Application Note

Wyatt Technology Corporation

Understanding Antibody and Viral Glycoprotein Interactions Using CG-MALS

Kathryn M. Hastie and Erica Ollmann Saphire†

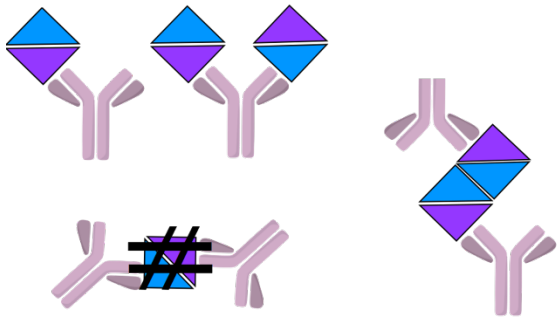
Summary

Viral glycoproteins are often the only antigen found on the viral surface and, as such, are key targets for neutralizing antibodies. Analyzing the type and affinity of the different molecular interactions that can occur is essential for understanding what makes a good neutralizing antibody and how these interactions can be utilized in post-exposure treatments as well as vaccine development.

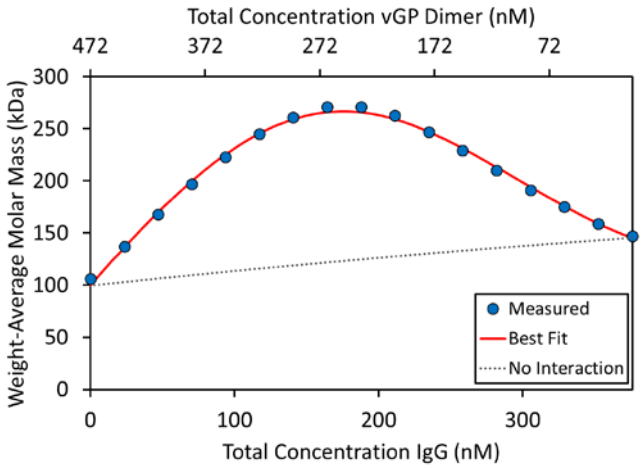
To this end, we first used size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) to determine the molar mass and native oligomeric state of a viral glycoprotein (vGP). In addition, MALS measured by the miniDAWN TREOS was combined with simultaneous concentration measurements by UV and dRI (Optilab T-rEX) to measure the glycan content of the vGP using Protein Conjugate Analysis.

Next, we employed composition-gradient multi-angle light scattering (CG-MALS) with the Wyatt Calypso II and miniDAWN TREOS to determine the stoichiometry and affinity of the glycoprotein:antibody interaction. The automated Calypso method consisted of a concentration gradient of glycoprotein to assess self-association properties as indicated by SEC-MALS and a dual-composition "crossover" gradient to assess hetero-association behavior.

The light scattering data were best fit in the CALYPSO software by an association model that accounts for two equivalent glycoprotein binding sites per antibody molecule and a meta-complex of two IgGs bound two dimers. Surprisingly, a model that provided for up to two antibody-binding sites on a single vGP dimer did not fit the data, suggesting the single epitope may lie near the dimeric interface.



Schematic of the measured binding stoichiometries for the interaction between a monoclonal antibody and dimeric viral glycoprotein (blue and purple triangles). Each Fab of the antibody was able to bind a separate vGP dimer, but two antibodies were not able to bind a single vGP dimer.



The measured weight-average molar mass of the solution increases as the vGP and IgG are combined at different ratios. The best fit to the CG-MALS data indicates that each Fab on the antibody can bind a vGP dimer with affinity K_D = 58 nM.

†erica@scripps.edu

WYATT TECHNOLOGY

I. Introduction

Viral glycoproteins (vGP) are essential for the attachment and fusion of virus and host cell membranes. They are also the primary targets of neutralizing antibody responses. Most surface vGPs are found as oligomeric complexes of dimers, trimers or tetramers. Whether multiple antibodies bind to these complexes and with what affinity is critical information when designing vaccines or creating a post-exposure antibody treatments. For example, Ebola virus GP is the target of current therapies and new IgGs are being investigated for their efficacy in therapeutic cocktails (Murin et al. 2014).

