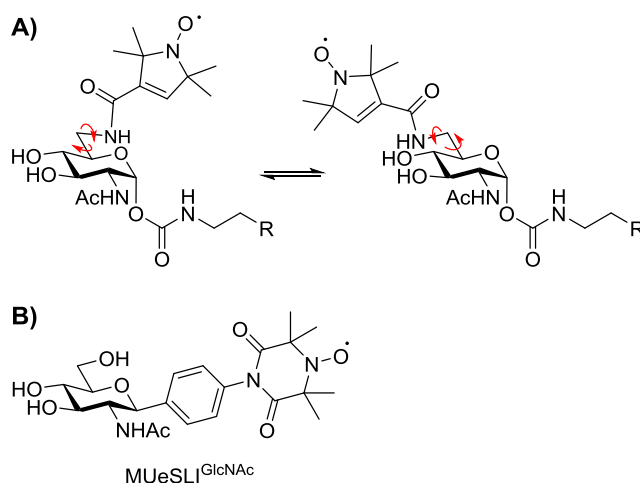
Multivalent ligand-receptor interactions, in particular carbohydrate-protein interactions, play key roles in biological processes, e.g., infection, adhesion and signaling.<sup>1-2</sup> Multivalent binding between several ligand and receptor moieties can increase the binding affinity by orders of magnitude compared to monovalent ligand binding, resulting in targeted and strong, yet reversible interactions.<sup>1, 3-4</sup> Proteins that specifically recognize and bind carbohydrates are called lectins. Many of them are relevant in health and disease and therefore important drug targets.<sup>5-6</sup> Commonly, the chelate effect, i.e., the spanning of several binding sites by a multivalent ligand, is considered as a major reason for the observed binding enhancement.<sup>7-9</sup>

Chelating binding of multivalent ligands requires that the scaffold or linker between the individual binding moieties fits the geometry of the receptor binding sites.<sup>2, 10-11</sup> With insufficient information about the target protein, a trial-and-error approach is the only way to find a suitable linker for a multivalent ligand. However, for the targeted design of high-potency multivalent carbohydrate ligands as effective bridging inhibitors, it is of prime importance to obtain information about the topology of the available binding sites of the target lectin.

Electron paramagnetic resonance (EPR) distance measurements have evolved into a powerful tool for the investigation of structure and conformational dynamics of proteins. Hence, the development of suitable spin labeling strategies fitting the large variety of applications is a highly active field of research.<sup>12-15</sup> Tailored spin-labeled ligands have proven valuable for studying the molecular details of protein-ligand interactions.<sup>16-17</sup> Thus, verification of a chelating binding mechanism in solution was achieved for the plant lectin wheat germ agglutinin (WGA), a well-investigated model system for multivalency, which forms a stable homodimer (35 kDa) and specifically binds N-acetylglucosamine (GlcNAc) at eight binding sites.<sup>18-20</sup> Divalent GlcNAc ligands carrying conformationally flexibly attached spin labels have been shown to

bridge adjacent binding sites using EPR distance measurements.<sup>21</sup> However, the attachment of a spin label via a flexible linker often results in broad EPR distance distributions, the interpretation of which is ambiguous in many cases, since they are influenced by conformations of the spin label side chain (Chart 1A).<sup>22</sup>

**Chart 1.** Spin-labeled ligands for EPR studies on proteins. A) A conformationally flexible spin label side chain as used previously,<sup>21</sup> B) MUESLI<sup>GlcNAc</sup>, structural formula.



