Electronic Supplementary Material (ESI) for ChemComm.
This journal is © The Royal Society of Chemistry 2023

Supporting Information for

O-GalNAc glycosylation affects the immunogenicity of receptor-binding domain (RBD) of SARS-CoV-2 spike protein

Yongheng Rong,[a] Xingyun Wang,[b] Weian Mao,[a] Min Chen,[a] Shengjun Wang,[c] Peng George Wang,[b] Yunjiao He[b*] & Yun Kong[a*]

[a]National Glycoengineering Research Center, Shandong Key Laboratory of Carbohydrate Chemistry and Glycobiology, State Key Laboratory of Microbial Technology, Shandong University, Qingdao, 266237, China.

[b]School of Medicine, Southern University of Science and Technology, Shenzhen, 518055, China.

[c]School of Health and Life Sciences, University of Health and Rehabilitation Sciences, Qingdao 266071, China

*Corresponding author: Yunjiao He, e-mail: heyj@sustech.edu.cn; Yun Kong, e-mail: kongyun@sdu.edu.cn

Table of Contents

Table S1.

Primers used in this study

Primers	Sequence
ppGalNAc-T2-for	5’-CCGGAATTCAAAAAGAAAGACCTTCATCAC-3’
ppGalNAc-T2-rev	5’-ATAAGAATGCGGCCGCCTGCTGCAGGTTGAGCGTGAA-3’
5’*AOX* I	5’-GACTGGTTCCAATTGACAAGC-3’

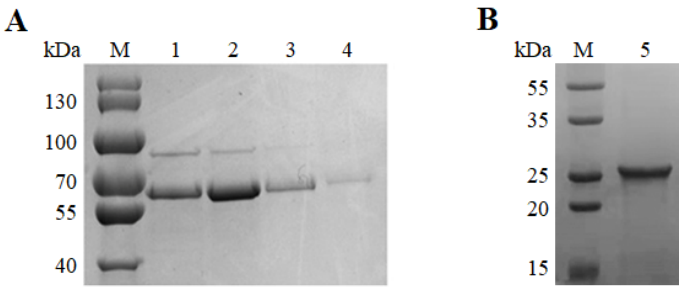

Figure S1. Coomassie-stained SDS-PAGE analysis of ppGalNAc-T2 (A) and RBD (B).

M: protein molecular marker, Lane 1: ppGalNAc-T2 eluted with 50 mM imidazole, Lane 2: ppGalNAc-T2 eluted with 100 mM imidazole, Lane 3: ppGalNAc-T2 eluted with 250 mM imidazole, Lane 4: ppGalNAc-T2 eluted with 500 mM imidazole, Lane 5: purified RBD protein

