Supplementary information for

Enhanced Transglycosylation Activity of Endo-F3 Mutant by Ligand-directed Localization

Xiangman Zou[1,2,6], Zhi Liu[2,3,6], Liya Liu[2], Wei Shi[2], Wanzhen Li[2,3], Zifen Guo[1*], Feng Tang[2]*, Wei Huang[2,4,5]*

[1]Institute of Pharmacy and Pharmacology, Hunan Province Cooperative Innovation Center for Molecular Target New Drug Study, Hunan Provincial Key Laboratory of Tumor Microenvironment Responsive Drug Research, University of South China, Hengyang, Hunan, 421001, China

[2]CAS Key Laboratory of Receptor Research, CAS Center for Excellence in Molecular Cell Science, Center for Biotherapeutics Discovery Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Pudong, Shanghai 201203, P. R. China

[3]School of Chinese Materia Medica, Nanjing University of Chinese Medicine, 210023, Nanjing, China

[4]University of Chinese Academy of Sciences, No.19A Yuquan Road, Beijing 100049, P. R. China

[5]School of Pharmaceutical Science and Technology, Hangzhou, Institute for Advanced Study, University of Chinese Academy of Sciences, Hangzhou 310024, P. R. China

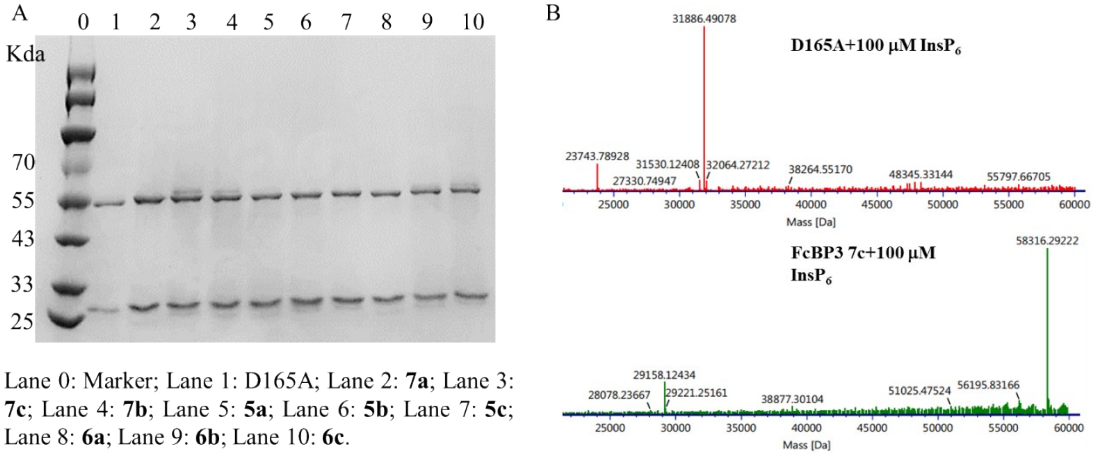
[6]theses authors contributed equally.

guozifen76@163.com (Zifen Guo)

tangfeng2013@simm.ac.cn (Feng Tang)

huangwei@simm.ac.cn (Wei Huang)

1. Supplementary figures:



Lane 0: Marker; Lane 1: D165A; Lane 2: **7a**; Lane 3: **7c**; Lane 4: **7b**; Lane 5: **5a**; Lane 6: **5b**; Lane 7: **5c**; Lane 8: **6a**; Lane 9: **6b**; Lane 10: **6c**.

Figure S1. Determination of conjugation site of FcBP. **A**, SDS-PAGE profile of D165A and FcBP-D165A conjugates. **B**, LC-MS analysis of free D165A and FcBP3-**7c** treated by 100 μM $InsP_6$ (inositol hexakisphosphate, a small molecule promoting CPD self-cleavage) at 4 °C for 2 h.

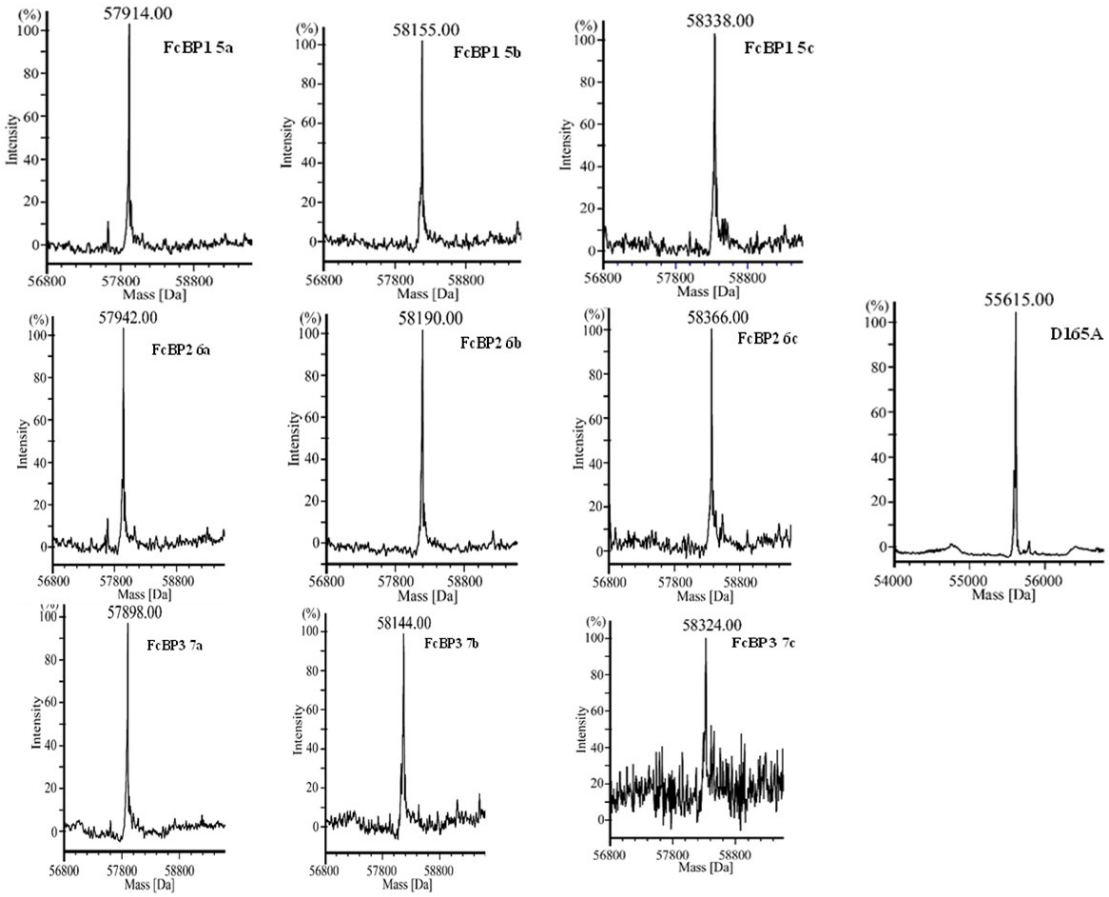


Figure S2. LC-MS analysis of D165A and FcBP-D165A conjugates.

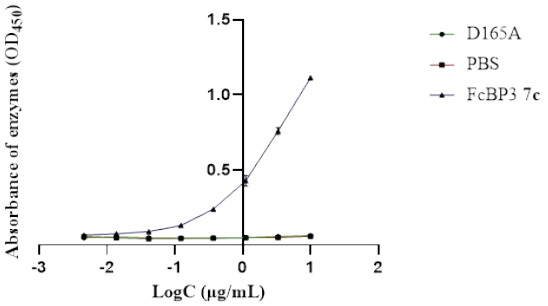


Figure S3. Affinity analysis of free D165A and FcBP3 **7c** to GN (F)-Rituximab. The primary concentration of enzymes was 2 μg/ml. And the x axis means the concentration of GN (F)-Rituximab.