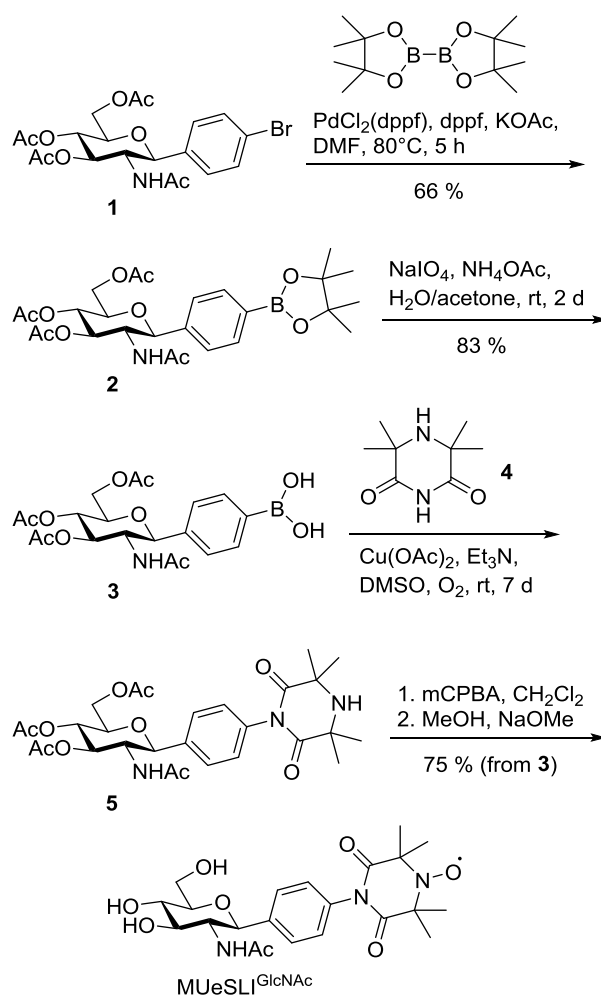
Here, we introduce a general strategy for the precise determination of distances between the binding sites of a multivalent protein with a Monovalent, conformationally Unambiguous, Spin-labeled Ligand (MUESLI). We demonstrate that owing to the defined spatial position of the nitroxide with respect to the binding site, EPR distance measurements can be interpreted directly in terms of the topology of accessible binding sites.

Various aspects are crucial for the design of a MUESLI. Firstly, the reliable determination of distances by EPR spectroscopy requires the unpaired electron to have a well-defined position in space. Secondly, the mutual orientation of two spin label molecules in the same nano-object should be randomized with respect to their interconnecting vector in order to avoid orientation selectivity. Both aspects are accounted for by conformationally unambiguous spin labels shown

for peptides and oligonucleotides.<sup>23-26</sup> One of the prime examples is the spin-labeled amino acid TOPP, which is characterized by a collinear alignment of the nitroxide N-O-bond and the C $\alpha$ -C $\beta$ -bond.<sup>23-24</sup> Thirdly, MUeSLI binding affinities should be weak in order to reduce multi-spin interactions arising from more than two spin-labeled ligands per protein. This applies for monovalent carbohydrate-ligands for lectins in general, where binding affinities in the mM range are no exception (compare SI, Figure S1).<sup>4</sup>

Taking the above into account, we designed and synthesized spin-labeled MUeSLI<sup>GlcNAc</sup> as a ligand for the model protein WGA (Chart 1B), carrying a conformationally unambiguous nitroxide spin label attached by a C-glycosidic bond to the anomeric carbon of GlcNAc. By this design, the position of the nitroxide is well-defined relative to the carbohydrate, which avoids broadening of the distance distribution due to linker flexibility.

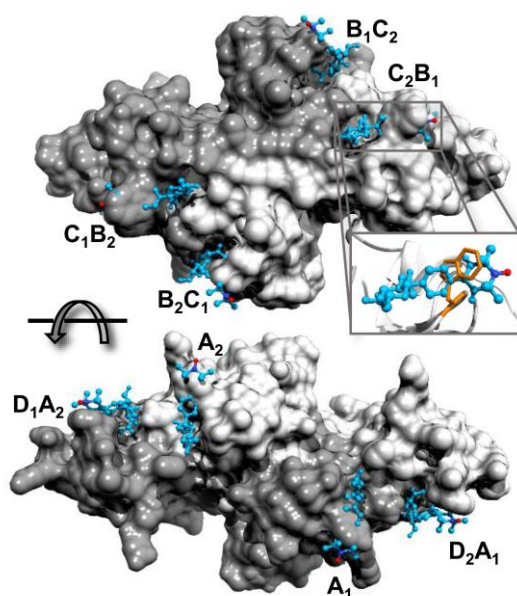
**Scheme 1.** Synthesis of the MUeSLI<sup>GlcNAc</sup>-ligand for WGA. dppf = 1,1'-ferrocenediyl-bis(diphenylphosphine)



We started synthesis of MUESLI<sup>GlcNAc</sup> (Scheme 1) from C-glycosyl bromobenzene 1, which was obtained from D-glucosamine hydrochloride in three steps as recently described.<sup>27</sup> Reaction with bis(pinacolato)diboron and the mild base potassium acetate under palladium catalysis<sup>28</sup> led to boronic acid pinacol ester 2 leaving the acetyl protecting groups untouched. Oxidative cleavage of 2 gave boronic acid 3 that served as starting material for a Chan-Lam coupling with amine 4 under conditions reported by Stoller et al.<sup>23</sup> to yield tetramethyldioxopiperazine 5. The spin label was introduced by oxidation with meta-chloroperoxybenzoic acid (mCPBA). Deacetylation delivered MUESLI<sup>GlcNAc</sup> in an overall-yield of 41 % starting from C-glycoside 1. The high purity of the compound is demonstrated in an HPLC chromatogram (SI, page 33).

