Structural remodeling of SARS-CoV-2 spike protein glycans reveals the regulatory roles in receptor binding affinity

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Significance

Glycans extensively cover the surface of SARS-CoV-2 spike (S) protein but the relationships between the glycan structures and the protein pathological behaviors remain elusive. Herein, we simplified and harmonized the glycan structures in the S protein receptor-binding domain and reported their regulatory roles in human receptor interaction. Opposite regulatory effects were observed and were determined by discrete glycan structures, which can be neutralized by the reported S309 antibody binding to the S protein. This report provides new insight into the mechanism of SARS-CoV-2 S protein infection as well as S309 neutralization.

Abstract:

Glycans of the SARS-CoV-2 spike protein are speculated to play functional roles in the infection processes as they extensively cover the protein surface and are highly conserved across the variants. To date, the spike protein has become the principal target for vaccine and therapeutic development while the exact effects of its glycosylation remain elusive. Experimental reports have described the heterogeneity of the spike protein glycosylation profile. Subsequent molecular simulation studies provided a knowledge basis of the glycan functions. However, there are no studies to date on the role of discrete glycoforms on the spike protein pathobiology. Building an understanding of its role in SARS-CoV-2 is important as we continue to develop effective medicines and vaccines to combat the disease. Herein, we used designed combinations of glycoengineering enzymes to simplify and control the glycosylation profile of the spike protein receptor-binding domain (RBD). Measurements of the receptor binding affinity revealed the regulatory effects of the RBD glycans. Remarkably, opposite effects were observed from differently remodeled glycans, which presents a potential strategy for modulating the spike protein behaviors through glycoengineering. Moreover, we found that the reported anti-SARS-CoV-(2) antibody, S309, neutralizes the impact of different RBD glycoforms on the receptor binding affinity. Overall, this work reports the regulatory roles that glycosylation plays in the interaction between the viral spike protein and host receptor, providing new insights into the nature of SARS-CoV-2. Beyond this study, enzymatic remodeling of glycosylation offers the opportunity to understand the fundamental role of specific glycoforms on glycoconjugates across molecular biology.

Covert art Legends

The glycosylation of the SARS-CoV-2 spike protein receptor-binding domain has regulatory effects on the receptor binding affinity. Sialylation or not determines the “stabilizing” or “destabilizing” effect of the

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glycans. (Protein structure model is adapted from Protein Data Bank: 6moj. The original model does not contain the glycan structure.)

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42 **Introduction:**

43 Host cell infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is initiated by the
44 interaction between the viral spike (S) proteins and the host cellular receptors, angiotensin-converting
45 enzyme 2 (ACE2).(1, 2) The S protein recognizes ACE2, mediates protease priming and then promotes
46 virus membrane fusion.(3) Because of its critical roles in the infection processes, the S protein has become
47 the principal target for vaccine and therapeutic development.(4, 5) Similar to other β-coronavirus
48 members, the SARS-CoV-2 S protein is heavily glycosylated.(6, 7) It contains 22 N-linked glycosylation sites
49 with 18 of them conserved from the SARS-CoV spike **(Figure 1A)**.(7, 8) The glycan moieties contribute
50 about 17% molecular weight to the native-state trimetric SARS-CoV-2 S protein, shielding approximately
51 40% of the protein surface.(9)

52 Glycosylation plays versatile roles in viral pathobiology.(10) For example, glycans are part of the cell entry
53 machinery of human immunodeficiency virus 1 (HIV-1). Deleting certain glycosylation sites from HIV-1
54 envelope protein reduces virus integrity and results in significantly decreased infectivity.(11) Similar
55 phenomenon in SARS-CoV-2 was reported recently by Casalino *et al*., where they found that the N165 and
56 N234 glycans on the S protein S1 subunit modulate the protein conformational transition required for
57 receptor binding.(12) Removal of these two glycosylation sites through mutation leads to 10-40%
58 reduction of ACE2 binding response, as supported by biolayer interferometry (BLI) analyses.(12) Another
59 well-known role of viral glycosylation is masking viral immunogenic epitopes from the host immune
60 systems by camouflaging them with host-derived glycans.(13, 14) This has proved to be a powerful
61 strategy to maintain the infectivity in coronavirus S protein, HIV-1 envelope protein, and influenza
62 hemagglutinin.(15-19) In SARS-CoV-2, the surface of the S protein is extensively covered by glycans,
63 indicating effective camouflaging effects against antibody recognition. However, a notable exception was
64 found in the receptor-binding domain (RBD).(9)