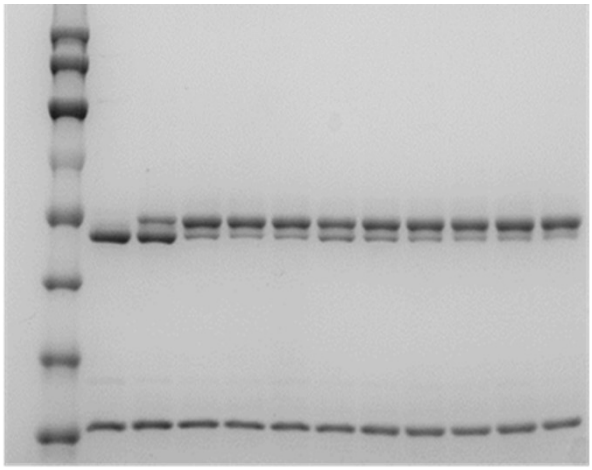


Figure S4. Transglycosylation effect of different enzymes for GN(F)-Rituximab (**9**). Reaction condition: 5 mg/ml GN(F)-Rituximab (**9)**, 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃ for 2 h; Lane 0: Marker; Lane 1: **9**; Lane 2: **9** + D165A; Lane 3: **9** + **7a**; Lane 4: **9**+**7c**; Lane 5: **9** + **7b**; Lane 6: **9** + **5a**; Lane 7: **9** + **5b**; Lane 8: **9** + **5c**; Lane 9: **9** + **6a**; Lane 10: **9** + **6b**; Lane 11: **9** + **6c**.

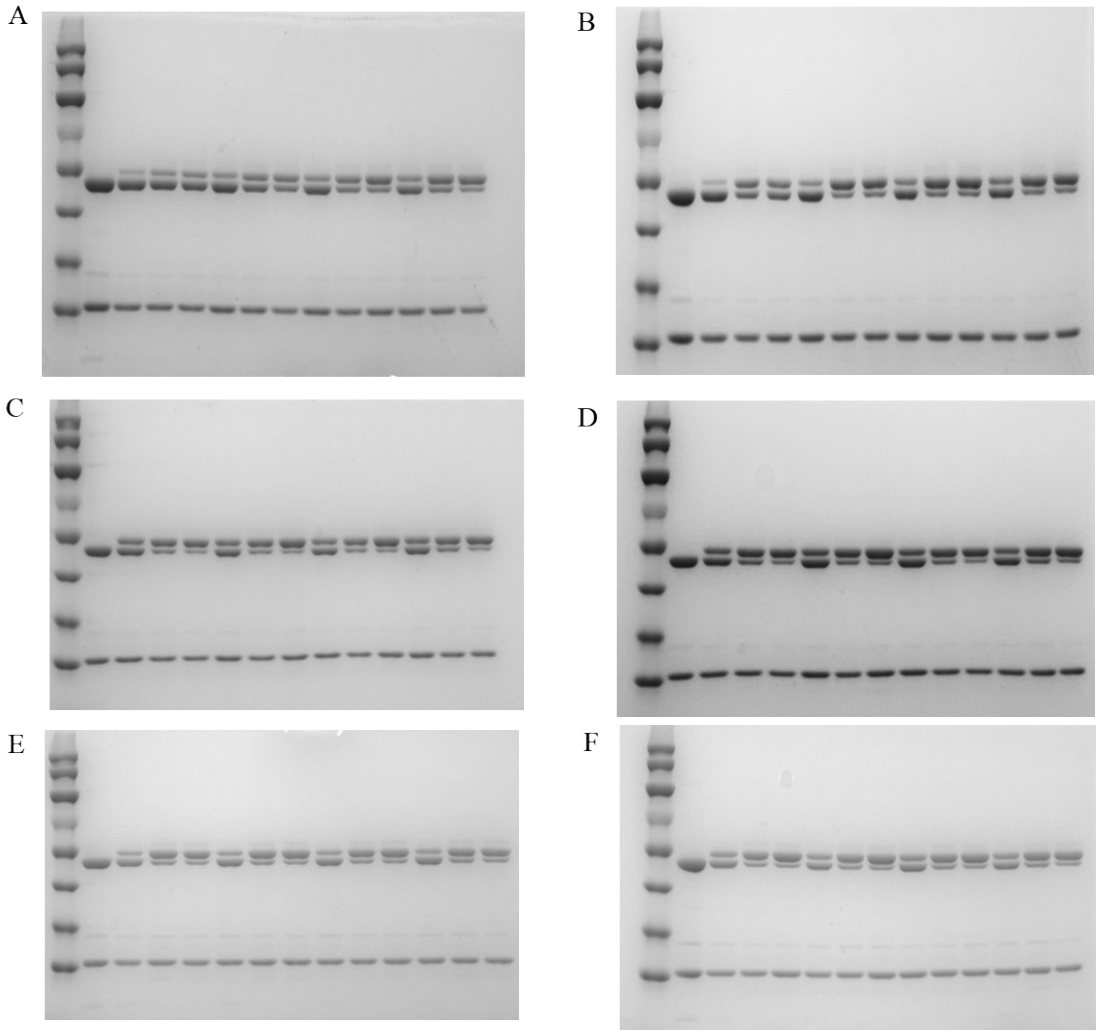


Figure S5. Screening of the enzyme concentration and pH. Reaction conditions were listed as follows: (**A**) 5 mg/ml GN(F)-Herceptin (**14**), 0.1 mg/ml enzymes, 1 mM Az-SCT-ox in 50 mM PB (pH 7.1) at 30 ℃. (B) 5 mg/ml GN(F)-Herceptin (**14**), 0.1 mg/ml enzymes, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**C**) 5 mg/ml GN(F)-Herceptin (**14**), 0.2 mg/ml enzymes, 1 mM Az-SCT-ox in 50 mM PB (pH 7.1) at 30 ℃. (**D**) 5 mg/ml GN(F)-Herceptin (**14**), 0.2 mg/ml enzymes, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**E**) 5 mg/ml GN(F)-Herceptin (**14**), 0.3 mg/ml enzymes, 1 mM Az-SCT-ox in 50 mM PB (pH 7.1) at 30 ℃. (**F**) 5 mg/ml GN(F)-Herceptin (**14**), 0.3 mg/ml enzymes, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 ℃.

Lane 0: Marker; Lane 1: **14**; Lane 2: **14** + D165A, 0.5 h; Lane 3: **14** + **5a**, 0.5 h; Lane 4: **14** + **5b**, 0.5 h; Lane 5: **14** + D165A, 1 h; Lane 6: **14** + **5a**, 1 h; Lane 7: 14 + **5b**, 1 h; Lane 8: **14** + D165A, 1.5 h; Lane 9: **14** + **5a**, 1.5 h; Lane 10: **14** + **5b**, 1.5 h; Lane 11: **14** + D165A, 2 h; Lane 12: **14** + **5a**, 2 h; Lane 13: **14** + **5b**, 2 h.