MUeSLI<sup>GlcNAc</sup> was spectroscopically characterized by continuous-wave (cw) EPR spectroscopy at X-band and W-band frequencies of 9 and 93.5 GHz, respectively (SI, Figures S1/2/3, Table S2). In order to demonstrate the universality of the synthetic approach, we prepared a C-glycosidically spin-labeled glucose derivate (MUeSLI<sup>Glc</sup>) (SI).

The goal of the EPR distance measurements in future applications is obtaining distance constraints as a basis for deducing suitable linker lengths in potential multivalent ligand architectures for lectins without a high-resolution structure available. In contrast, in the current proof-of-concept study, the EPR distance constraints can be predicted from a crystal structural model: Previously, Schwefel et al. elucidated the binding mode of GlcNAc-containing ligands in all eight binding sites of WGA by X-ray crystallography, which serves to validate the results of this proof-of-concept study.<sup>29</sup> The eight binding sites of WGA are designated A<sub>1</sub>, A<sub>2</sub>, B<sub>1</sub>C<sub>2</sub>, B<sub>2</sub>C<sub>1</sub>, C<sub>1</sub>B<sub>2</sub>, C<sub>2</sub>B<sub>1</sub>, D<sub>1</sub>A<sub>2</sub>, and D<sub>2</sub>A<sub>1</sub>, where numerated capitals refer to protein domains contributing aromatic and polar residues to the binding sites, respectively.<sup>18-20</sup> These are pairs of four unique sites due to the C2-symmetry of the dimer, located at the monomer-monomer interface of the dimer. EPR distance constraints were predicted by determining the mutual distances between all MUeSLI<sup>GlcNAc</sup> molecules (O-atoms of the nitroxide moieties) from an exact overlay of eight MUeSLI<sup>GlcNAc</sup> onto a crystal structural model of WGA with all binding sites simultaneously occupied by GlcNAc-ligands (Figure 1 and SI, Figure S4).



**Figure 1.** Overlay of the WGA dimer crystal structure (surface representation, dark and light gray indicating different monomeric chains)<sup>29</sup> with eight MUESLI<sup>GlcNAc</sup> (light blue ball-and-stick model, N- and O-atoms of the nitroxide spin labels colored blue and red, respectively). The inset shows a steric clash between MUESLI<sup>GlcNAc</sup> in binding pocket C<sub>2</sub>B<sub>1</sub> and Trp 107 (orange) of a WGA protomer. Due to dimer symmetry, the same situation applies for binding pocket C<sub>1</sub>B<sub>2</sub>.

Results are summarized in Table 1. Due to the C<sub>2</sub>-symmetry of the WGA dimer, two similar distances are expected for pairs of symmetrically equivalent binding sites. Since there are no considerable conformational changes in WGA upon binding of various GlcNAc-based ligands,<sup>29</sup> uncertainties in these distances are small. We estimate them at ~2 Å (see SI, Figure S5).

**Table 1.** Distance constraints in nm between the O-atoms of the nitroxide radicals predicted from overlaying the crystal structural model of WGA (PDB 2X52)<sup>29</sup> with MUESLI<sup>GlcNAc</sup> using UCSF Chimera.<sup>30</sup>

	C <sub>1</sub> B <sub>2</sub>	C <sub>2</sub> B <sub>1</sub>	B <sub>1</sub> C <sub>2</sub>	B <sub>2</sub> C <sub>1</sub>	D <sub>1</sub> A <sub>2</sub>	D <sub>2</sub> A <sub>1</sub>	A <sub>1</sub>
C <sub>1</sub> B <sub>2</sub>							
C <sub>2</sub> B <sub>1</sub>	4.8 <span style="color: green;">■</span>						
B <sub>1</sub> C <sub>2</sub>	3.8 <span style="color: red;">●</span>	2.3 <span style="color: orange;">◆</span>					