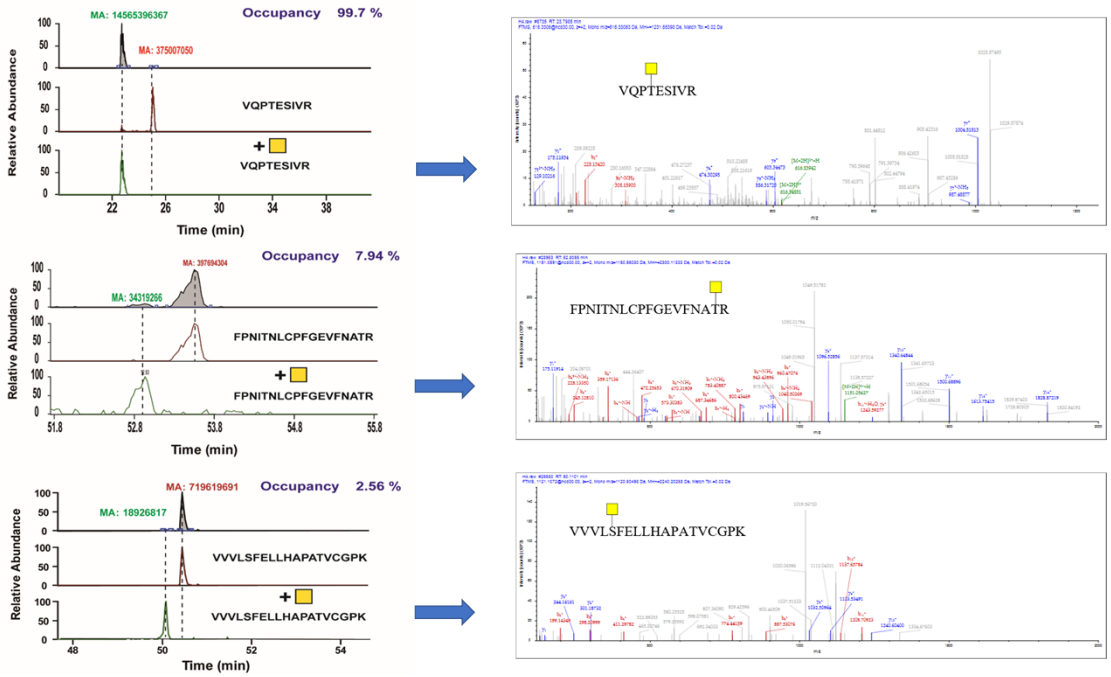

Figure S2. LC-MS/MS spectra analysis of *O*-glycosylation sites of Tn-RBD glycoprotein.

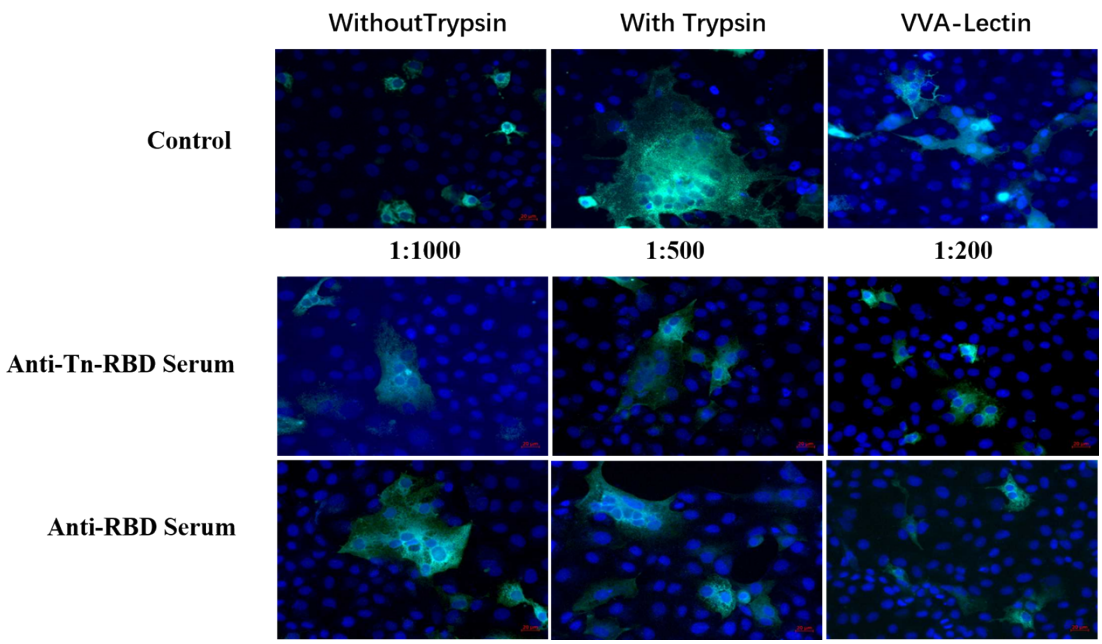

Figure S3. Anti-RBD, anti-Tn-RBD sera and lectin affect trypsin-induced cell syncytium formation. Vero E6 cells were transfected with the furin recognition mutation of SARS-CoV-2 S-protein mutants (R685A)-GFP. After 48 h, the cell culture medium was changed to DMEM (no serum), treated with antibodies or VVA lectin, and incubated for 1 h at 37°C. The cells were then treated with trypsin at 15 mg/mL for 2 h at 37°C or left untreated. Cells were fixed with 4% paraformaldehyde (PFA) and stained with DAPI. Syncytia is formed by fusion of an infected cells with neighboring cells leading to the formation of multi-nucleate enlarged cells. Thus, the number of enlarged cells is reduced when antibody sera/lectins are present.