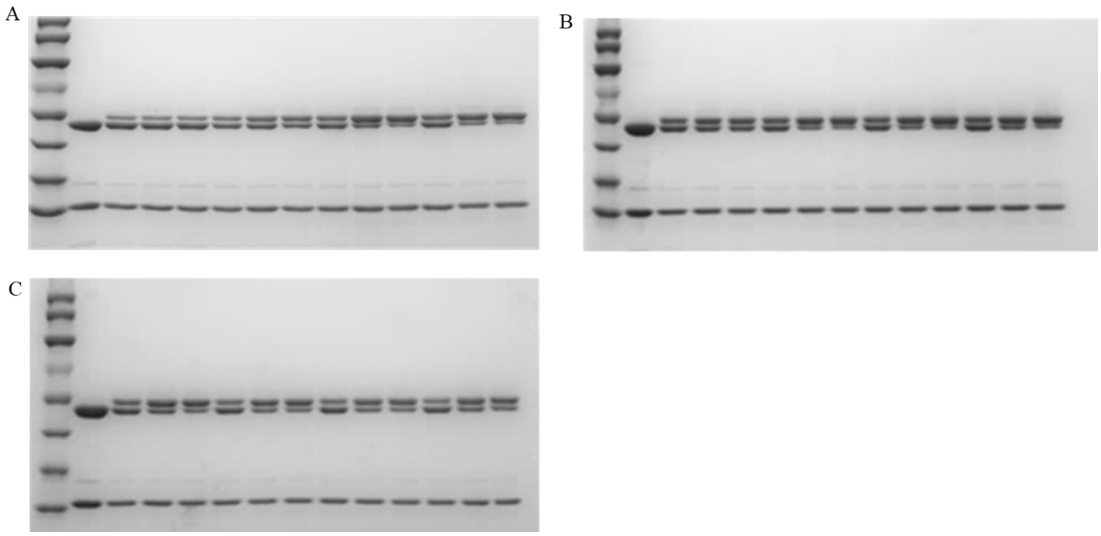


Figure S6. Screening of the reaction temperature. (**A**) 5 mg/ml GN(F)-Herceptin (**14**), 0.2 mg/ml enzyme, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at **25 ℃**. (**B**) 5 mg/ml GN(F)-Herceptin (**14**), 0.2 mg/ml enzyme,1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at **30 ℃**. (**C**) 5 mg/ml GN(F)-Herceptin (**14**), 0.2 mg/ml enzyme, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at **37 ℃**.
Lane 0: Marker; Lane 1: **14**; Lane 2: **14** +D165A, 0.5 h; Lane 3: **14** + **5a**, 0.5 h; Lane 4: **14** + **5b**, 0.5 h; Lane 5: **14** + D165A, 1 h; Lane 6: **14** + **5a**, 1 h; Lane 7: **14** + **5b**, 1 h; Lane 8: **14** + D165A, 2 h; Lane 9: **14** + **5a**, 2 h; Lane 10: **14** + **5b**, 2 h; Lane 11: **14** + D165A, 3 h; Lane 12: **14** + **5a**, 3 h; Lane 13: **14** + **5b**, 3 h.

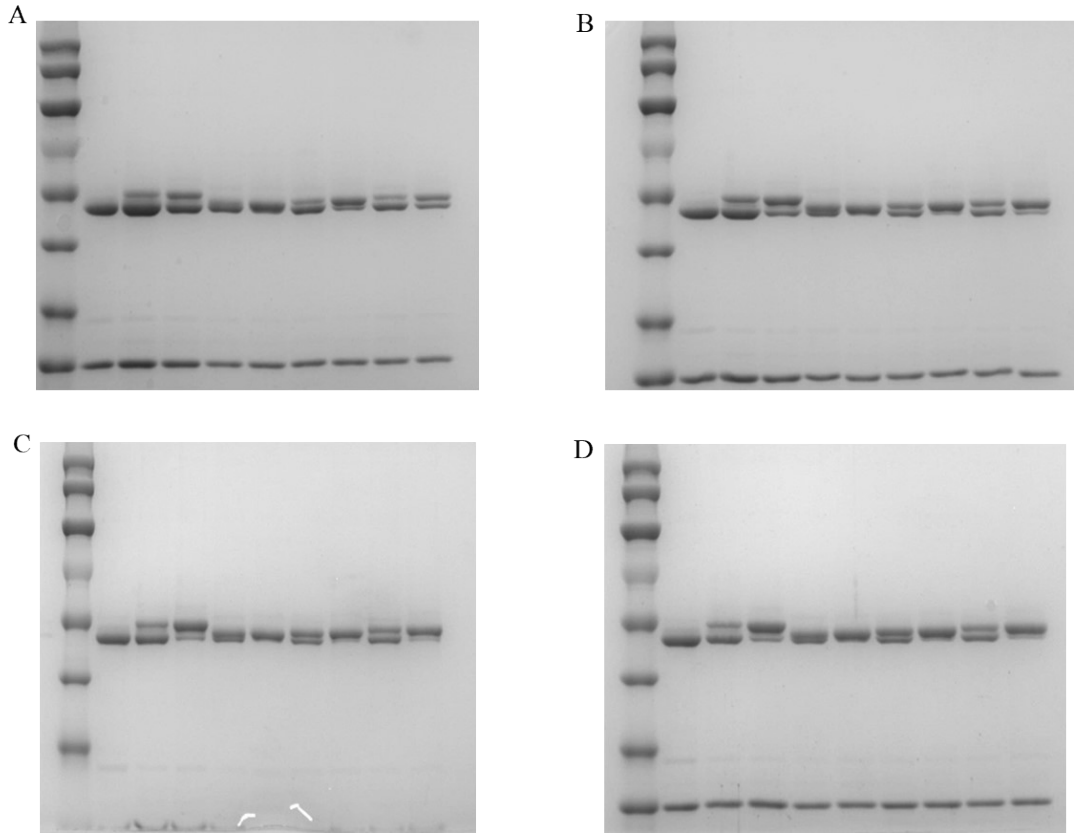


Figure S7. Transglycosylation effect of different substrates for GN(F)-Rituximab (**9**). Reaction

conditions: 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 1 mM oxazoline in 50 mM PB (pH 7.4) at 30 ℃. (**A**) 15 min; (**B**) 30 min; (**C**) 1 h; (**D**) 2 h; Lane 0: Marker; Lane 1: **9**; Lane 2: **9** + D165A + SCT-ox; Lane 3: **9** + **6c** + SCT-ox; Lane 4: **9** + D165A + Man_3-ox; Lane 5: **9** + **6c** + Man_3-ox; Lane 6: **9** + D165A + GN_2M_3-ox; Lane 7: **9** + **6c** + GN_2M_3-ox; Lane 8: **9** + D165A + CT-ox; Lane 9: **9** + **6c** + CT-ox.

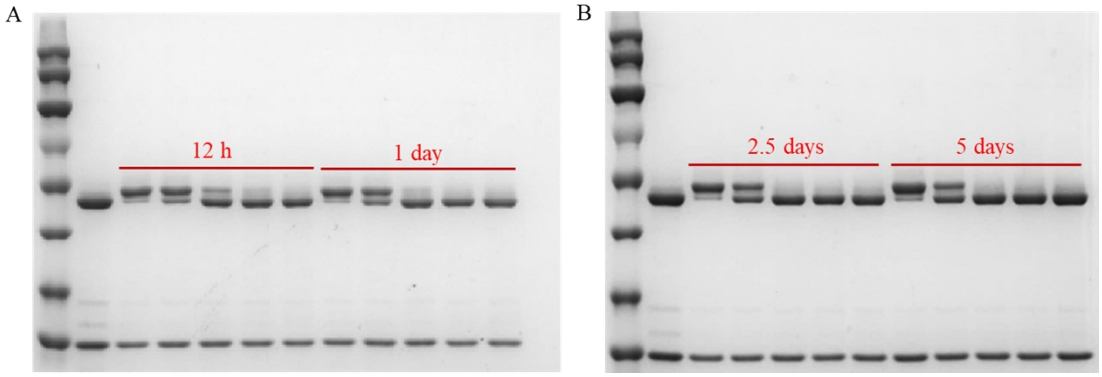


Figure S8. Stability analysis of the glycoengineering antibody. 3.31 mg/ml G2F-Rituximab (**10b**) and FcBP2-PEG_8-SMCC-D165A (**6c**) in 1X PBS at 37 ℃.