65 The S protein RBD contains 222 residues (R319 to F541 residues) with two glycosylation sites located at
66 N331 and N343. Strong ACE2 interactions were found in the receptor-binding motif (S438 to G504
67 residues) through hydrogen bonds and salt bridges, as revealed by crystal structures.(20, 21) **(Figure 1B)**
68 Despite its crucial role in ACE2 binding, the RBD has the lowest coverage of glycan shielding among the
69 entire protein, which makes it vulnerable to immune recognization.(12, 13) One possible explanation to
70 this phenomenon is the existence of the "up and down" conformational change of the spike protein during
71 the cell entry processes, where the RBD remains buried by the heavily glycosylated S1 unit (the "down"
72 state) during trafficking for immune evasion until it engages ACE2 at the infection interface (turning into
73 the "up" state).(3, 22) This strategy minimizes the exposure time of RBD to the surrounding local
74 environment, reducing the probability of immune recognition. However, this explanation makes the roles
75 of the RBD glycans even more intriguing, especially for the glycans at N343 that is very close to the
76 receptor-binding motif (RBM).(20) We suspect that the RBD glycans could have roles additional to glycan
77 shielding in the S protein-ACE2 interactions.

78 In-depth probing of glycans' functions during viral infection is challenging, largely due to the lack of
79 strategies to control glycan structures and minimize their micro-heterogeneity.(23-26) As a result,
80 molecular dynamics (MD) simulation has become the predominant approach for studying glycan
81 biochemistry; yet support from experimental data is in great demand.(27) In this work, we report the
82 strategies to harmonize the glycans of SARS-CoV-2 S protein RBD into controlled glycoforms, as well as
83 subsequent binding affinity measurement between human ACE2 and the glycoengineered RBD. By the

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designed combinations of glycoengineering enzymes that we have characterized, we successfully transformed the S protein RBD glycans into 1) glycoforms with harmonized terminal glycan species; and 2) structure-defined single glycoforms.(28) This work reveals the regulatory roles of S protein RBD glycan in receptor binding: a double-edged sword that can either stabilize or destabilize RBD-ACE2 interactions. In combination with their roles in glycan shielding, these insights lay the foundations for modulating the S protein's nature through glycan remodeling, which may create new strategies for vaccine and therapeutic design.