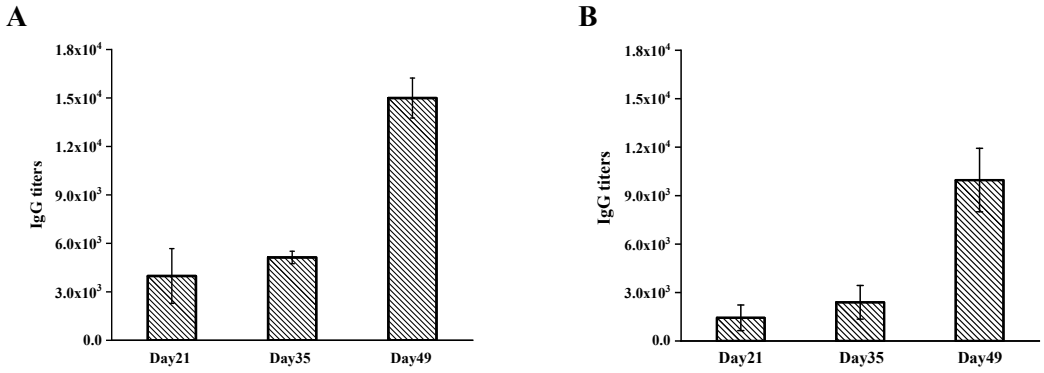

Figure S4. Titers of IgG antibodies of mice immunized with Tn-RBD.

Titers of IgG antibodies of mice immunized with Tn-RBD detected by Tn-RBD (A). Titers of IgG antibodies of mice immunized with Tn-RBD detected by RBD (B).

Figure S5. Amino acid sequences alignment of spike protein RBD from SARS-CoV-2 Wuhan strain, Delta variant (B.1.617.2) and Omicron variant (B.1.1.529).

Amino acids with white background indicate mutation positions on RBD and red arrows indicate identified *O*-glycosylation sites in this study.

Bacterial strains, media and chemicals

Pichia pastoris GS115 (his4^{-}) and pPIC9K vector were purchased from Invitrogen (Thermo Fisher Scientific). *Escherichia coli* DH5α and BL21 (DE3) chemically competent cells were purchased from Vazyme (Nanjing, China). 2×*ApexHF* HS DNA polymerase was purchased from Accurate Biotechnology (Changsha, China). Murine anti-His monoclonal antibody and HRP-conjugated secondary antibody were got from Genscript Bio-Technology (Nanjing, China). HRP-conjugated goat anti-mouse IgM, IgG, IgG1, IgG2b, IgG2c and IgG3 were purchased from Abcam. Biotinylated vicia villosalectin (VVA) was bought from Vector Laboratories. The sugar nucleotides were purchased from Glycogene (Wuhan, China). All other chemicals and solvents were purchased from Sangon-Biotech (Shanghai, China).

Cloning, expression, and purification of human ppGalNAc-T2

Full-length gene fragment of human ppGalNAc-T2 was cloned from A549 cDNA. Then the DNA fragment with the depletion of the cytoplasmic and transmembrane parts of ppGalNAc-T2 was cloned into the *Eco*R I and *Not* I restriction sites of the pPIC9K plasmid with the addition of a His tag at the C-terminal. The primers used were as shown in Table S1. Resultant clones, named pPIC9k-sol-*ppGalNAc-T2*, was selected by ampicillin and confirmed by DNA sequencing which was performed by Tsingke Bio-Technology (Qingdao, China). The plasmid was linearized with *Sac*I and introduced into *Pichia pastoris* GS115 strain via the Gene PulserXcell Electroporation System (Bio-Rad). The multicopy insert of transformants were selected with MD plates and subsequently YPD plates containing different concentrations of geneticin (0.5 mg/mL, 1 mg/mL, 2 mg/mL and 4 mg/mL). The geneticin-resistant clones were confirmed by PCR with 5'-*AOX*I primer and ppGalNAc-T2-rev reverse primer, the positive clones from 4 mg/mL geneticin plates were selected for the expression.