Composition-gradient multi-angle light scattering (CG-MALS) quantifies the change in apparent weight-average molar mass (M_w) as a function of composition and allows for the analysis reversible associations in solution. With CG-MALS, the affinity and stoichiometry of various protein-protein associations can be determined in solution. Here, we use CG-MALS to characterize the interaction between a dimeric viral glycoprotein (vGP) and a neutralizing antibody (IgG).

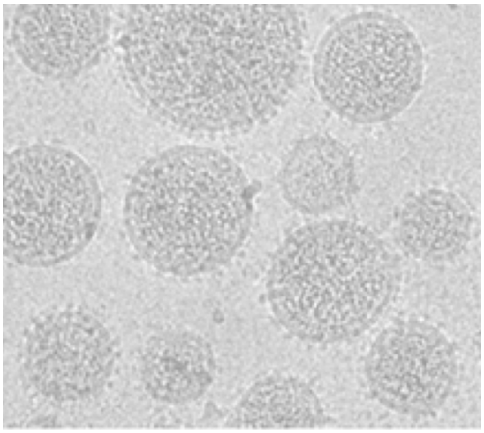


Figure 1: Electron micrograph of viral particles studded with glcyoproteins

II. Materials and Methods

Reagents

vGP was produced recombinantly from insect cells and affinity purified prior to SEC-MALS. IgG was produced recombinantly in mammalian cells from plasmids encoding the heavy and light chains and purified by Protein A affinity capture. All experiments were performed in 25 mM Tris, pH 7.5, 150mM NaCl (TBS).

SEC-MALS and Protein Conjugate Analysis

SEC-MALS experiments were performed using an S200 10/30 column (GE Healthcare) and an AKTA purifier FPLC in-line with the miniDAWN TREOS MALS detector and Optilab T-rEX differential refractive index detector. Data collection and analysis were performed with ASTRA software. Protein conjugate analysis in ASTRA was used to determine the protein fraction for vGP and a corrected extinction coefficient and dn/dc to account for the glycan content.

Determination of Equilibrium Constants

CG-MALS experiments were done with a Calypso II composition gradient system to prepare different compositions of buffer, glycoprotein and antibody and deliver to the miniDAWN TREOS and an online UV detector (GE). Polycarbonate (Millipore) filter membranes with 0.1 µM pore size were installed in the Calypso for sample and buffer filtration. vGP and IgG were diluted to a stock concentration of 50 µg/mL in TBS. Each solution was filtered to 0.02 µM using Anotop (Whatman) syringe filters and loaded on the Calypso II.

The automated Calypso method consisted of a single component gradient designed to assess self-association of the vGP and a dual-component "crossover" gradient to assess hetero-association. For each composition, 0.7 mL of protein solution was injected into the UV and MALS detectors and allowed to come to equilibrium within the MALS flow cell. For the GP self-association gradient the flow was stopped for 60 s and for the hetero-association gradient the flow was stopped for 300 s. Data collection and analysis were performed with the CALYPSO software. The extinction coefficient and dn/dc obtained by SEC-MALS for the conjugate were used to analyze the CG-MALS data.

III. Results and Discussion

Glycoprotein Characterization

Prior to analysis by CG-MALS, vGP was first analyzed by SEC-MALS to determine the average molecular weight of the glycoprotein and oligomeric state. By combining the measured UV and RI concentration signals, Protein Conjugate Analysis could be performed to determine the glycan content of vGP (Figure 2). For both injections shown in Figure 2, the glycan made up ~17% of the mass of vGP. This characterization also provided a corrected extinction coefficient and dn/dc value for future CG-MALS studies.

The molar mass measured by MALS increased as a function of eluting concentration (injection volume), which suggests an equilibrium association with fast kinetics (Figure 2). In this case, the apex molecular weight (M_P) for the 100 µL injection was 104 kDa at an eluting concentration of 66 µg/mL, and the M_P for the 400 µL injection was 118 kDa at an eluting concentration of 199 µg/mL (Figure 3). The vGP has a predicted monomer mass 47 kDa, with nine potential glycosylation sites, adding ~10 kDa in glycan mass. Thus, the molecular mass calculated for the glycoprotein using SEC-MALS corresponds to a native dimer, and the increase in molar mass as a function of concentration may suggest an equilibrium association of the dimers into higher order species.