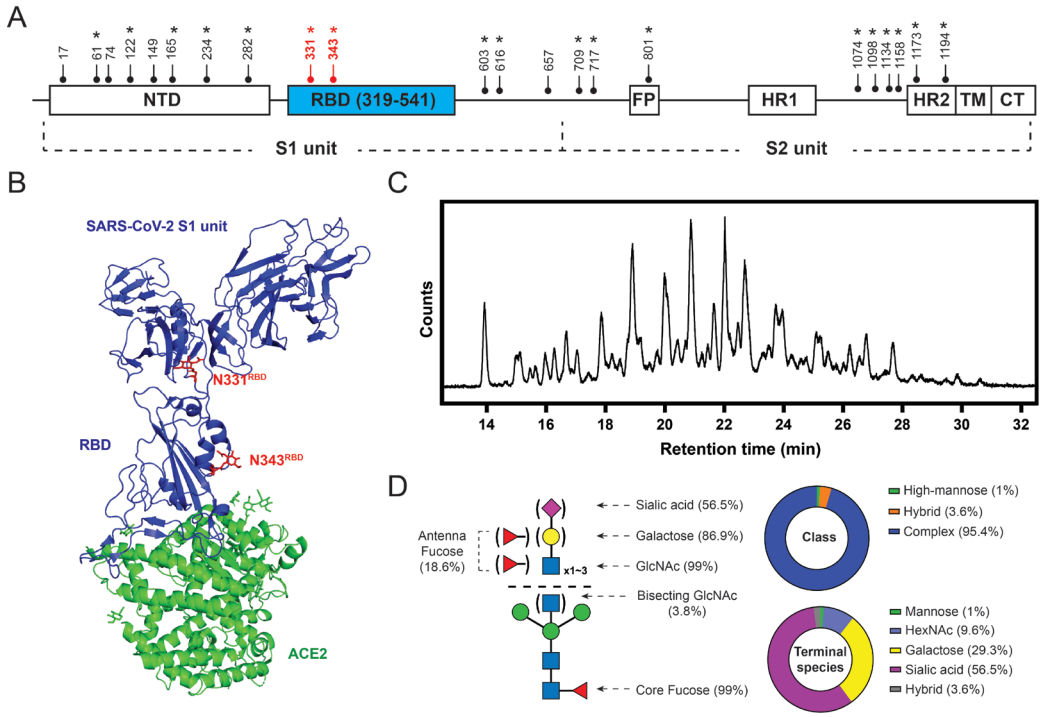

Figure 1. SARS-CoV-2 S protein glycosylation. (A) Glycosylation sites are distributed throughout the S protein structures. Star symbols (*) indicate conserved glycosylation sites with SARS-CoV S protein. NTD: N-terminal domain; RBD: receptor-binding domain; FP: fusion peptide; HR: heptad repeat; TM: transmembrane region; CT: cytoplasmic domain fusion. (B) Structure of the S protein S1-ACE2 complex with highlighted RBD glycosylation sites in red (Protein Data Bank (PDB): 7a92). (C) HILIC chromatogram of glycans collected from HEK293-expressed S protein RBD used in this study. (D) Composition of S protein RBD glycosylation described in relative abundance of glycoform(s) with each property. Left: graphic representative of the RBD glycopattern. Upper right: composition of glycan classes. Bottom right: composition of terminal saccharide species. 56.5% of the RBD glycan population contains at least one terminal sialic acid; 29.3% of the population exhibits at least one terminal galactose without having any sialic acid; 9.6% of the population terminating with GlcNAc without having any galactose and sialic acid.

Results

Dissecting the glycoforms of SARS-CoV-2 S protein RBD

Glycans in the S protein RBD comprise N-glycan species.(6, 7) Their biosynthesis occurs in the endoplasmic reticulum (ER) and continues in the Golgi in a species-, cell-, protein-, and site-specific manner. N-glycans

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97 share a common core structure made of two *N*-Acetylglucosamine (GlcNAc) and three mannose (Man)
98 residues. The core further extends into a myriad of glycoforms through the activity of glycosidases and
99 glycosyltransferases. It has been known that different glycoforms could lead to different regulatory effects
100 to protein-protein or cell-cell interactions.(29)

101 Given that glycan formation is sensitive to the expression conditions, we first analyzed the glycoforms of
102 the recombinant RBD (expressed from HEK293, GenScript Z03483) used in this study by isolating the
103 glycans using PNGase F and profiling them by liquid-chromatography mass spectrometry (LC-MS) analysis.
104 (**Figure 1C**) Our substrate RBD exhibits heterogeneous glycoforms composed of complex-type species
105 (95.4%), with three antennae the highest number we observed, and small amount of high-mannose (1%)
106 and hybrid-type (3.6%) species. **(SI-Data)** As summarized in **Figure 1D**, GlcNAc, galactose, and sialic acid
107 (SA) appear as the terminal monosaccharide at the non-reducing ends of N-glycans with relative
108 abundances of 9.6%, 29.3%, and 56.5%, respectively. Over 99% of the RBD glycans contain the core fucose
109 and 18.6% of the glycoforms have additional fucose located at the complex-type glycan antennae, as
110 supported by glycosidase treatment experiments. **(Figure S1)** *N*-Acetylgalactosamine (GalNAc), the
111 epimer of GlcNAc, likely exists in the RBD glycans since *N*-Acetylglucosaminidase (GlcNAcase) alone was
112 not able to remove all the *N*-acetylhexosamine (HexNAc) residues, unless GalNAcase was also added.
113 **(Figure S2)** In addition, retention time comparison using glycan standards suggested that a small amount
114 of bisecting N-glycan species exist, as indicated by FA2B glycoform ($[Man]_3[GlcNAc]_5[Fuc]_1$). **(Figure S3)**
115 We note that our analyses based on isolated glycans did not provide site-specific information for the
116 glycosidic linkages. The reported numbers here are averaged results from the glycan populations at N331
117 and N343.

