# CHARACTERIZATION OF THE INTERACTIONS BETWEEN HIGH MANNOSE GLYCANS AND A NOVEL PROKARYOTIC LECTIN USING MASS SPECTROMETRY-BASED APPROACHES

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## OBJECTIVE

Characterize the specificity, affinity constants, and kinetics of the binding between released N-linked glycans and a novel Recombinant Prokaryotic Lectin, RPL-Man2.

## INTRODUCTION

- Lectins are a class of carbohydrate-binding proteins with specificity towards the monosaccharide subunits of glycoforms
- High mannose glycans are a type of oligosaccharide found on glycoproteins and are associated both with immunogenicity and half-life of therapeutic antibodies
- Lectins have been used in SPR, chromatographic, and microarray formats to assess the glycosylation profile of glycoproteins
- Understanding the binding interactions between lectins and glycans can provide insights into the accuracy and precision of glycosylation profile measurements in these different formats, allowing for optimization towards a more precise solution for the measurement of glycan profile

## METHODS

**Catch-and-Release Native MS**

- Recombinant Prokaryotic Lectin Man2 (RPL-Man2, GlycoSelect Ltd) was buffer exchanged into 50 mM ammonium acetate and incubated with variable concentrations of Waters *Rapi*Fluor-MS™ High Mannose Test Standard for 30 minutes
- Glycan-lectin complexes were introduced to the gas phase via Native Spray Ionization and analyzed on a SYNTAPT™ XS Mass Spectrometer
- Glycans were released from the lectin complexes via collision induced dissociation and the resultant glycan ion quantified for determination of the amount of glycan bound

**Affinity Pull-down**

- Carboxylate-modified polystyrene beads were alternately coupled to tris-NTA amine using a 1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide crosslinker or directly to the RPL
- The tris-NTA functionalized beads were saturated with Ni2+, washed and incubated with RPL-Man2
- *Rapi*Fluor-MS tagged high mannose glycans were incubated with the RPL-Man2 beads over 15 seconds to 20 minutes, washed, and glycans released with a methyl-mannose competitive eluent. Glycans were analyzed on a Xevo™ G2-XS Mass Spectrometer coupled to an ACQUITY I-Class UPLC.

## RESULTS: CAR-MS

Catch-and-release (CaR) MS experiments were used to assess the binding behavior of RPL-Man2 free in solution. Bound glycan was released in the gas phase from the RPL complex under varying initial glycan concentrations. Exemplary spectra for the undissociated and dissociated complex are shown in Figure 1, from which it can be observed that the complexity of the mixture prohibited resolution of individual glycan-RPL complexes.

Energy-resolved MS plots were generated for the RPL-glycan complexes to determine if this approach was suitable for determination of $K_d$ values. Critically, the complex must be stable enough to survive the electrospray process without significant dissociation of the glycans. The optimal collisional energy for full dissociation of the glycan from the RPL was determined to be 50V, affording the use of a relatively high cone voltage to skim non-specifically bound glycan from the complex in the source region. The complex was then quadrupole isolated, dissociated, and the abundance of the resultant free glycan measured.

Dissociation curves were generated for 5 µM RPL-Man2 in complex with *Rapi*Fluor-MS labeled glycans (man5 - man11) and are shown in Figure 2. Association constants were generated by fitting Equation 2 to each dissociation curve and are listed in Table 1. First, it is assumed that the concentration of the RPL-glycan complex [PL] is equal to the intensity of the released glycan at a given glycan concentration, divided by the maximally observed released glycan signal from the highest concentration studied. A valency of 4 was empirically found to provide the best fits. Equation 1 is then substituted into Equation 2, the relationship between the association constant Ka and the equilibrium concentrations of the protein [P], the glycan [L], and the complex [PL]. Equation 3 is then generated from a non-linear regression analysis.

Equation 1:

$$R = \frac{[PL]}{P_t} = \frac{I_{L,obs}}{I_{L,obs,max}}$$

Equation 2:

$$K_a = \frac{[PL]}{[P][L]}$$

Equation 3:

$$R = \frac{(1+(K_a \times [S])+(K_a \times [P])) - \sqrt{(-(1+(K_a \times [S])+(K_a \times [P]))^2 - 4 \times {K_a}^2 \times [S] \times [P])}}{2 \times K_a \times [P]}$$

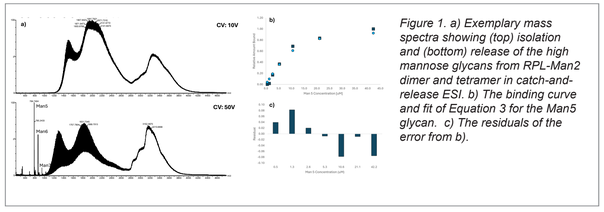


*Figure 1. a) Exemplary mass spectra showing (top) isolation and (bottom) release of the high mannose glycans from RPL-Man2 dimer and tetramer in catch-and-release ESI. b) The binding curve and fit of Equation 3 for the Man5 glycan. c) The residuals of the error from b).*

