UNIVERSITY OF GEORGIA
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# The Role of ACE2 Receptor Glycosylation in Modulating SARS-CoV-2 Spike Affinity

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## Introduction

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) viral infection causes a variety of symptoms from mild to fatal. While vaccinations and therapeutics are available, currently ~1% of cases result in death globally (W.H.O.). It is known that SARS-CoV-2 infection requires the receptor binding domain (RBD) on the SARS-CoV-2 Spike glycoprotein (Spike) to interact with the glycosylated human angiotensin-converting enzyme 2 (ACE2) receptor of the host (Hoffmann et al., 2020; Tai et al., 2020; Zhou et al., 2020). Structural analysis of the Spike-ACE2 complex, combining glycoproteomic data and molecular dynamic simulations, revealed that ACE2 glycosites interact with Spike glycosites to shield binding which may influence overall binding affinity (Fig.1) (Zhou et al., 2020). Concomitantly, structural studies show interactions between human monoclonal antibodies and Spike glycosites. Differential glycosylation on both host receptor proteins and viral spike proteins could potentially modulate the ability of SARS-CoV-2 to bind to host cells and evade antibody recognition.

Thus, we propose that glycosylation of ACE2 and Spike modulate their binding affinity. Here, we generated point mutations that remove single glycosylation sites on ACE2 and Spike and quantified their binding using biolayer interferometry (BLI). Further, the Spike RBD has rapidly evolved and resulted in many variants of concern (V.O.C.), such as Delta. Emerging strains are proposed to enhance infectivity and evade humoral immunity (Caniels et al., 2021). Thus, we analyzed the impact of Spike glycosylation and V.O.C. mutations on the binding of Spike to Covid-19 patient-derived monoclonal antibody, CR3022. This research will improve our understanding of the role glycosylation plays in Spike interactions, potentially leading to enhancements in vaccine design and therapeutic interventions.

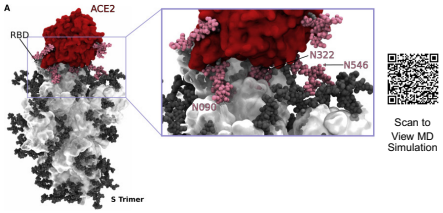


**Figure 1. ACE2 *N* –glycans located near the RBD of SARS-CoV-2 Spike protein**. Molecular dynamic simulation displays ACE2 (red) glycoforms N90, N322, and N546 (pink) interact with the Spike glycoprotein near the receptor binding domain (RBD) (Zhou et al., 2020)

## Experimental Techniques

ACE2 and Spike proteins were expressed in HEK293 S(GnTI-) cells to constrain glycosylation to the oligomannose form. Site-directed mutagenesis was performed to obtain glycosylation and V.O.C. mutants. CR3022 human IgG1 mAb was obtained from Cell Signaling (#37475). Spike was immobilized to an amine-reactive second generation (AR2G) biosensor (Sartorius) via an EDC/NHS coupling reaction, followed by quenching with ethanolamine. The shift in wavelength between immobilized Spike and unbound ACE2 is measured by the BLI Octet Red 384 instrument to generate a response. From a concentration gradient of ACE2 and response, a saturation curve is generated and fitted to derive direct binding constants ($K_D$) based on a 1:1 binding model [$Y = B_{max} * X / (K_D + X)$] using GraphPad Prism 9.0.

