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SEAL by NMR: Glyco-based Selenium-labeled Affinity Ligands detected by NMR spectroscopy[†]

Christoffer Hamark,^[a] Jens Landström,^[a] and Göran Widmalm^{*,[a]}

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Abstract: We report a method for the screening of interactions between proteins and selenium-labeled carbohydrate ligands. SEAL by NMR is demonstrated with selenoglycosides binding to lectins where the selenium nucleus serves as an NMR-active handle and reports on binding through ⁷⁷Se NMR spectroscopy. In terms of overall sensitivity, this nucleus is comparable to ¹³C NMR while the NMR spectral width is ten times larger, yielding little overlap in ⁷⁷Se NMR spectroscopy, even for similar compounds. The studied ligands are singly-selenated bioisosteres of methyl glycosides for which straightforward preparation methods are at hand and libraries can readily be generated. The strength of the approach lies in its simplicity, sensitivity to binding events, the tolerance to additives and the possibility of having several ligands in the assay. This study extends the increasing potential of selenium in structure biology and medicinal chemistry. We anticipate that SEAL by NMR will be a beneficial tool for the development of selenium-based bioactive compounds, such as glycomimetic drug candidates.

Protein structure is a central theme in biology and it is experimentally resolved at atomic resolution, in particular by two techniques, viz., X-ray crystallography^[1] and nuclear magnetic resonance (NMR) spectroscopy^[2]. Biological functions of proteins are largely governed by their interactions with ligand molecules. There are countless incentives for the study of such complexes and consequently a large variety of methodologies exists depending on the information sought, including biochemical, biophysical and *in silico* methods.^[3] The power of NMR spectroscopy in this research has been widely recognized^[4,5] and several approaches and experiments are available^[6–9]. Ligand-based NMR experiments have been shown to be particularly helpful for screening, even for high throughput approaches, and prominent examples are the STD NMR^[10], WaterLOGSY^[11], trNOESY^[12] and ILOE^[13]. The problem of unresolved resonances in screening assays involving larger groups of ligands is a limitation of these ¹H NMR-based experiments. A way to circumvent this is to exploit NMR-active heteronuclei for detection at a wider spectral range and fluorine has proven to be suitable for this purpose. Hence, a growing amount of attention has been drawn to ¹⁹F NMR-based interaction experiments over the last decade.^[14–18]

Another nucleus receiving increased interest within the field of structure biology is selenium. This element occurs naturally in

the human proteome (selenoproteome) in the form of selenocysteine^[19] and it is also found as selenium-containing metabolites^[20] and pharmaceuticals^[21]. In addition to its abundance, selenium bears physical properties beneficial for structure analysis, which has been exploited in X-ray crystallography not only for selenoproteins but also for co-crystals with selenium-labeled ligands, such as selenium-containing carbohydrates.^[22,23] Furthermore, the isotope ⁷⁷Se (7.6% natural abundance) is NMR-active being a spin-½ nucleus, enabling high-resolution NMR experiments to be carried out. It has been used in protein NMR, *inter alia* as a surrogate for sulfur, the latter being devoid of the spin-½ isotope.^[24] The transverse relaxation time (T₂) of ⁷⁷Se is significantly influenced by chemical shift anisotropy (CSA). The contribution to the total T₂ of this CSA term, which is dependent on the global correlation time (τ_c), becomes larger when a selenium-containing ligand is associates to e.g. a protein receptor.^[25] Moreover, due to CSA, ⁷⁷Se chemical shifts are highly sensitive to the details of local chemical environment which is displayed by its large resonance range, ~3000 ppm. This range is over ten times wider

Figure 1. Schematic and CFG representation of compounds 1 - 5 studied using SEAL by NMR. Models of the lectins were generated by the PyMol software (The PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC) and originate from X-ray crystal structures (WGA^[30]; PDB ID: 2UVO; ConA^[32]; PDB ID: 5CNA; PNA^[33]; PDB ID: 2PEL). The ligands are represented as 3D-CFG.^[34]

[a] C. Hamark, Dr J. Landström, Prof. Dr. Göran Widmalm
Arrhenius Laboratory
Department of Organic Chemistry
Stockholm University, S-106 91 Stockholm, Sweden
E-mail: gw@organ.su.se

Figure 2. 1D ^{77}Se NMR spectra of compounds 1 - 5 in the absence and presence of lectin: a,b) 2 and 5 at 298 K in the absence and presence of WGA (15:1 ligand-protein ratio), respectively; c,d) 3 and 4 at 310 K in the absence and presence of ConA (6:1 ligand-protein ratio), respectively; e,f) 4 and 5 at 298 K in the absence and presence of PNA (15:1 ligand-protein ratio), respectively. Compound 1 is present as a reference, in an insert-tube in all experiments.

than for ^{13}C NMR and the sensitivity at natural abundance (receptivity, abundance and nuclear Overhauser effect taken together) is quite similar.^[26] In this communication we describe the use of selenoglycosides as bioisosteres of a number of carbohydrate ligands for detection of their protein interactions by ^{77}Se NMR spectroscopy.