## RESULTS: CAR-MS

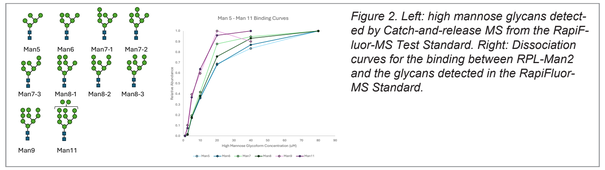


*Figure 2. Left: high mannose glycans detected by Catch-and-release MS from the RapiFluor-MS Test Standard. Right: Dissociation curves for the binding between RPL-Man2 and the glycans detected in the RapiFluor-MS Standard.*

## RESULTS: PULL DOWN

RPLs were immobilized on polymer beads using the RPL His-tag. Kinetics of binding were studied following binding and competitive elution with mannose. Glycan species were identified by mass spectrometry and quantified by the fluorescence from the *Rapi*Fluor-MS Tag. The resulting kinetics curves for on-binding were exceedingly similar and representative curves using different concentrations of glycan are shown in Figure 3 for the Man5 glycoform. Determination of $K_d$ for an immobilized protein is given by the relationship between $k_{on}$, the association rate, and $k_{off}$, the dissociation rate and is shown in Equation 4. The apparent association rate, $k_{on,app}$, is the measured association from kinetic analysis and is a function of $k_{on}$ and $k_{off}$, as shown in Equation 5.

Equation 4:

$$Kd = \frac{k_{off}}{k_{on}}$$

Equation 5:

$$kon, app = k_{on}\, x\, C + k_{off}$$

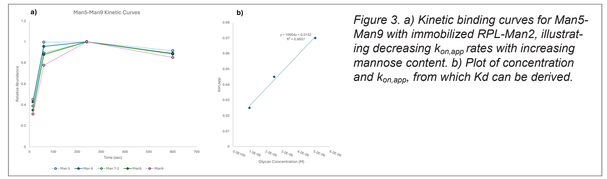


*Figure 3. a) Kinetic binding curves for Man5-Man9 with immobilized RPL-Man2, illustrating decreasing $k_{on,app}$ rates with increasing mannose content. b) Plot of concentration and $k_{on,app}$, from which Kd can be derived.*

| Glycoform | Kd uM (CaR-MS) | Kd (Pull Down) |
|---|---|---|
| Man5 | 5.0 | 1.4 |
| Man6 | 4.0 | 1.1 |
| Man7-1 Man7-2 | 1.4 | 0.79/2.0 |
| Man8 | 2.0 | 4.4 |
| Man9 | 0.10 | 1.1 |
| Man11 | 0.080 | NA |

*Table 1. $K_d$ values for in-solution and immobilized RPL -Man2 and RapiFluor-MS Man5-Man11.*

## DISCUSSION

To date, characterization of the binding behavior of Recombinant Prokaryotic Lectins has been restricted to intact proteins, limiting the fundamental understanding of the performance of the lectins at the individual glycan level.[1] Immobilization can restrict access to binding sites, elevating observed dissociation constants. Thus, the in-solution and immobilized affinity characteristics of RPL-Man2 with a *Rapi*Fluor-MS glycan mixture was studied. In-solution measurements indicate binding constants ranging from 5 µM to 80 nM for Man5-Man11, respectively. Interestingly, the Man7 glycoform was found to have a lower $K_d$ than the Man8 glycoform. Although both Man7 and Man8 can exist as three isomeric species, shown in Figure 2, Man7 is observed as two distinct chromatographic peaks in HILIC chromatography, suggesting that it may be more structurally diverse. One explanation for this behavior is that one of these isomers binds interacts more weakly with the RPL-Man2 binding sites.

Affinity analysis using the kinetic rates of association between the RPL-Man2 protein and Man5-Man11 yielded dissociation constants in the range of 0.8 to 4 µM. Surprisingly, while the solution phase assay indicated stronger binding with increasing mannose content, the kinetics-based approach on the immobilized RPL resulted in relatively equivalent dissociations constants across the range of high mannose glycans. Variation between the two assays may be a consequence of the different methodologies used for the analyses, but may also reflect the impact of immobilization, if surface immobilization at the His-tag of the RPL-Man2 orients the protein favorably. Additional study using non-specific immobilization approaches will be examined to evaluate if this is the case.

## CONCLUSIONS

- **Relatively low dissociation constants were determined for the interaction between *Rapi*Fluor-MS high mannose glycans (Man5-Man11) in both solution phase and immobilized experiments**
- **Solution-phase affinity analysis using CaR-MS yielded variable dissociation constants for the interaction between *Rapi*Fluor-MS high mannose glycans and RPL-Man2, with the higher mannose species exhibiting tighter binding**
- **Kinetic analysis of His-tag immobilized RPL-Man2 and *Rapi*Fluor-MS Man5-Man11 glycan yielded relatively consistent Kd values for the range of high mannose glycans explored, a consequence of both $k_{on}$ and $k_{off}$ values decreasing with increasing mannose content**

**References**

1. S. Fernandez-Poza, A. Padros, R. Thompson, Lucy Butler, Meez Islam, J.A. Mosely, James H. Scrivens, Muhammad F Rehman, Muhammad Safwan Akram, Tailor-made recombinant prokaryotic lectins for characterisation of glycoproteins,. Analytica Chimica Acta, Volume 1155,2021,338352,ISSN 0003-2670.



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