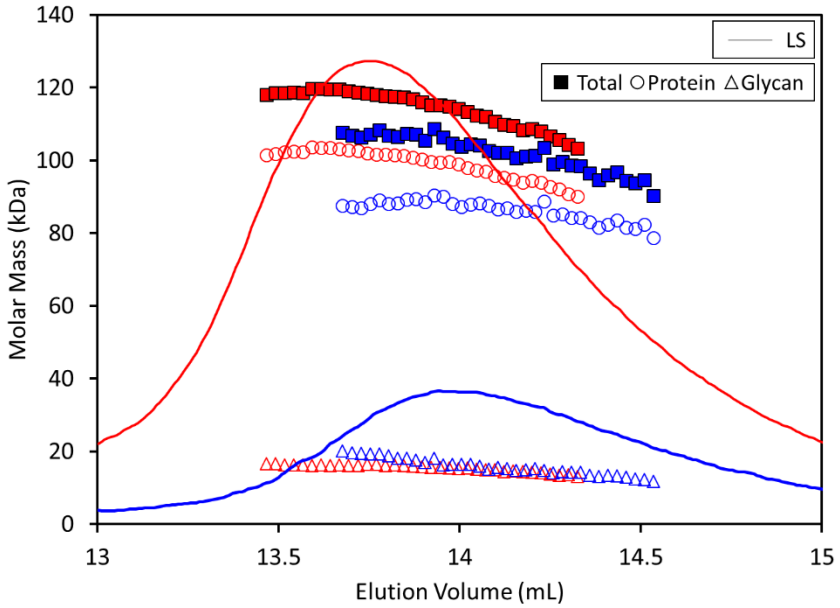


Figure 2: SEC-MALS data with Protein Conjugate Analysis of 400 µl (red) and 100 µl (blue) injections of vGP. When 400 µl of sample was run over the column, the M_P of vGP was calculated as 118kDa (82.5% protein content), corresponding to a dimer. When 100 µl of the same concentration was run over the column, the M_P of vGP was calculated as 104 kDa (83% protein content), suggesting the dimer forms with fast kinetics.

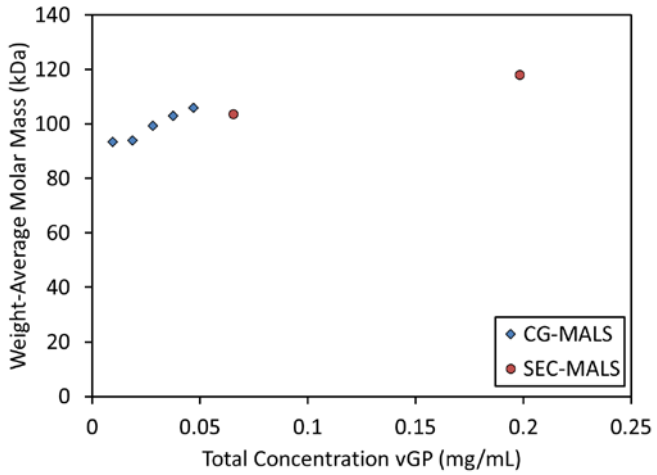


Figure 3: The weight-average molar mass of vGP increases as a function of concentration in CG-MALS experiments, suggesting equilibrium self-association, in agreement with SEC-MALS data

A similar concentration-dependent increase in molar mass was observed in CG-MALS experiments with vGP. A single-species concentration gradient for vGP, consisting of five concentrations from 9.4 µg/mL to 47 µg/mL, showed the molar mass increasing from 94 kDa to 106 kDa (Figure 3). This suggests vGP is a native dimer under the experimental conditions with a very small fraction of tetramer (dimers of dimers). However, the contribution of this species was so minor that this term was not incorporated into the best fit model. Instead, the GP sample was treated as a native, non-associating dimer.

Equilibrium Association of IgG and vGP

Automated Calypso methods measured multiple protein compositions to investigate the potential interactions present in a solution of vGP and anti-vGP monoclonal antibody (IgG). The data combined light-scattering and concentration measurements at 5 concentrations of vGP for self-association and at 17 combinations of vGP and IgG for hetero-association.

vGP was treated as a native dimer with measured molar mass of 100 kDa, which agreed well with the M_w obtained by SEC-MALS (Figure 2). The molar mass of the antibody was measured as 145 kDa, which agreed well with the formula weight mass of 144.84 kDa. The binding of the IgG to vGP was evident in the measured weight-average molar mass (M_w) during the crossover gradient (Figure 4). We observed an increase in weight-average molar mass (M_w) corresponding to complex formation, and the measured M_w increased more than two-fold compared to the M_w for a mixture of non-interacting proteins (Figure 4, gray dotted line).