Selenoglycosides have found their use as versatile glycosyl donors in carbohydrate chemistry for quite some time and this has led to the development of robust methods for their creation.^[27] Inspired by this, we reasoned that selenoglycosides, singly selenated at the reducing end of the carbohydrates, could serve as non-perturbing bioisosteres of natural epitopes, selenium acting as reporter atom for detection by ^{77}Se NMR spectroscopy. Earlier studies suggested that it would be possible to apply such an approach in carbohydrate-protein interaction studies.^[28,29]

To this end we investigated the interactions of a number of selenated monosaccharides and one disaccharide with three different lectins, see Figure 1. Lectins are non-enzymatic carbohydrate-binding proteins widely found in nature. Wheat Germ Agglutinin (WGA), Concanavalin A (ConA) and Peanut Agglutinin (PNA) are well studied, commercially available, model lectins that possess different carbohydrate-ligand specificity, orthogonal with respect to our ligands. As indicated in Figure 1, WGA binds selectively to *N*-acetyl-D-glucosamine, ConA to α -D-mannose and PNA to D-galactose and the affinities (K_D) are in the range of mM to μM according to literature data.^[30] Our approach is to study combinations of the selenoglycosides in the absence (blank) and presence of protein, employing otherwise identical conditions, and directly monitoring different parameters that change upon complexation by regular proton decoupled 1D ^{77}Se NMR spectra, see Figure 2. In order to display the effects of ligand-binding unbiased from medium effects, all the experiments were carried out with methyl 1-seleno- β -D-glucopyranoside (GlcSeMe, 1) as a non-interfering reference compound present in an NMR insert-tube. In addition to ^{77}Se NMR spectroscopy, binding and selectivity were tested for each sample using STD NMR experiments^[10] as a control, see Figure S1. Together with sensitivity, the enabling of such STD NMR control is the reason why ligand-protein ratios on the order of 15-5:1 (see experimental section) was chosen in the study.

The lectins were mixed with one compound that was anticipated to bind and one expected non-binder; in the case of PNA one additional competitive binder was present, see Figure 2. For the three systems investigated, conspicuous changes in ligand ^{77}Se NMR parameters appeared as a result of protein addition. Especially linewidths and resonance intensity changed affectedly for binders as compared with non-binders. Chemical shift differences were also monitored. WGA was added to methyl 2-*N*-acetamido-2-deoxy-1-seleno- β -D-glucopyranoside (GNAcSeMe, 2) and methyl β -D-galactopyranosyl-(1 $\rightarrow$ 4)-1-seleno- β -D-glucopyranoside (LacSeMe, 5) (Figure 2a and b). The GNAcSeMe resonance experienced an 86% decrease in intensity, a line-broadening of 14.7 Hz and a downfield chemical shift change of ~ 17 Hz compared with the reference sample. LacSeMe however, did not show any significant line-broadening or chemical shift change and only a marginal ($<10\%$) intensity

decrease in the presence of protein. This result implies interactions only between WGA and GNAcSeMe as would be expected from the behavior of the unmodified glycosides. The mannose-binding lectin ConA was mixed with methyl 1-seleno- α -D-mannopyranoside (ManSeMe, **3**) in the presence of the anticipated non-ligand methyl 1-seleno- β -D-galactopyranoside (GalSeMe, **4**) (Figure 2c and d). In this case, the ManSeMe resonance intensity was reduced by 51% and broadened by 8.5 Hz whereas for GalSeMe, we did not observe any changes upon protein addition. PNA was mixed with GalSeMe, LacSeMe and GNAcSeMe (Figure 2e and f) and competitive binding was indicated for GalSeMe and LacSeMe with intensity effects of 38% and 19%, respectively, as well as line-broadening of 2.7 and 1.9 Hz, respectively. The non-binding GNAcSeMe showed only a small reduction in intensity (11%) and less than 1 Hz line-broadening. The chemical shift changes for the ligands studied in combination with ConA and PNA were all < 5 Hz.

The lectin-selenoglycoside interactions studied herein follow the selectivity anticipated from the natural bioisosteres and this was effectively demonstrated with SEAL by NMR. The observed changes in ligand resonance intensity and line-broadening are interdependent and stem from the differential transverse relaxation (T_2) properties of the proteins and the small carbohydrate ligands. Bound molecules will relax faster displaying broader linewidths (for ^1H methyl resonances see Figures S2-S4) as compared to non-binders due to the dynamic exchange with the protein receptor; especially considering the CSA contribution to ^{77}Se T_2 relaxation time.