118 <u>The RBD glycans can stabilize RBD-ACE2 interaction</u>

119 We used BLI to study the interaction between the S protein RBD and ACE2 expressed from HEK293. The
120 RBD with native glycoforms binds to ACE2 with an equilibrium dissociation constant (K_D) of 99 ± 12 nM,
121 similar to earlier reports.(30) The terminal saccharides of mature glycans often regulate the biochemical
122 properties and functions of glycoconjugates. A good example comes from the human blood group
123 antigens that are classified by their terminal residue species.(31) Therefore, we aimed to harmonize the
124 RBD glycan termini and investigate their potential effect on the RBD-ACE2 interaction. First, we created
125 glycoengineered RBD glycoprotein with all N-glycans ending in terminal HexNAc by incubating the native
126 RBD with α2-3/6/8 neuraminidase, β1-4 galactosidase, and α1-2,3/4 fucosidases. **(Figure 2, Table S1)**
127 Interestingly, we found that the resulting substrate, named tHexNAc-RBD, had improved binding affinity
128 to ACE2 with a K_D value of 47 ± 8 nM. **(Figure 3A-B)** Direct comparison of ACE2 binding curves showed a
129 20% increase in binding response in tHexNAc-RBD compared to the native RBD. **(Figure S4)** Furthermore,
130 we prepared glycoengineered RBD bearing (i) the core glycan ($[Man]_3[GlcNAc]_2[Fuc]_1$, core-RBD), and (ii)
131 species terminating with galactose (tGal-RBD) by introducing *N*-Acetylglucosaminidase/*N*-
132 Acetylhexosaminidase cocktail and Galactosyltransferase to the reactions, respectively. Similar to the
133 tHexNAc-RBD, having glycans terminating with mannose and galactose slightly improved the ACE2 binding
134 affinity relative to the native RBD. This result suggests that certain glycoforms can facilitate RBD-ACE2
135 interactions.

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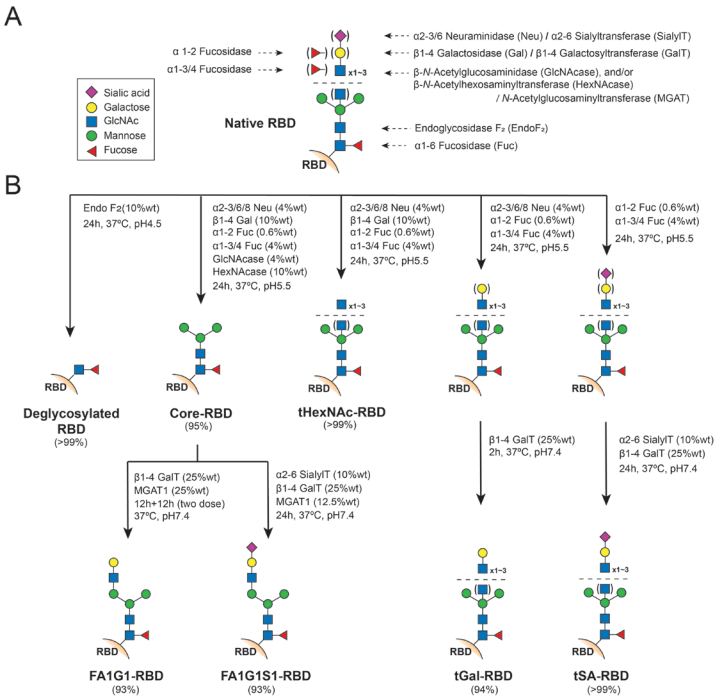

Figure 2. Scheme of the S protein RBD glycan remodeling routes. (A) Enzymes used in this work and their corresponding saccharide targets. (B) Preparation of the S protein RBD with controlled glycoforms using enzymatic reactions. The yield indicates estimated populations of the desired glycoform(s).