Recombinant yeast clones were grown at 30°C in 50 mL BMGY until the OD600 reached 2-5. Cells were harvested and cultured in BMMY with pH 6.5 for 3-4 days at 25°C and 1% methanol (v/v) was added to the culture every 24 h. The fermentation culture was collected and the secreted recombinant proteins were purified using Ni^{2+}-NTA agarose. The cell-free supernatant was loaded onto the Ni^{2+}-NTA column pre-equilibrated with binding buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10 mM imidazole). After washed with 10 column volumes of binding buffer, the target proteins were eluted with elution

buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 250 mM imidazole). The fractions containing the purified target proteins were desalted and stored at -80°C.

Cloning, expression, and purification of RBD

The RBD domain (residues Arg319–Phe541) of the SARS-CoV-2 spike protein was cloned into the pET21a expression vector (Invitrogen) with a C-terminal 6×His tag for purification. The construct was transformed into bacterial BL21 (DE3)-pLysS competent cells and a single colony was cultured in Luria Broth (LB) media for protein expression. The bacterial pellet was lysized using a high-pressure homogenizer; the target protein was present in inclusion bodies, which were washed with 2 M urea buffer and then solubilized in 8 M urea–containing buffer (50 mM Tris, pH 9.0, 8 M urea, 10 mM beta-ME). The denatured protein was purified through Ni^{2+}-affinity chromatography and size exclusion chromatography under denaturing conditions. Protein refolding was performed through fast dilution in refolding buffer (50 mM Tris, pH 9.0, 0.4 M arginine, 5 mM GSH, 0.5 mM GSSG) to decrease the concentration of urea.

Enzymatic synthesis of Tn-RBD glycoprotein

The preparation of Tn-RBD glycoprotein was carried out by *in vitro* glycosylation reaction. The recombinant unglycosylated RBD expressed in *E. coli* was used as the substrate. The reaction was operated as follows: recombinant RBD protein (1 mg), UDP-GalNAc (5 mM), Tris-HCl buffer (25 mM, pH 7.5), $MnCl_2$ (10 mM) and 5 μg ppGalNAc-T2. The reaction mixtures were incubated at 37°C for 4 h with shaking at 180 rpm. Additional UDP-GalNAc was added and the mixture was incubated for another 4 h at 37°C. The obtained Tn-RBD glycoprotein was stored -80°C after purification.

SDS-PAGE, western blot, lectin blot and migration analyses of RBD glycosylation *in vitro*

The recombinant unglycosylated and glycosylated RBD samples were run on 12% SDS-PAGE gels with DTT reduction, and transferred onto PVDF membranes for 90 min. For western blot, after blocked in 5% BSA for 1 h, the membranes were incubated with anti-His-tag antibody at 4°C overnight. Blots were detected with ECL Western Blotting Substrate (Thermo Fisher Scientific) following incubation with HRP-conjugated secondary antibody for 1 h at room temperature. The steps of VVA-lectin blot are the same as western blot except that the blocking buffer is changed to 1% polyvinylpyrrolidone. RBD samples were run on SDS-PAGE gel. Measure the distances from the top of the separating gel to the

bands marked with different protein molecular weights and make a standard curve between the distance and protein molecular weight. Measure the distances from the top of the separating gel to the unglycosylated and glycosylated RBD samples and the change of migration can be estimated from the standard curve.