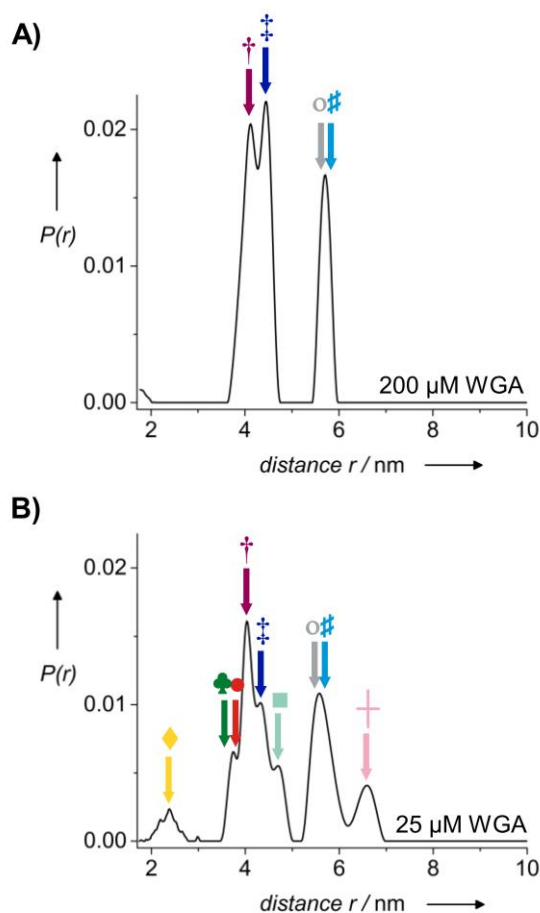
B <sub>2</sub> C <sub>1</sub>	2.2 ♦	3.8 ●	4.0 †				
D <sub>1</sub> A <sub>2</sub>	3.7 ♣	6.5 †	5.7 ○	4.5 ‡			
D <sub>2</sub> A <sub>1</sub>	6.5 †	3.7 ♣	4.5 ‡	5.8 ○	5.8 #		
A <sub>1</sub>	5.3	3.9	3.7	5.3	4.5	1.8	
A <sub>2</sub>	3.8	5.3	5.2	3.6	1.9	4.5	3.6

EPR distance measurements were performed using double electron-electron resonance (DEER) spectroscopy, also known as PELDOR (pulsed electron double resonance), which gives access to distances between spin labels separated by 1.8 up to 16 nm in favorable cases.<sup>31-34</sup> It is therefore ideally suited to explore the binding site topology of lectins like WGA, where all expected distances lie in a range between 1.8 up to 6.5 nm (Table 1). DEER experiments were performed at Q-band frequency (34 GHz) at 2-fold and 16-fold molar excess of MUESLI<sup>GlcNAc</sup> (400 μM) over WGA (200 and 25 μM, respectively) in shock-frozen deuterated aqueous solution with 20 % (v/v) [D<sub>8</sub>]glycerol. The phase memory time of the DEER samples is ~ 9 μs (SI, Figure S6). Resulting DEER modulation depths are low and multi-spin interactions were neglected (SI, Figure S7). Additionally, a DEER experiment with pure MUESLI<sup>GlcNAc</sup> ligand was performed, where no dipolar oscillations could be detected (SI, Figure S8). This result confirms that the small modulation depths obtained with MUESLI<sup>GlcNAc</sup> in the presence of WGA result from dipolar couplings between bound ligand molecules. Distance distributions were extracted by a model-free analysis with Tikhonov regularization using DEERAnalysis 2016.<sup>35</sup>

The complexity of the binding situation with up to eight possible binding sites per WGA dimer is reflected in the resulting distance distributions (Figure 2). In order to ensure their reliability and avoid over-interpretation we performed repetitive measurements of identical samples, independent experiments with individually prepared samples, validation of the data analysis procedure (influence of background, noise and the regularization parameter,<sup>36</sup> SI, Figures S7/9/10), independent analysis of subsets of the 2D DEER data (SI, Figure S11) and excluded

the possibility of orientation selection (SI, Figure S12). The analysis is extraordinary robust and yields complex but reliable distance information.