Recently published simulation studies by Mehdipour *et al.* provided a plausible explanation to our observation.(32) In addition to the S protein RBD, ACE2 is also a heavily glycosylated protein.(33) Both the RBD glycan at N343 ($N343^{RBD}$) and the ACE2 glycan at N322 ($N322^{ACE2}$) are close to the binding interface with a distance of only 31.3 Å between the two glycans (Asn side chains, **Figure 3C**). Mehdipour and co-workers investigated the ACE2 glycan dynamics and found that the $N322^{ACE2}$ glycans interact with the RBD protein backbone as well as the $N343^{RBD}$ glycans. Modeling analysis indicated that these interactions generate 250-360 kJ/mol binding force, which contributes to about one-third of the RBD-ACE2 binding energy (890-1040 kJ/mol).(32) In other words, the RBD glycans interact with ACE2, which explains why remodeling their structures led to different binding affinities. To examine this explanation, we measured the ACE2 binding affinity of deglycosylated RBD prepared by endoglycosidase F2 treatment.(34, 35) **(Figure S5)** An apparent reduction in binding response was found with a K_D value of 110 ± 3.3 nM, a weaker affinity than that of native RBD. **(Figure 3B)** Moreover, when the ACE2 N-glycans were removed, a dramatic decrease of binding affinity was observed. **(Figure S6)** In combination with the published simulation study, our result suggested that the RBD glycans can stabilize ACE2 binding, likely through the interactions with the $N322^{ACE2}$ glycans or the ACE2 backbone.

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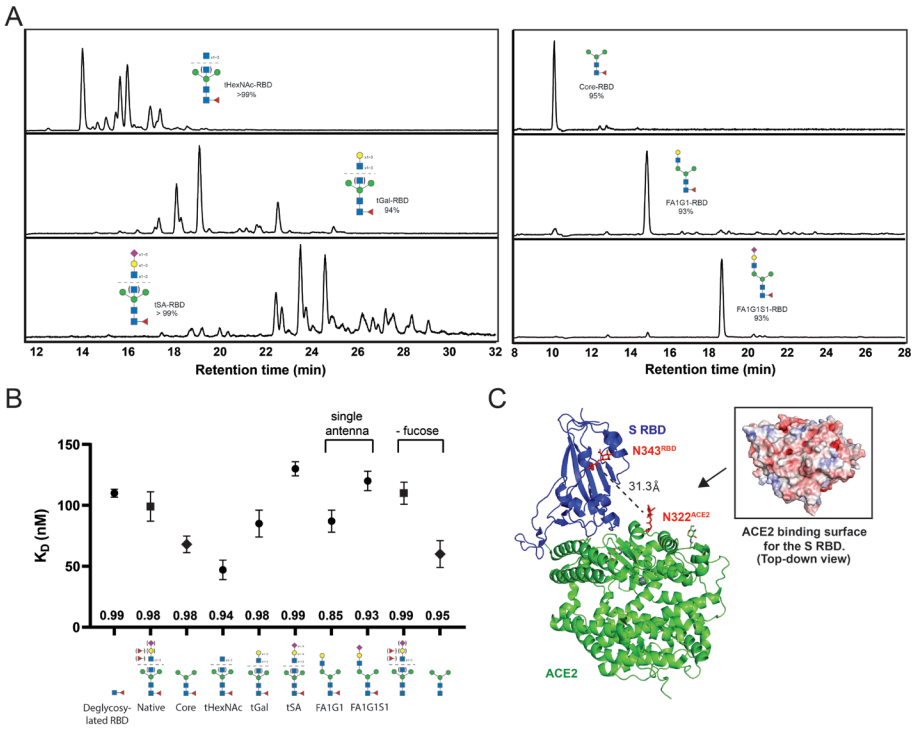

Figure 3. Glycans of the S protein RBD affect ACE2 binding affinity. (A) Chromatograms of glycans collected from glycoengineered RBD in this study. Left: the RBD with harmonized terminal glycan species. Right: the RBD substrates with single mono-antennary glycoforms. (B) Binding affinity measurement (BLI) between human ACE2 and the S protein RBD with controlled glycoforms. The numbers located at the bottom of each data point indicate the R^2 value of curve fitting. Error bars: standard error of mean. (C) Structure of the RBD-ACE2 complex revealed potential interaction between the RBD N343 glycan and ACE2 glycans/amino acid backbone (PDB: 6m0j). Insertion displays the electrostatic potential of ACE2 surface that potentially interacts with RBD N343 glycan. Red: negatively charged; blue: positively charged.