(**A**) 12 h and 1 day. Lane 0: Marker; Lane 1: **9**; Lane 2: **10b**, 12 h; Lane 3: **10b** + 0.01 mg/ml **6c**, 12 h; Lane 4: **10b** + 0.05 mg/ml **6c**,12 h; Lane 5: **10b** +0.1 mg/ml **6c**, 12 h; Lane 6: **10b** + 0.2 mg/ml **6c**, 12 h; Lane 7: **10b**, 1 day; Lane 8: **10b** + 0.01 mg/ml **6c**, 1 day; Lane 9: **10b** + 0.05 mg/ml **6c**, 1 day; Lane 10: **10b** + 0.1 mg/ml **6c**, 1 day; Lane 11: **10b** + 0.2 mg/ml **6c**, 1 day. (**B**) 2.5 days and 5 days. Lane 0: Marker; Lane 1: **9**; Lane 2: **10b**, 2.5 days; Lane 3: **10b** + 0.01 mg/ml **6c**, 2.5 days; Lane 4: **10b** + 0.05 mg/ml **6c**, 2.5 days; Lane 5: **10b** + 0.1 mg/ml **6c**, 2.5 days; Lane 6: **10b** +0.2 mg/ml **6c**, 2.5 days; Lane 7: **10b**, 5 days; Lane 8: **10b** + 0.01 mg/ml **6c**, 5 days; Lane 9: **10b** +0.05 mg/ml **6c**, 5 days; Lane 10: **10b** + 0.1mg/ml **6c**, 5 days; Lane 11: **10b** + 0.2 mg/ml **6c**, 5 days.

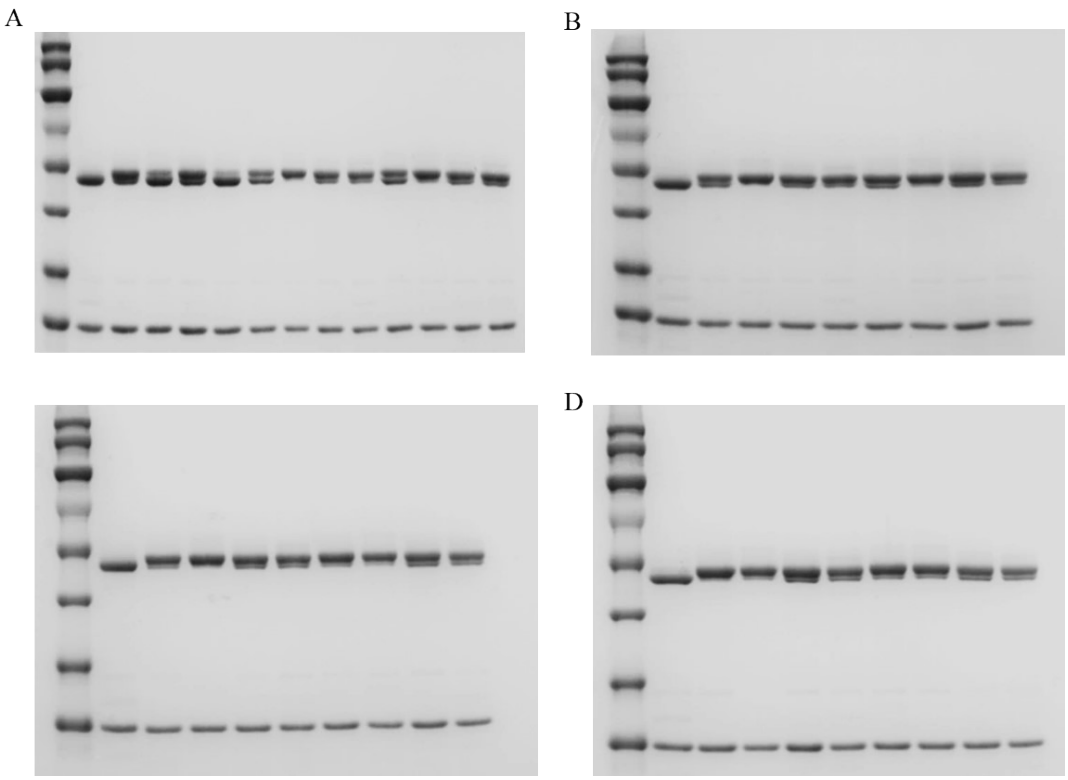


Figure S9. One-pot glycoengineering of IgGs by FcBP-D165A conjugates. 4 mg/ml WT-Rituximab (**8**) and 0.1 mg/ml or 0.2 mg/ml FcBP2-PEG$_8$-SMCC-D165A (**6c**) or D165A were added in 1X PBS at 37 ℃ for 24 h. Then, a half of solution was supplied with 1 mM CT-ox, while the left solution supplied with 1 mM CT-ox and 0.1 mg/ml **6c** or D165A for transglycosylation evaluation (30℃). (**A**) Lane 0: Marker; Lane 1: **9**; Lane 2: 0.1 mg/ml D165A + **8**, 24 h; Lane 3: 0.1 mg/ml **6c** + **8**, 24 h; Lane 4: 0.2 mg/ml D165A + **8**, 24 h; Lane 5: 0.2 mg/ml **6c** + **8**, 24 h; Lane 6: 0.1 mg/ml **6c** + **8** + 1 mM CT-ox, 15 min; Lane 7: 0.1 mg/ml **6c** + **8** + 1 mM CT-ox + 0.1 mg/ml **6c**, 15 min; Lane 8: 0.1 mg/ml D165A + **8** + 1 mM CT-ox, 15 min; Lane 9: 0.1 mg/ml D165A + **8** + 1 mM CT-ox + 0.1 mg/ml D165A, 15 min; Lane 10: 0.2 mg/ml **6c** + **8** + 1 mM CT-ox, 15 min; Lane 11: 0.2 mg/ml **6c** + **8** + 1 mM CT-ox + 0.1 mg/ml **6c**, 15 min; Lane 12: 0.2 mg/ml D165A + **8** + 1 mM CT-ox, 15 min; Lane 13: 0.2 mg/ml D165A + **8** + 1 mM CT-ox + 0.1 mg/ml D165A, 15 min.
(B) 30 min; **(C)** 1 h; **(D)** 2 h. Lane 0: Marker; Lane 1: **9**; Lane 2: 0.1mg/ml **6c** + **8** + 1 mM CT-ox; Lane 3: 0.1 mg/ml **6c** + **8** + 1 mM CT-ox+0.1 mg/ml **6c**; Lane 4: 0.1 mg/ml D165A + **8** + 1 mM CT-ox; Lane 5: 0.1 mg/ml D165A + **8** + 1 mM CT-ox + 0.1 mg/ml D165A; Lane 6: 0.2 mg/ml **6c** + **8** + 1 mM CT-ox; Lane 7: 0.2 mg/ml **6c** + **8** + 1 mM CT-ox + 0.1 mg/ml **6c**; Lane 8: 0.2 mg/ml D165A + **8** + 1 mM CT-ox; Lane 9: 0.2 mg/ml D165A + **8** + 1 mM CT-ox + 0.1 mg/ml D165A.

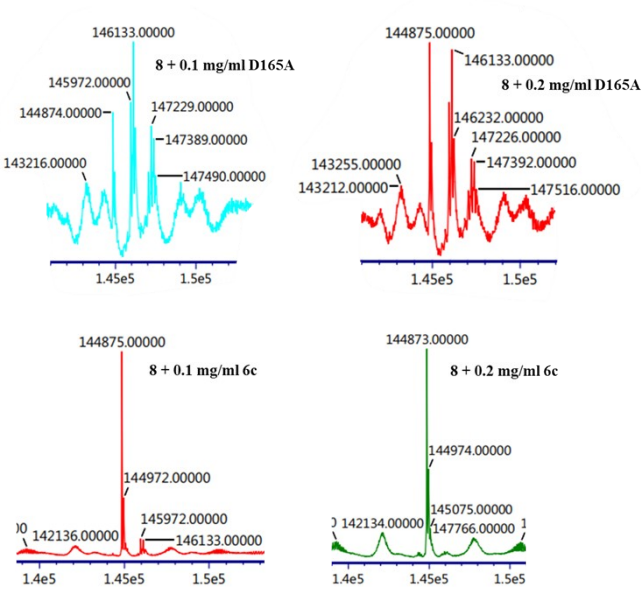


Figure S10. LC-MS analysis of the preparation of one-pot glycoengineering of IgGs by FcBP-D165A conjugates.