The distance distributions of MUESLI<sup>GlcNAc</sup> in 2-fold and 16-fold molar excess over WGA dimer are shown in Figures 2A and 2B, respectively. Both distributions feature a variety of different peaks in the accessible distance range. However, distances around 5.2 nm as predicted for MUESLI<sup>GlcNAc</sup> bound to binding sites A<sub>1</sub> and A<sub>2</sub> are missing from both distributions (compare SI, Figure S10), suggesting that in solution MUESLI<sup>GlcNAc</sup> does not bind to A<sub>1</sub> and A<sub>2</sub>. This is in accordance with previous findings, where A<sub>1</sub> and A<sub>2</sub> are suggested to exhibit weakest ligand binding affinity in solution, most likely due to the lack of polar amino acids in these particular binding sites.<sup>20</sup>



**Figure 2.** Distance distributions  $P(r)$  of MUESLI<sup>GlcNAc</sup> (400  $\mu$ M) in 2-fold (A) and 16-fold (B) molar excess over WGA dimer. Arrows indicate contributions to the distance distributions as discussed in the text, and colored symbols connect them to the predicted distances (Table 1).

The distance distribution recorded with a 2-fold molar excess of MUESLI<sup>GlcNAc</sup> over WGA (Figure 2A) reveals four major contributions (arrows). The peak at 4.0 nm can be attributed to spin labels in binding sites  $B_1C_2$  and  $B_2C_1$ . The contribution at 4.5 nm corresponds to the distances between one MUESLI<sup>GlcNAc</sup> in the  $B_{1/2}C_{2/1}$  site paired with one MUESLI<sup>GlcNAc</sup> in the  $D_{2/1}A_{1/2}$  sites. Correspondingly, at 5.8 nm a prominent peak appears, representing the distances between  $D_1A_2$  and  $D_2A_1$ . This peak coincides with distances of pairs in  $D_{1/2}A_{2/1}$  and  $B_{1/2}C_{2/1}$ . A steric clash between the rigid spin label side chains of MUESLI<sup>GlcNAc</sup> in  $C_1B_2$  and  $C_2B_1$  and the Trp 107 residues of both WGA protomers is predicted in the crystal structural overlay (Figure 1). Indeed, contributions expected for occupied  $C_{1/2}B_{2/1}$  sites are not detected at 2-fold excess of MUESLI<sup>GlcNAc</sup>. This absence of the corresponding distances is in agreement with the steric hindrance of binding to these sites.

Adding a 16-fold molar excess of MUESLI<sup>GlcNAc</sup> to WGA results in a distance distribution with significantly more distinct features (Figure 2B). The additional contributions at 2.2, 3.7, 3.8, 4.8 and 6.5 nm are consistent with occupation of the binding sites  $C_{1/2}B_{2/1}$  (compare Table 1, columns  $C_{1/2}B_{2/1}$ ). The sterical hindrance in the  $C_{1/2}B_{2/1}$  sites does not completely knock out the binding sites, but significantly depresses their affinity. Accordingly, increased MUESLI<sup>GlcNAc</sup> concentration reveals  $C_{1/2}B_{2/1}$  functionality.

Strikingly, MUESLI<sup>GlcNAc</sup> reflects the most relevant binding sites and inter-binding site distances of WGA. Despite its monovalent nature, MUESLI<sup>GlcNAc</sup> provides direct insights into the complex binding site topology of multivalent WGA. Said monovalent nature allows

straightforward and easy ligand design. Owing to the well-defined distances, individual distance contributions can be separated from each other, making the rational design of a multivalent ligand possible without a high-resolution structure of the receptor.

In summary, we report the design and synthesis of the conformationally unambiguously spin-labeled ligand MUESLI<sup>GlcNAc</sup>. We show that together with EPR distance measurements this tailored spin label is suited for accessing the binding site topology of the multivalent target protein WGA. All experimental contributions to the complex distance distributions can be conclusively assigned to distances predicted from a crystal structural analysis. This demonstrates that MUESLI is a tool for extracting the topology of multivalent binding sites in a simple and elegant way. The MUESLI approach is generally applicable to arbitrary multivalent systems with low-affinity monovalent ligands, enabling the targeted design of high-potency multivalent ligands without any structural information available beforehand. Transfer of the MUESLI design to other ligand molecules makes it a universal tool for investigating multivalent receptor geometries.

## ASSOCIATED CONTENT

### **Supporting Information.**

The following files are available free of charge.

Synthesis, characterization of compounds, EPR experimental details and analysis, additional EPR experiments (PDF)

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## Notes

The authors declare no competing financial interests.

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