154

155 <u>Glycans with terminal sialic acid destabilize RBD-ACE2 interactions</u>

156 Sialic acids are often involved in biomolecular interactions because they are usually found at glycan non-
157 reducing end termini. For example, binding to sialic acid and subsequent release by neuraminidase are
158 required events for influenza virus infection.(36) By incubating α2-6 sialyltransferase with the tGal-RBD,
159 we introduced sialic acids to the terminus of the RBD glycans. The resulting tSA-RBD contains
160 heterogeneous glycoforms with over 99% of the population bearing at least one terminal sialic acid.
161 **(Figure 3A, SI-Data)** Unexpectedly, BLI measurement showed that the tSA-RBD has lower binding affinity
162 to ACE2 (K_D = 130 ± 6 nM) compared to the Native and deglycosylated RBD. We reasoned this result could
163 be attributed to the electrostatic repulsion between the $N343^{RBD}$ glycan and the ACE2 surface.
164 Computational calculation of electrostatic potential has shown that the ACE2 surface is predominantly
165 negative, including the area that $N343^{RBD}$ glycan engages.(37) **(Figure 3C)** Therefore, sialylated glycoforms,
166 which are negatively charged, could cause electrostatic repulsion and destabilize RBD-ACE2 interactions.
167 Given that the native RBD has a heterogeneous glycan profile, the existence of the sialylated glycan
168 species possibly negates the stabilizing effects resulting from other glycoforms, which explains the weaker
169 ACE2 binding affinity in native RBD over non-sialylated glycoforms. Recent reports of site-specific glycan
170 mapping have also indicated that both native $N343^{RBD}$ and $N322^{ACE2}$ glycosylation sites have a low content

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171 of sialylated glycan species, further implying that sialylation may not be favored for RBD-ACE2
172 interactions.(7, 33)

173 Another possible explanation of the reduced ACE2 binding affinity found in tSA-RBD is the increased steric
174 hindrance caused by sialylation. Having over-constructed glycan structures nearby the receptor-binding
175 motif could prevent the S protein from approaching ACE2. To test this possibility, we constructed
176 harmonized mono-antennary glycans on RBD, as confirmed by LC-MS analysis **(Figure 3A, S7, SI-Data)**.
177 The FA1G1-RBD bears an octa-saccharide with only one antenna connected to the α1-3-linked core
178 mannose. This was achieved by incubating the core-RBD with *N*-acetylglucosaminyltransferase I (also
179 known as MGAT1) and galactosyltransferase in one pot. Similarly, the FA1G1S1-RBD contains an additional
180 sialic acid and was prepared by introducing sialyltransferase to the reaction. BLI analyses showed that the
181 FA1G1-RBD has improved ACE2 binding affinity compared to native RBD; by contrast, FA1G1S1-RBD
182 showed weaker binding than native RBD, which is consistent with the data found earlier. **(Figure 3B)** This
183 result suggested that steric hindrance is not the major cause of the ACE2-binding affinity reduction found
184 in the tSA-RBD. Instead, the electrostatic repulsion is more likely the reason. Our results, together, suggest
185 that the RBD glycans have regulatory effects on the RBD-ACE2 interactions: non-sialylated glycoforms
186 reinforce the binding while sialylated glycoforms destabilize it.

187 <u>Fucose in the RBD glycans does not have apparent impacts on ACE2 binding</u>

188 Glycosidase treatment provided direct evidence that the core structure of the complex-type RBD glycans
189 is fucosylated because afucosylated core was not detected. **(Figure 3A, S1)** The core fucose linked to the
190 reducing end GlcNAc has been a hot target of interest for pharmaceutical research because of its
191 regulatory effects on protein-protein interactions, such as immunoglobulin G (IgG) and its receptors.(38)
192 To investigate the role of fucosylation in the RBD-ACE2 interaction, we prepared partially defucosylated
193 RBD using α1-6 fucosidase to target the core fucose. A 10-day reaction converted approximately 45%
194 native glycans into non-fucosylated forms. **(Figure S8)** It is known that the activity of this enzyme is
195 glycoform-dependent, where lower structural complexity leads to higher enzyme activity.(28) Therefore,
196 we used the core-RBD as the substrate for the reaction and successfully remove fucose from over 90% of
197 the core glycans. **(Figure S8)** BLI measurement showed that both defucosylated RBD substrates have no
198 significant difference in ACE2 binding affinity compared to their parent species. **(Figure 3B)** This result is
199 not surprising because the core fucose is shielded close to the RBD peptide backbone, which minimizes
200 its probability of interacting with ACE2 or ACE2 glycans. In addition to the core-fucose, approximately
201 18.6% RBD glycans contain α1-2 and α1-3/4 fucose which can be removed by corresponding fucosidases.
202 **(Figure S1)**. Their removal did not result in a significant difference in ACE2 binding affinity (K_D = 110 ± 14
203 nM).