## Spike and ACE2 Glycoforms

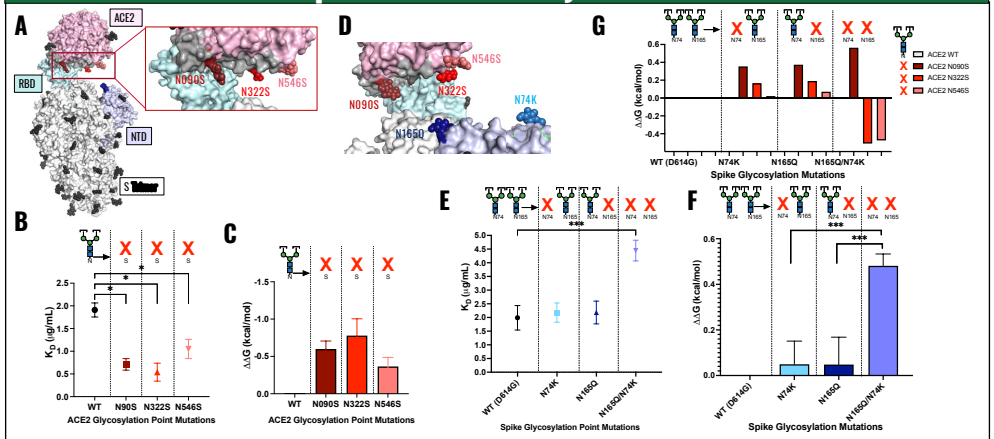


**Figure 2. Glycosylation of ACE2 and Spike modulate binding.** (A) Modeled structure of glycosylated ACE2 bound to glycosylated SARS-CoV-2 Spike trimer (PDB 7KJ2). (B) ACE2 glycosylation point mutations dissociation constants ($K_D$) Spike WT (D614G) and (C) relative binding energy calculated from $\Delta\Delta G = RTln(K_{d1}/K_{d}^{ACE2\ WT})$ derived from BLI. (D) Spike N165 and N74 glycan position near ACE2-Spike RBD interaction site. (E) Spike glycosylation point mutations dissociation constants ($K_D$) ACE2 WT and (C) relative binding energy calculated from $\Delta\Delta G = RTln(K_{d1}/K_{d}^{Spike\ (D614G)})$ derived from BLI. (G) Relative binding energy of Spike glycosylation mutants binding ACE2 glycosylation mutants using $\Delta\Delta G = RTln(K_{d1}/K_{d}^{Spike\ (D614G)})$ * p<0.05, ** p<0.005, *** p<0.0005 for Dunnett's multiple comparisons test relative to ACE2 WT, Spike WT, and ACE2 mutants, respectively.

## Spike – ACE2 Binding Affinity

| SARS-CoV-2 Variant | RBD Point Mutations |
|---|---|
| Victoria (A.1) | D614G |
| Alpha (B.1.1.7) | D614G/N501Y |
| Beta (B.1.351) | D614G/N501Y/E484K/K417N |
| Sub Beta | D614G/K417N |
| Gamma (P.1) | D614G/N501Y/E484K/K417T |
| Zeta (P.2) | D614G/E484K |
| Theta (P.3) | D614G/N501Y/E484K |
| Epsilon (B.1.429) | D614G/L452R |
| Iota (B.1.1.526) | D614G/E484K/S477N |
| Delta/Kappa (B.1.617) | D614G/E484Q/L452R |

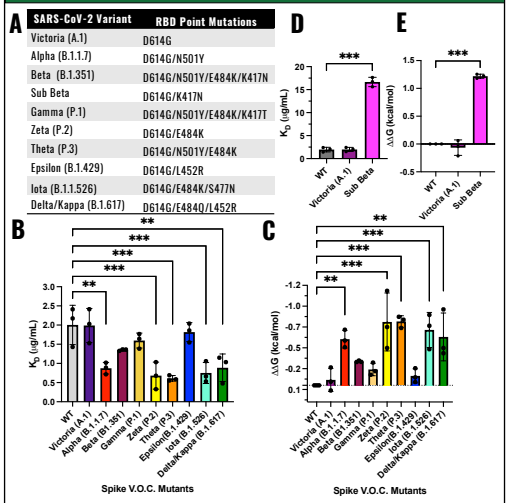


**Figure 3. SARS-CoV-2 Variants of Concern Spike glycoprotein binding to ACE2 WT.** Spike WT was expressed and purified with each set as a control. (A) Spike V.O.C. according to strain and associated RBD point mutations. (B& D) Binding constants and calculated from saturation curves based on 1:1 specific binding. (C & E) Relative binding energy calculated from $\Delta\Delta G = RTln(K_{d1}/K_{d2}^{WT})$. * p<0.05, ** p<0.005, *** p<0.0005 for Dunnett's multiple comparisons test relative to Spike WT.