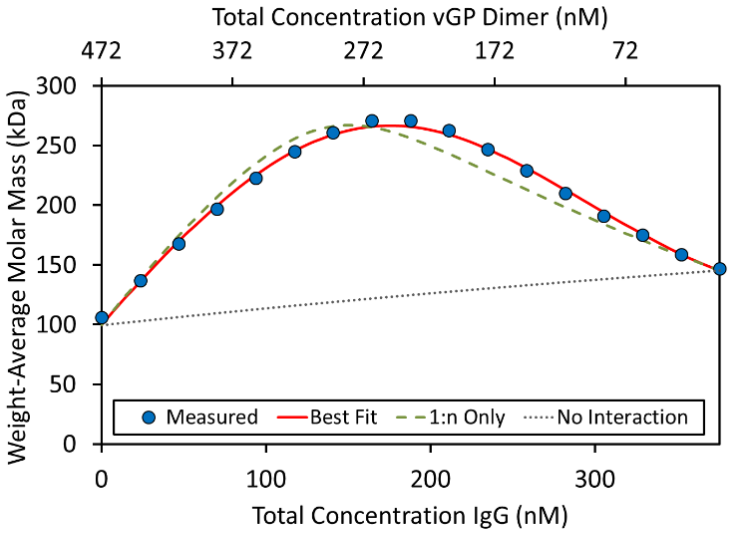


Figure 4: Weight-average molar mass for each composition of the hetero-association gradient. The formation of IgG:vGP complexes results in an increase in measured molar mass. The best fit (red line) requires higher order association than simply (IgG)(GP) and $(IgG)(GP)_2$, shown by the "1:n Only" curve (green dashed line). The molar concentration of GP refers to the dimer concentration.

The light scattering intensity and concentration data where analyzed to determine the binding affinity and stoichiometry for the interaction between vGP and IgG (Figure 5, left). The data were best described by an association model which included the following:

1. Two vGP-binding sites per antibody molecule, such that each antibody could bind one or two vGP dimers with equivalent affinity
2. A higher order complex of two antibodies bound to two vGP dimers

Importantly, an association model that incorporated a term for two equivalent antibody-binding sites per vGP dimer did not fit the data. This suggests the antibody epitope may lie at or near the dimeric interface and that steric hindrance prevents a second IgG molecule from binding. Furthermore, a model that neglects the higher order complexes described in #2 above does not fit the data (Figure 4, green dashed line), which implies there may be a small fraction of higher-order oligomers (most probably a dimer of dimers) to which multiple antibodies can bind.

The best fit yielded equilibrium association constants for the interaction between IgG and the vGP dimer as follows:

$$K_{A\,1,1} = \frac{[(IgG)(vGP)]}{[IgG][vGP]} = 3.44 \times 10^7\ M^{-1}$$

$$K_{A\,1,2} = \frac{[(IgG)(vGP)_2]}{[IgG][vGP]^2} = 2.95 \times 10^{14}\ M^{-2}$$

These correspond to a single-site binding affinity K_D = 58 nM. The equilibrium association constant for the $(IgG)_2(GP)_2$ interaction was calculated as $K_{A\,2,2} = 1.63 \times 10^{21}\ M^{-3}$, which corresponds to an average single-site binding affinity, K_D = 85 nM.

The calculated molecular weights and K_A were used to determine the concentration for each species in solution during the crossover gradient (Figure 5, right). The maximum concentration of both the (IgG)(vGP) and $(IgG)_2(vGP)_2$ species occurred when the total concentration of vGP dimer was equal to the total concentration of IgG: $[vGP]_{total} = [IgG]_{total}$ = 209 nM. Although these two species peaked at the same composition of IgG and vGP, the fraction of (IgG)(GP) was more than six times that of the $(IgG)_2(GP)_2$ (Figure 5, right). The maximum concentration of $(IgG)(vGP)_2$ occurred where the $[vGP]_{total} = 2[IgG]_{total}$ = 290 nM.