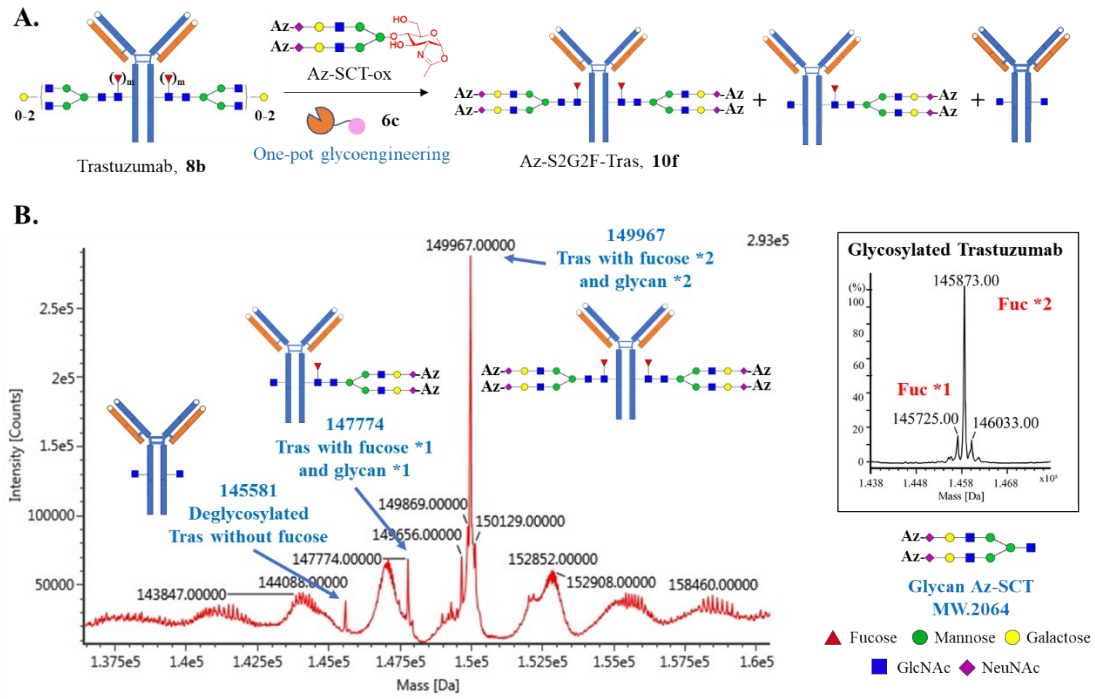


Figure S11. Synthesis of Az-S2G2F-Trastuzumab and LC-MS analysis of the Az-S2G2F-Trastuzumab and the deglycosylated trastuzuamb. Owing to the quite low percentage of non-core-fucosylated IgG, the mass spectra of deglycosylated IgG only exhibited one major peak with 2 fucose and one small peak with 1 fucose, but without peak without fucose (see black box). We could clearly see that the molecular weight of deglycosylayed IgG without core-fucose (145581) didn't change after transglycosylation, and the molecular weight of deglycosylated IgG with 1 fucosylation shifted to 147774 (equals to 145581 + 146 (MW of fucose) + 2046 (MW of Az-SCT-ox)), and the

molecular weight of deglycosylated IgG with 2 fucosylation shifted to 149967 (equals to 145581 + 2 * 146 (MW of fucose) + 2 * 2046 (MW of Az-SCT-ox)), indicating that the non-fucosylated heavy chain can't be re-glycosylated by FcBP-D165A **6c**.

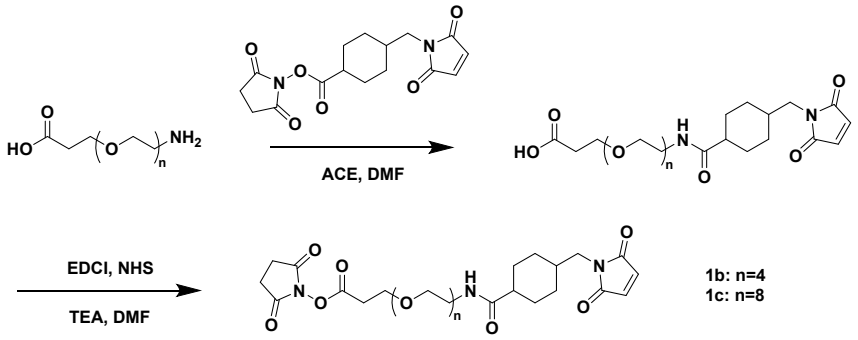


Figure S12. Synthetic scheme of linker **1b** and **1c.**

*General Procedure for Synthesis of linker **1b** and **1c**.*

Acid-PEG-Amino (10 μmol, 1.0 eq), SMCC (12 μmol, 1.2 eq), Et_3N (2.8 μl, 2.0 eq) and DMF (97.2 μl) were added to a tube successively at r.t. for 2 h and monitored by HPLC and LC-MS. The mixture was subjected to semi-preparation HPLC purification (Method A). The fractions containing the products were collected and lyophilized to obtain the product as a white powder. Then, the harvested Acid-PEG-MCC (10 μmol, 1.0 eq), NHS (20 μmol, 2.0 eq), EDCI (20 μmol, 2.0 eq), Et_3N (2.8 μl, 2.0 eq) and DMF (97.2 μl) were added to a tube successively at r.t. for 6 h and monitored by HPLC and LC-MS. The mixture was subjected to semi-preparation HPLC for purification (Method A). The fractions containing the products were collected and lyophilized to obtain **1b** and **1c** as a white powder respectively.

2. Supplementary original figures:

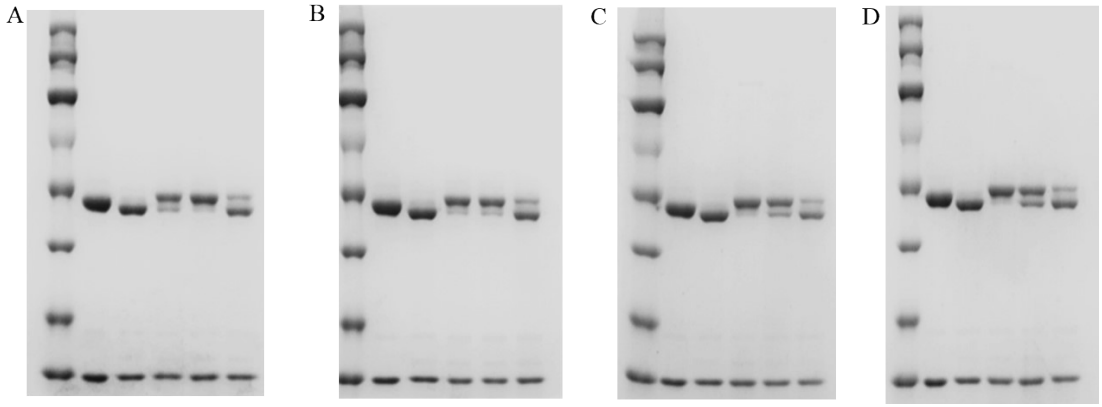


Figure S13. Transglycosylation effect of different enzymes for GN(F)-Rituximab (**9**). Reaction condition: 5 mg/ml GN(F)-Rituximab (**9**), 0.15 mg/ml Endo S mutant D233Q/0.05 mg/ml Endo S2 D184M/0.15 mg/ml Endo F3 D165A, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**A**) 30 min; (**B**) 1 h; (**C**) 2 h; (**D**) 3 h; Lane 0: Marker; Lane 1: **8**; Lane 2: **9**; Lane 3: **9** + **D233Q**; Lane 4: **9** + **D184M**; Lane 5: **9** + **D165A**.