Immunogenicity assessment

Test mice (BALB/c strain, 8-10 weeks old) groups were established randomly. 30 μg (100 μL) unglycosylated or glycosylated RBD proteins (PBS as control) were injected subcutaneously into mice under the scruff as an emulsion in complete Freund's adjuvant on day 0. Boosters were given subcutaneously under the scruff mixed with incomplete Freund's adjuvant on day 14, 28 and 42. 100-200 μL blood samples were collected from the orbital sinuses of mice on days 0, 21, 35, 49, and 63. The samples were centrifuged at 1200×g for 20 min after clotting and the serum samples were stored at -20°C until further analysis. All animal experiments were conducted by the guidance of the Animal Care and Use Committee of Shandong University.

ELISA analysis

Titers of antibodies were determined from the mouse serum samples by ELISA. 96-well microtiter plates were coated with various antigens (100 ng/well, 100 μL) in carbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$) at 4°C overnight. After antigen coating, the plates were washed with PBST (200 μL/well) for three times and blocked with 1% BSA in PBS (100 μL/well) for 1 h at room temperature. Then the plates were washed with PBST and incubated with serial dilutions of sera in 0.1% BSA/PBS (100 μL/well) at 37°C for 2 h. After the plates were washed three times, HRP-conjugated antibodies were added at an appropriate dilution. Subsequently, the plates were incubated at 37°C for 1 h and washed for three times. Add TMB working solution (100 μL/well), incubate for 15 minutes and protect from light, and then add 0.5 M sulfuric acid (50 μL/well) to stop the reaction. The absorbance was measured at 450 nm with BioTek Cytation™ 5. All samples were performed in triplicate.

Bio-layer interferometry (BLI)

The binding affinities of hACE2 with RBD and Tn-RBD were performed by BLI on an Octet Red 96 instrument (ForteBio, USA) using Ni-NTA biosensors. The experiment followed a four-step sequential assay at 25°C. First, 200 μL of diluted RBD samples and buffer were applied in a 96-well plate, and 10

μg/mL His-tagged ACE2 diluted with 20 mM PBS was loaded on biosensors. The biosensors were dipped into PBS for 300 s to reach baseline, then incubated with 3-fold serial diluted RBDs (from 1500 nM to 6.2 nM) in PBS for association, and PBS for dissociation. Results were analyzed by ForteBio Data Analysis software. The K_D value of RBD or Tn-RBD binding affinity to hACE2 was calculated from the binding curves based on the global fit to a 1:1 Langmuir binding model with an R^2 value of ≥ 0.95. The kinetically derived affinities were calculated as $K_D = k_{dis}/k_{on}$. Binding experiments were performed at 25°C. Data were analyzed using Octet Data Analysis Software 9.0 (ForteBio, Menlopark, CA, USA).

Trypsin-induced cell syncytium formation

Vero-E6 cells (ATCC, Cat#CRL-1586) were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% heat-inactivated FBS (GIBCO). Vero-E6 cells were transfected with the SARS-CoV-2 S-protein mutants (R685A)-GFP by Lipofectamine 3000 (Thermo Fisher Scientific). After 48 h, the cell culture medium was changed to DMEM without FBS, treated with different dilutions of serum, and incubated for 2 h at 37°C. The cells were then treated with Trypsin at 15 μg/mL for 2 h at 37°C. The cells untreated with serum and trypsin were used as control. Cells were fixed with 4% paraformaldehyde (PFA) and stained with 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstaining for 2 h at room temperature. Images were acquired under a Zeiss AXIO Imager A1 microscope in 10×, 20×, and 40× view.

SARS-CoV-2 surrogate virus neutralization ELISA

The hACE2 (His-tag) and the diluted 20× mouse sera were mixed in a 96-well plate pre-coated with RBD expressed from HEK293 cells, and incubated for 1 h at room temperature. Then the HRP conjugated anti-His-tag antibody was added and incubated for another 1 h at room temperature. Subsequently, the plates were washed for three times. Add TMB working solution (100 μL/well) and incubate for 15 minutes protect from light, and then add 0.5 M sulfuric acid (50 μL/well) to stop the reaction. The absorbance was measured at 450 nm with BioTek Cytation™ 5. All samples were performed in triplicate.

The formula is as follows:

$$Inhibition\% = 1 - \frac{OD\ value\ of\ sample}{OD\ value\ of\ negative\ control} * 100$$