204 <u>Monoclonal Antibody S309 neutralizes the regulatory effects of the RBD glycans</u>

205 Neutralizing antibodies have presented effective therapeutics for viral infection treatment.(39) An
206 antibody, named S309, isolated from a SARS-CoV-infected patient was reported to have strong cross-
207 neutralization activity on SARS-CoV-2 through the induction of antibody-dependent cell cytotoxicity
208 (ADCC) and antibody-dependent cellular phagocytosis (ADCP).(40) S309 has a distinct RBD binding
209 interface from that of ACE2. It recognizes a SARS-CoV-2 S protein RBD epitope consisting of two regions,
210 residues 356-361 and residues 440-444. Notably, the S309 binding interface sandwiches the $N343^{RBD}$
211 glycosylation site and shows potential interaction with the glycan structure, especially the core fucose.(40)
212 **(Figure 4A)** To investigate this interaction, we measured the binding affinity between S309 and the RBD

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with differently engineered glycan structures. BLI analyses showed strong RBD-S309 interaction with K_D values at nanomolar scale. However, no significant difference was found when the RBD glycan bore different terminal saccharide species, so as the removal of the core fucose. **(Figure 4B)** This result suggested that the N343^{RBD} glycan is not essential for the RBD-S309 binding. The RBD-S309 interaction is likely established on the amino acid epitope of the RBD.

Despite the N343^{RBD} glycan is not involved in the antibody binding, its structure has been sterically hindered by the S309-RBD interaction, which could restrict its probability of interacting with ACE2 and/or ACE2 glycans. **(Figure 4C)** To test this possibility, we measured the ACE2 affinity of the RBD samples that were bound to S309. As expected, comparable binding affinities ($K_D \approx 90$ nM) were observed on the native RBD, tHexNAc-RBD and tSA-RBD that were saturated with S309. **(Figure 4D)** Namely, S309 neutralizes the regulatory effect of the RBD glycan. Given that the native RBD glycans tend to stabilize ACE2 interaction owing to the low content of sialylated glycoforms, the RBD-S309 binding could compromise this stabilizing effect and reduce the infectivity of the virus, which presents a new inhibitory mechanism in S309 neutralization.