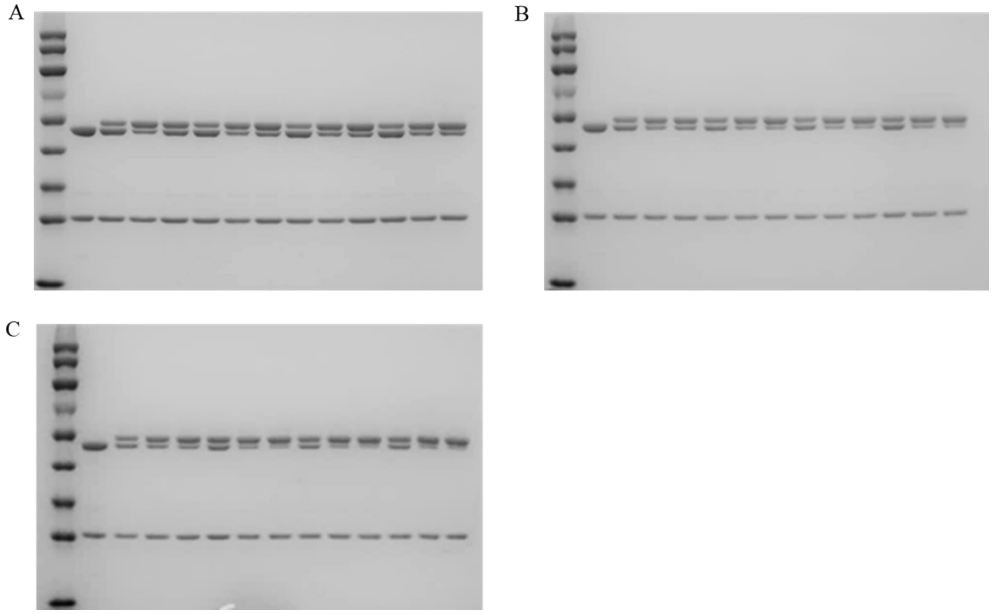


Figure S14. Optimization of the substrate concentration. (**A**) 5 mg/ml GN(F)-Herceptin (**14**), 0.2 mg/ml enzyme, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**B**) 5 mg/ml GN(F)-Herceptin (**14**), 0.2mg/ml enzyme, 1.5 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**C**) 5 mg/ml GN(F)-Herceptin (**14**), 0.2 mg/ml enzyme, 2 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. Lane 0: Marker; Lane 1: **14**; Lane 2: **14** + D165A, 0.5 h; Lane 3: **14** + **5a**, 0.5 h; Lane 4: **14** + **5b**, 0.5 h; Lane 5: **14** + D165A, 1 h; Lane 6: **14** + **5a**, 1 h; Lane 7: **14** + **5b**, 1 h; Lane 8: **14** + D165A, 1.5 h; Lane 9: **14** + **5a**, 1.5 h; Lane 10: **14** + **5b**, 1.5 h; Lane 11: **14** + D165A, 2 h; Lane 12: **14** + **5a**, 2 h; Lane 13: **14** + **5b**, 2 h.

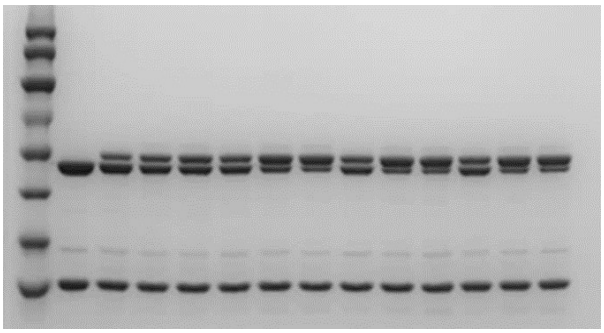


Figure S15. Transfer efficiency of conjugates with distinct linker length. 5 mg/ml GN(F)-Herceptin (**14**), 0.2 mg/ml enzyme, 1 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. Lane 0: Marker; Lane 1: **14**; Lane 2: **14** + D165A, 0.5 h; Lane 3: **14** + **5b**, 0.5 h; Lane 4: **14** + **5c**, 0.5 h; Lane 5: **14** + D165A, 1 h; Lane 6: **14** + **5b**, 1 h; Lane 7: **14** + **5c**, 1 h; Lane 8: **14** + D165A, 2 h; Lane 9: **14** + **5b**, 2 h; Lane 10: **14** + **5c**, 2 h; Lane 11: **14** + D165A, 3 h; Lane 12: **14** + **5b**, 3 h; Lane 13: **14** + **5c**, 3 h.

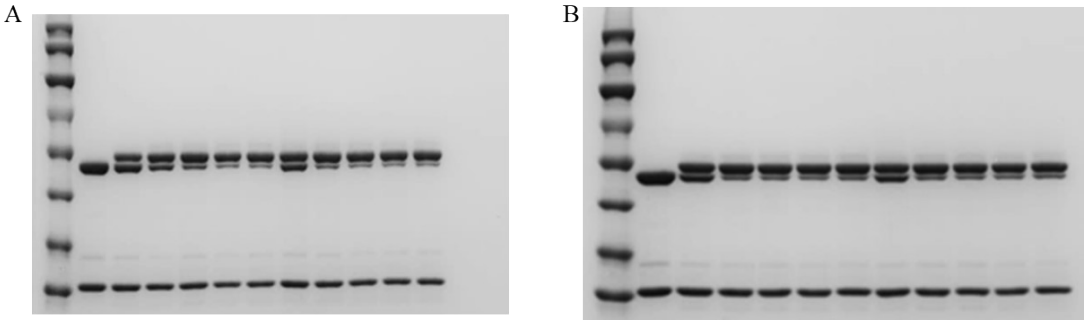


Figure S16. Transfer efficiency of conjugates with different mutation and distinct linker length. 5 mg/ml GN(F)-Herceptin (**14**), 0.3 mg/ml enzyme, 2 mM Az-SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**A**) Lane 0: Marker; Lane 1: **14**; Lane 2: **14** + D165A, 0.5 h; Lane 3: **14** + **5b**, 0.5 h; Lane 4: **14** + **5c**, 0.5 h; Lane 5: **14** + **6b**, 0.5 h; Lane 6: **14** + **6c**, 0.5 h; Lane 7: **14** + D165A, 1 h; Lane 8: **14** + **5b**, 1 h; Lane 9: **14** + **5c**, 1 h; Lane 10: **14** + **6b**, 1 h; Lane 11: **14** + **6c**, 1 h; (**B**) Lane 0: Marker; Lane 1: **14**; Lane 2: **14** + D165A, 2 h; Lane 3: **14** + **5b**, 2 h; Lane 4: **14** + **5c**, 2 h; Lane 5: **14** + **6b**, 2 h; Lane 6: **14** + **6c**, 2 h; Lane 7: **14** + D165A, 3 h; Lane 8: **14** + **5b**, 3 h; Lane 9: **14** + **5c**, 3 h; Lane 10: **14** + **6b**, 3 h; Lane 11: **14** + **6c**, 3 h.