The particularly profound effects seen for the WGA-GNAcSeMe interactions might seem unintuitive at first, given the fact that the unmodified GNAcOMe is a weak binder ($K_D = 2.5$ mM).^[30] However, comparing x-ray crystal structures of the natural ligands for the lectins studied^[31–33] (see Figure 1), it is clear that only in the case of WGA the anomeric oxygen (position for selenium substitution) is in close proximity to a protein amino acid residue in the binding pocket. This is revealed by the fact that the chemical shift differences between the GNAcSeMe in presence and absence of WGA is the largest seen in the study (~17 Hz) indicating an altered chemical environment between free and bound state. Due to this prominent chemical shift change, the T_2 relaxation can be considerably affected and the linewidths broadened additionally by an exchange term $R_{2,\text{ex}}$. Besides the change in chemical shift, this term depends on the populations of the free and bound state as well as the rate of the exchange. $R_{2,\text{ex}}$ can contribute significantly to the transverse relaxation rate R_2 even at weak affinities and small fractions of bound ligand if the chemical shift change is sufficiently large, which might be the case for GNAcSeMe. There is, however, a possibility that the affinity is increased by the incorporation of selenium. With reference to theoretical studies, it has been suggested that selenium could enhance protein binding and in particular interact efficiently with aromatic amino acid residues in proteins.^[34,35] A molecular docking simulation was performed with WGA and GNAcSeMe as ligand (see Figure S5) and it showed that the selenomethyl group is in close proximity to the indole group of a tryptophan residue, just as for the anomeric oxygen in the crystal structure with the natural ligand. The strong

effect seen for the WGA-binding of GNAcSeMe in ^{77}Se NMR is proposed to be a result of the particular interaction of the selenium atom and the protein which enhances the affinity and generates an additional broadening of the linewidth due to $R_{2,\text{ex}}$. To the best of our knowledge, experimental evidence of such an interaction has not been reported and this finding will be highly interesting to study in more detail.

In this communication we have presented a method for the direct study of protein interactions by ligand-detected ^{77}Se NMR spectroscopy. We foresee that SEAL by NMR will be useful for the screening of selenium-containing ligands in general, the selenium atom acting as an NMR-handle, reporting on binding. The technique which is operationally straightforward could be extended with an R_2 relaxation-filter^[36] or be used in a competition-based approach with a ^{77}Se -labeled weak ligand acting as a spy molecule^[7,37,38]. It should be particularly suitable for ligand screening with a large number of compounds and it is also tolerant to various conditions, otherwise challenging for ^1H NMR-based experiments, such as the presence of H_2O , buffers, detergents, additives or even whole cell systems.

Experimental Section

Compounds. The lectins were purchased as lyophilized powder from Sigma-Aldrich (St Louis, MO, USA). For all analyses they were kept in D_2O solutions with 50 mM sodium phosphate buffer at pH 6.8 and 100 mM NaCl. For ConA and PNA solutions 1 mM of CaCl_2 and MgCl_2 were added. Protein concentrations were measured with a BioSpec-nano (Shimadzu corp., Tokyo, Japan) spectrophotometer. The syntheses of compounds **1** - **5** will be described elsewhere.

NMR spectroscopy. All NMR experiments were carried out on a Bruker Avance III 600 MHz spectrometer equipped with a 5 mm Z-gradient BBO-probe. 1D ^{77}Se NMR spectra were recorded with a waltz-16 inverse-gated proton decoupling scheme and acquisition was carried out with 80 k transients in addition to 64 dummy scans. A tip angle of $\sim 70^\circ$, a spectral width of 2.7 kHz and 1 k data points were employed, yielding an acquisition time of 0.19 s, followed by a relaxation delay of 0.04 s. Prior to Fourier transformation the FIDs were zero-filled to 8 k data points and multiplied with a 5 Hz exponential line-broadening factor. WGA experiments were performed at 298 K with concentrations for compound **2** and **5** of 3.5 and 3.6 mM, respectively, and a protein concentration of 30 μM corresponding to a ligand-protein ratio of 15:1 per primary binding site. ConA experiments were performed at 310 K with concentrations for compounds **3** and **4** of 2.9 mM and a protein concentration of 126 μM corresponding to a ligand-protein ratio of 6:1 per binding site. PNA experiments were performed at 298 K with concentrations for compounds **2**, **4** and **5** of 1.4, 1.6 and 1.5 mM, respectively, and a protein concentration of 54 μM corresponding to a ligand-protein ratio of 7:1 per binding site. The NMR insert-tube, used in all experiments, contained compound **1** with a concentration of 25 mM in addition to buffer solution, *vide supra*; spectra were externally referenced to Me_2Se , $\delta_{\text{Se}} = 0.0$.

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Keywords: ^{77}Se NMR • protein-ligand interactions • selenoglycosides • lectins • SEAL

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Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

⁷⁷Seeing the interactions between oligosaccharide ligands and proteins are possible by using selenium-labeled bioisosteres. This method exploits ⁷⁷Se NMR spectroscopy for the study of carbohydrate-lectin complexes.

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