## Spike – mAb Binding Affinity

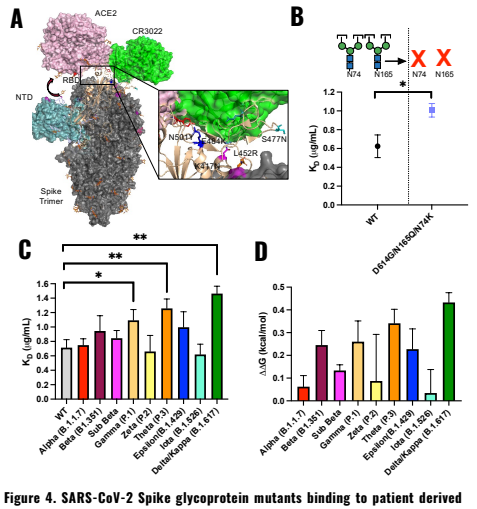


**Figure 4. SARS-CoV-2 Spike glycoprotein mutants binding to patient derived mAb CR3022**. (A) Superimposition of CR3022-Spike and ACE-2 Spike with modeled Spike V.O.C. mutations (PDB 7A5S, 7KJ2). (B) Binding constants calculated from binding curves for Spike N-glycosylation double mutant and (C) Spike V.O.C. (D) Relative binding energy calculated from $\Delta\Delta G = RTln(K_{d1}/K_{d2})$. (D) Spike V.O.C. according to strain and associated RBD point mutations. * p<0.05, ** p<0.005, *** p<0.0005 for Dunnett's multiple comparisons test relative to Spike WT.

## Discussion

Our previous studies identified the glycan profiles of both Spike and ACE2 through MS and MD simulations, which led to the question of whether glycans modulate their interaction. To address this question, Spike and ACE2 mutants were generated using single point mutations in the N-X-T/S motif that prevented glycans from being added to those sites. When ACE2 glycosites N90, N322, and N546 are removed, a significant increase in binding affinity for Spike is observed (Fig 2B & C). This agrees with predictions from MD simulations that display ACE2 N90, N322, and N546 inhibit binding to Spike (Fig. 1). Alternatively, the Spike glycosylation site N74 and N165 mutations displayed a significant decrease (Fig. 2D-F), which indicates that these N-glycans enhance ACE2 WT binding and supports previous findings of their role in stabilizing the Spike "open" conformation. To determine how changes in these specific ACE2 and Spike glycans influence binding, matrix-based binding by BLI was performed. Spike N74K/N165Q decreased affinity in ACE2 N090S, whereas it increased with ACE2 N322S and N546S (Fig2. G). This observation further supports structural studies that ACE2 N90 glycan interaction with the Spike RBD in the active state. Additionally, this data supports that Spike N74 and N165 interact directly with ACE2 N546 and N165 directly.

To determine the binding affinities between Spike V.O.C.'s (Fig. 3A) and ACE2, we obtained purified Spike RBD mutants from our collaborators and measured their binding constants to ACE2 using BLI (Fig. 3B-E). A statistically significant increase in binding affinity was observed for V.O.C. mutants containing N501Y, E484K, L452R mutations (Fig 3B&C). Interestingly, the K417N mutation, which is a part of the Beta variant, showed a statistically significant decrease in binding (16.3 μg/mL) (Fig3.D& E). When comparing relative binding energies, K417N appears to be energetically compensated for with N501Y and E84K mutations within the Beta variant (Fig3. C &E).

Next, we analyzed the binding between Spike and the patient-derived mAb CR3022. We observed an almost two-fold decrease in binding affinity to with Spike N74 and N165 removal (Fig. 4B), which reinforces their role in stabilizing the Spike "open" conformation for CR3022 binding the Spike RBD. Interestingly, Spike V.O.C. mutants containing the N501Y, E484K/Q, and L452R mutations dramatically decrease in binding CR3022 (Fig. 4C &D), which supports studies of antibody escape in emerging strains, such as Omicron which contains the following mutations.

## Conclusions

**Key Findings:**

- ACE2 N90, N322, N546 glycans are unfavorable and support its role in "glycoshielding" from Spike binding.
- Spike N74 and N165 glycans are favorable for binding ACE2 and unfavorable for CR3022 mAb binding which is consistent with structural data supporting its role in the stabilizing Spike "open" conformation..
- The unfavorable interaction between Spike N74 and N165 glycan removal and ACE2 N90 glycan removal is indicative of ACE2 N90 glycan direct interaction with the Spike RBD in the "open" state.
- ACE2 N322 and N546 glycan removal and Spike N74 and N165 glycan removal are favorable for binding, which correlate with MD simulations and structural analyses suggesting direct glycan-glycan interactions.
- Circulating variants of concern of SARS-CoV-2 containing mutations N501Y, E484K, and L452R are energetically favorable for ACE2 binding.
- Alternatively, mutations containing a combination of N501Y, E484K, and L452R are unfavorable for CR3022 mAb binding.

**Future Directions:**

- Evaluate ACE2 glycosylation mutations binding affinity to Spike V.O.C. RBD mutants.
- Determine if ACE2 glycosylation affects Spike neutralization by CR3022 by competition assays, and similarly with therapeutic mAbs.

## Acknowledgments

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