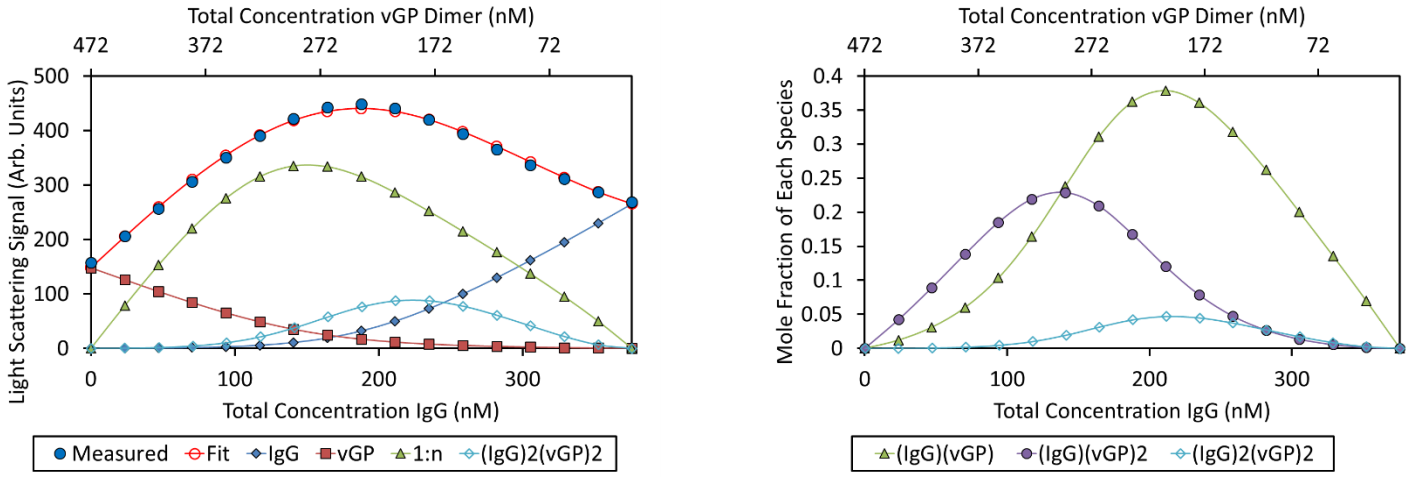


Figure 5: Best fit of CG-MALS data (left) and mole fraction of each species (right). Left: The CG-MALS hetero-association data was best fit to a model wherein each Fab of the IgG bound to the vGP dimer with equivalent affinity. The best fit (⊖) to the measured light scattering data (●) is made up of the sum of the contributions of the free monomer IgG, free vGP dimer, IgG bound to one or two vGP dimers ("1:n"), and a higher order complex of two IgG molecules bound to two vGP dimers. Right: The light scattering data are converted to molar concentration of each complex. The fraction of unbound IgG monomer and vGP dimer have been left off for clarity.

IV. Conclusions

Size-exclusion chromatography, combined with multi-angle light scattering and differential refractive index detection analysis allowed us to determine unambiguously the total molecular weight, protein component weight, and glycan content for a recombinantly expressed viral glycoprotein. Using this technique, we also show the vGP forms higher order associations that dissociate with fast kinetics.

CG-MALS was then used to quantify each species present in a solution of vGP and antibody. We expected one IgG molecule to bind up to two GP dimers with equal affinity as has previously been seen many times with other IgG-antigen interactions. However, we were surprised to find the converse was not true: one vGP dimer did not bind two IgG molecules simultaneously. Thus, using CG-MALS, we were able to understand how this antibody interacts with its antigen to greater detail than was previously known. This technique has not only given us valuable information regarding the affinity of each type of interaction but has also provided information regarding the potential epitope for the antibody, both of which are essential for formulating post-exposure treatments or designing antigens for vaccine production.

V. References

Murin CD, Fusco ML, Bornholdt ZA, Qiu X, Olinger GG, Zeitlin L, Kobinger GP, Ward AB, Saphire EO. "Structures of protective antibodies reveal sites of vulnerability on Ebola virus." *Proc Natl Acad Sci USA*. 2014. **111**(48):17182-17187.