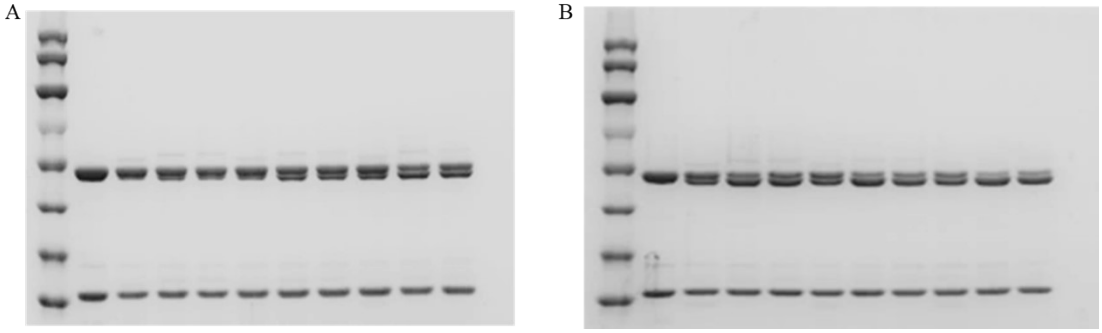


Figure S17. Cleavage efficiency of free D165A and conjugates. (**A**) 5 mg/ml WT-Herceptin (**15**) and 0.2 mg/ml enzymes in 50 mM PB (pH 6.5) at 37 ℃. Lane 0: Marker; Lane 1: **15**; Lane 2: **15** + D165A, 0.5 h; Lane 3: **15** + **5a**, 0.5 h; Lane 4: **15** + **5b**, 0.5 h; Lane 5: **15** + D165A, 1 h; Lane 6: **15** + **5a**, 1 h; Lane 7:**15** + **5b**, 1 h; Lane 8: **15** + D165A, 2 h; Lane 9: **15** + **5a**, 2 h; Lane 10: **15** + **5b**, 2 h; (**B**) 5 mg/ml WT-Herceptin (**15**) and 0.2 mg/ml enzymes in 50 mM PB (pH 6.5) at 37 ℃. Lane 0: Marker; Lane 1: **15**; Lane 2: **15** + D165A, 3 h; Lane 3: **15** + **5a**, 3 h; Lane 4: **15** + **5b**, 3 h; Lane 5: **15** + D165A, 4 h; Lane 6: **15** + **5a**, 4 h; Lane 7: **15** + **5b**, 4 h; Lane 8: **15** + D165A, 6 h; Lane 9: **15** + **5a**, 6 h; Lane 10: **15** + **5b**, 6 h.

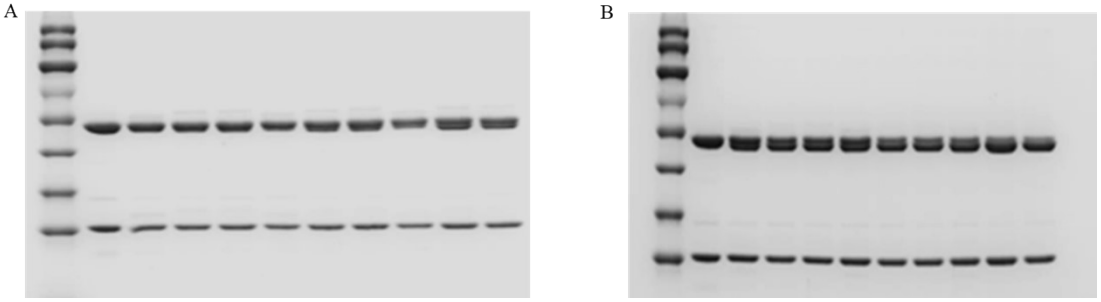


Figure S18. Cleavage efficiency of free D165A and conjugates. (**A**) 5 mg/ml WT-Herceptin (**15**) and 0.3mg/ml enzymes in 50 mM PB (pH 6.5) at 37 ℃. Lane 0: Marker; Lane 1: **15**; Lane 2: **15** + D165A, 0.5 h; Lane 3: **15** + **5a**, 0.5 h; Lane 4: **15** + **5b**, 0.5 h; Lane 5: **15** + D165A, 1 h; Lane 6: **15** + **5a**, 1 h; Lane 7: **15** + **5b**, 1 h; Lane 8: **15** + D165A, 2 h; Lane 9: **15** + **5a**, 2 h; Lane 10: **15** + **5b**, 2 h; (**B**) 5 mg/ml WT-Herceptin (**15**) and 0.3mg/ml enzymes in 50 mM PB (pH 6.5) at 37 ℃. Lane 0: Marker; Lane 1: **15**; Lane 2: **15** + D165A, 4 h; Lane 3: **15** + **5a**, 4 h; Lane 4: **15** + **5b**, 4 h; Lane 5: **15** + D165A, 6 h; Lane 6: **15** + **5a**, 6 h; Lane 7: **15** + **5b**, 6 h; Lane 8: **15** + D165A, 12 h; Lane 9: **15** + **5a**, 12 h; Lane 10: **15** + **5b**, 12 h.

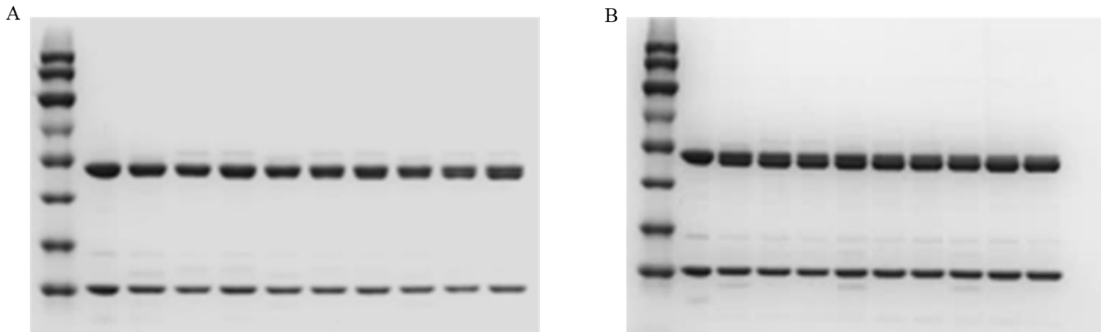


Figure S19. Cleavage efficiency of free D165A and conjugates. (**A**) 5 mg/ml WT-Herceptin (**15**) and 0.5 mg/ml enzymes in 50 mM PB (pH 6.5) at 37 ℃. Lane 0: Marker; Lane 1: **15**; Lane 2: **15** + D165A, 0.5 h; Lane 3: **15** + **5a**, 0.5 h; Lane 4: **15** + **5b**, 0.5 h; Lane 5: **15** + D165A, 1 h; Lane 6: **15** + **5a**, 1 h; Lane 7: **15** + **5b**, 1 h; Lane 8: **15** + D165A, 2 h; Lane 9: **15** + **5a**, 2 h; Lane 10: **15** + **5b**, 2 h; (**B**) 5 mg/ml WT-Herceptin (**15**) and 0.5 mg/ml enzymes in 50 mM PB (pH 6.5) at 37 ℃. Lane 0: Marker; Lane 1: **15**; Lane 2: **15** + D165A, 4 h; Lane 3: **15** + **5a**, 4 h; Lane 4: **15** + **5b**, 4 h; Lane 5: **15** + D165A, 6 h; Lane 6: **15** + **5a**, 6 h; Lane 7: **15** + **5b**, 6 h; Lane 8: **15** + D165A, 12 h; Lane 9: **15** + **5a**, 12 h; Lane 10: **15** + **5b**, 12 h.

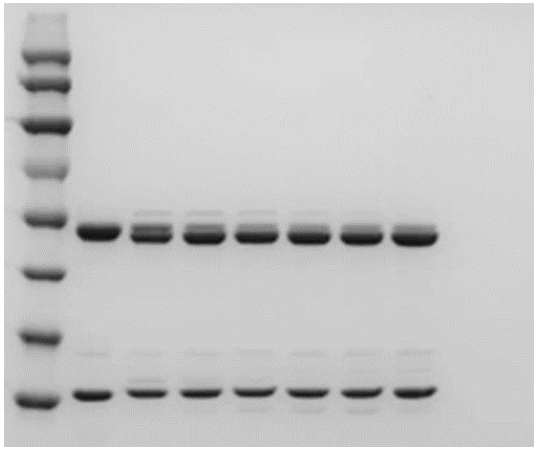


Figure S20. Cleavage efficiency of WT Endo F3. 5mg/ml WT-Herceptin (**15**) and 0.5mg/ml WT-F3 were incubated in 50 mM PB (pH 6.5) at 37 ℃. Lane 0: Marker; Lane 1: **15**; Lane 2: 0.5 h; Lane 3: 1 h; Lane 4: 2 h; Lane 5: 4 h; Lane 6: 6 h; Lane 7: 12 h.