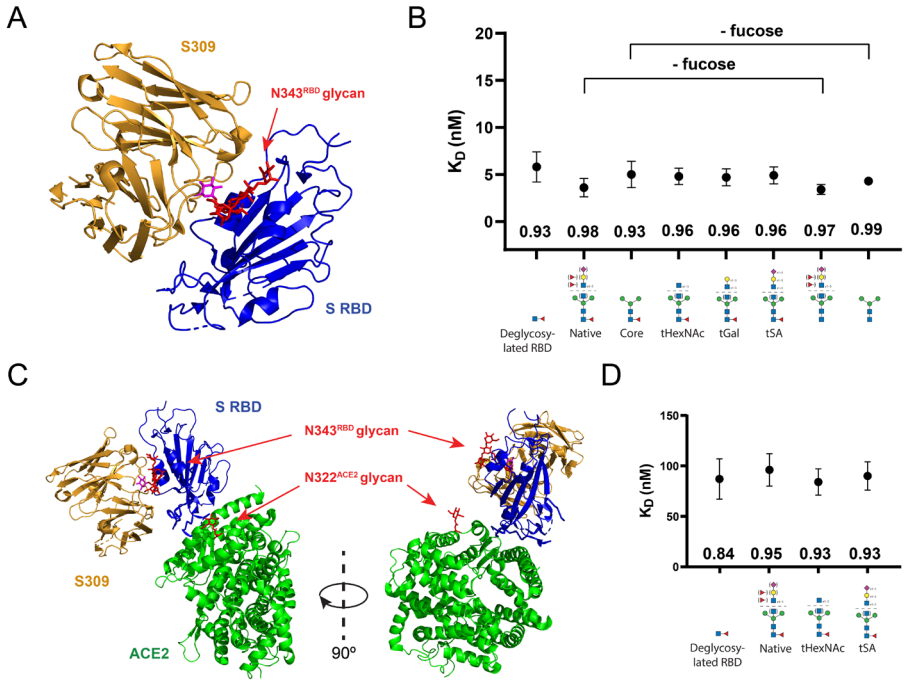

Figure 4. Antibody S309 neutralizes the regulatory effect of the RBD glycans. (A) The N343^{RBD} glycan is located at the RBD-S309 binding interface. (PDB: 6wps) The core fucose is highlighted in pink. (B) RBD glycans are not involved in S309 binding. No significant difference was observed in the binding affinity analysis. (C) The RBD-S309 interaction hinders the accessibility of the N343^{RBD} glycan to ACE2 and ACE2 glycans. The composite model was generated using the RBD-S309 cryo-EM structure (PDB: 6wps) reported by Pinto *et al.* and the RBD-ACE2 crystal structure (PDB: 6m0j) reported by Lan *et al.*[20, 39] (D) The RBD-S309 binding neutralizes the regulatory effect of the RBD glycan on ACE2 interaction. The numbers located at the bottom of each data point indicate the R^2 value of curve fitting. Error bars: standard error of mean.

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230 **Discussions**

231 The COVID-19 pandemic has caused numerous deaths worldwide. While the emerging vaccines have
232 partially relieved the immediate suffering, chronic symptoms could have persisted in many who have
233 contracted the virus.(41) With the rapid evolution of SARS-CoV-2, a comprehensive understanding of the
234 virus pathology is in urgent demand for developing vaccines and effective therapeutics to cease the spread
235 of the virus variants.(42-44)

236 Glycans play versatile structural and functional roles in protein biochemistry, but it has remained
237 historically challenging to elucidate the phenotypic roles of discrete glycoforms. Given the proximity of
238 the RBD glycans to the binding interface with ACE2, investigating their roles in its biological properties
239 could provide insights into the infection mechanism.(7, 8) We have used an enzymatic remodeling
240 approach to remove the native glycosylation microheterogeneity and decorate the S protein RBD with
241 specific glycan structures in order to study their impact on receptor binding.(28) We found that the
242 neutrally charged RBD glycans can stabilize ACE2 binding, likely through the interaction with ACE2 glycans,
243 as suggested by published simulation studies.(32) Remarkably, this effect is reversed when negatively
244 charged sialic acids are present on the RBD glycans, a destabilizing force caused by electrostatic repulsion.
245 The dissociation constants measured herein varied approximately 3-fold, based solely on the displayed N-
246 glycan structures. The results suggest that SARS-CoV-2 infectivity could be altered by manipulating the
247 content of sialic acid on the RBD glycans. For example, neuraminidase inhibitors could mitigate the viral
248 infection by halting the removal of sialic acid from the spike protein. Although anti-influenza
249 neuraminidase inhibitors have proven to be inactive for SARS-CoV-2 treatment, strategies that regulate
250 the neuraminidase and sialyltransferase activities on the RBD glycans are still worth investigating.(45)

251 In addition, we found that the binding of S309 neutralizes the regulatory effects of the RBD glycan,
252 preventing the potential stabilization of ACE2 interaction through the glycan structure. Given that the
253 glycosylation sites of the RBD are highly conserved across its evolution reported to date, glycans could
254 provide new handles for developing strategies to combat the emerging virus variants **(Figure S9).** For
255 example, glycan remodeling could be applied to protein subunit vaccines to maximize the mimicry of viral
256 glycoproteins and improve the immune response. Generally speaking, this work demonstrates that it is
257 possible to efficiently screen the biological behavior of discrete protein glycoforms, an approach that will
258 greatly advance our fundamental knowledge of glycobiology and reinforce our ability to design targeted
259 strategies to defeat disease.

260 **Data Availability**

261 All data are available upon reasonable request to the corresponding authors.

262 **Author contributions**

263 Method development for glycan remodeling, binding affinity measurements, LC-MS analysis, and data
264 analysis was performed by Y-P. H. MALDI-ToF analysis was performed by D. M. and V. S. All the authors
265 were involved in the design of the research and manuscript drafting.

266 **Competing interests**

267 The authors declare no competing interests.

268 **Acknowledgment**

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