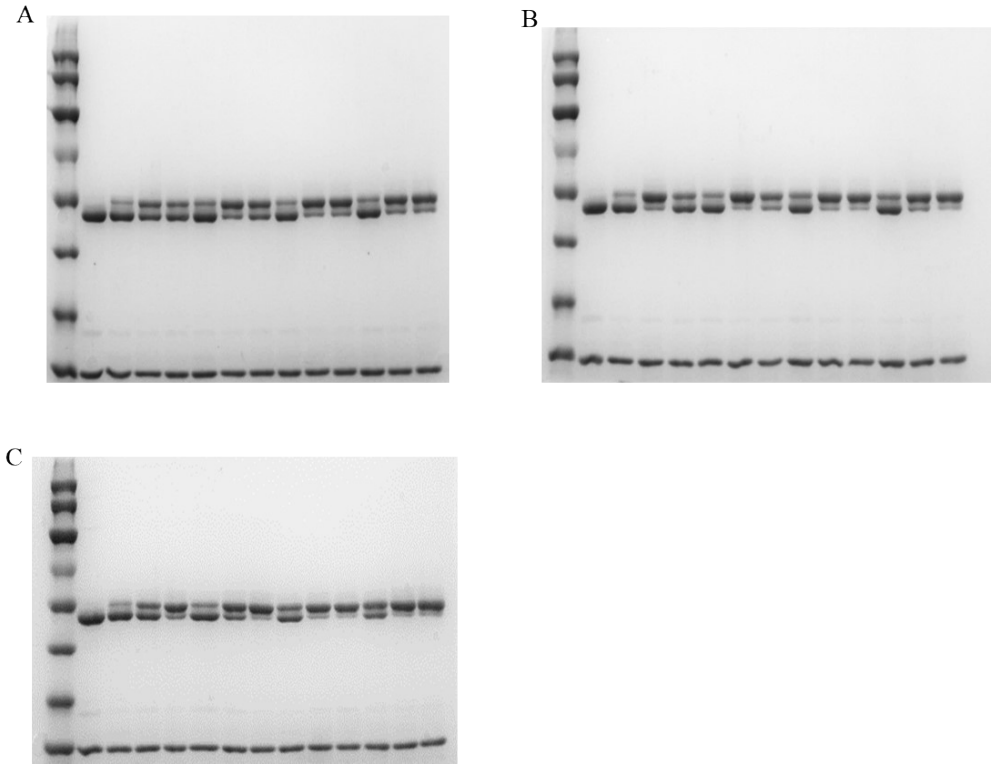


Figure S21. Transglycosylation effect of different enzymes for GN(F)-Rituximab. Reaction conditions were listed as follows: (**A**) 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 0.5 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**B**) 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**C**) 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 2 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. Lane 0: Marker; Lane 1: **9**; Lane 2: **9** + D165A, 15 min; Lane 3: **9** + **6b**, 15 min; Lane 4: **9** + **6c**, 15 min; Lane 5: **9** + D165A, 30 min; Lane 6: **9** + **6b**, 30 min; Lane 7: **9** + **6c**, 30 min; Lane 8: **9** + D165A, 1 h; Lane 9: **9** + **6b**, 1 h; Lane 10: **9** + **6c**, 1 h; Lane 11: **9** + D165A, 2 h; Lane 12: **9** + **6b**, 2 h; Lane 13: **9** + **6c**, 2 h.

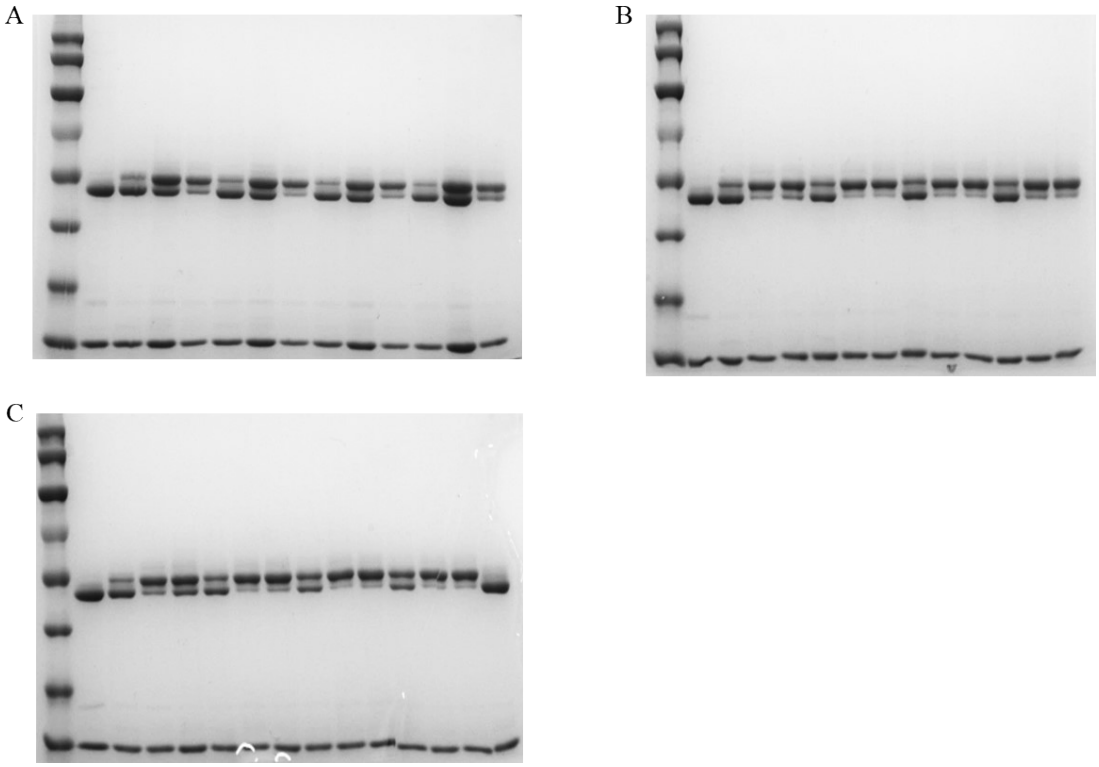


Figure S22. Transglycosylation effect of different enzymes for GN(F)-Rituximab (**9**). Reaction conditions were listed as follows: (**A**) 5 mg/ml GN(F)-Rituximab (**9**), 0.2 mg/ml enzyme, 0.5 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**B**) 5 mg/ml GN(F)-Rituximab (**9**), 0.2 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**C**) 5 mg/ml GN(F)-Rituximab (**9**), 0.2 mg/ml enzyme, 2 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. Lane 0: Marker; Lane 1: **9**; Lane 2: **9** + D165A, 15 min; Lane 3: **9** + **6b**, 15 min; Lane 4: **9** + **6c**, 15 min; Lane 5: **9** + D165A, 30 min; Lane 6: **9** + **6b**, 30 min; Lane 7: **9** + **6c**, 30 min; Lane 8: **9** + D165A, 1 h; Lane 9: **9** + **6b**, 1 h; Lane 10: **9** + **6c**, 1 h; Lane 11: **9** + D165A, 2 h; Lane 12: **9** + **6b**, 2 h; Lane 13: **9** + **6c**, 2 h.

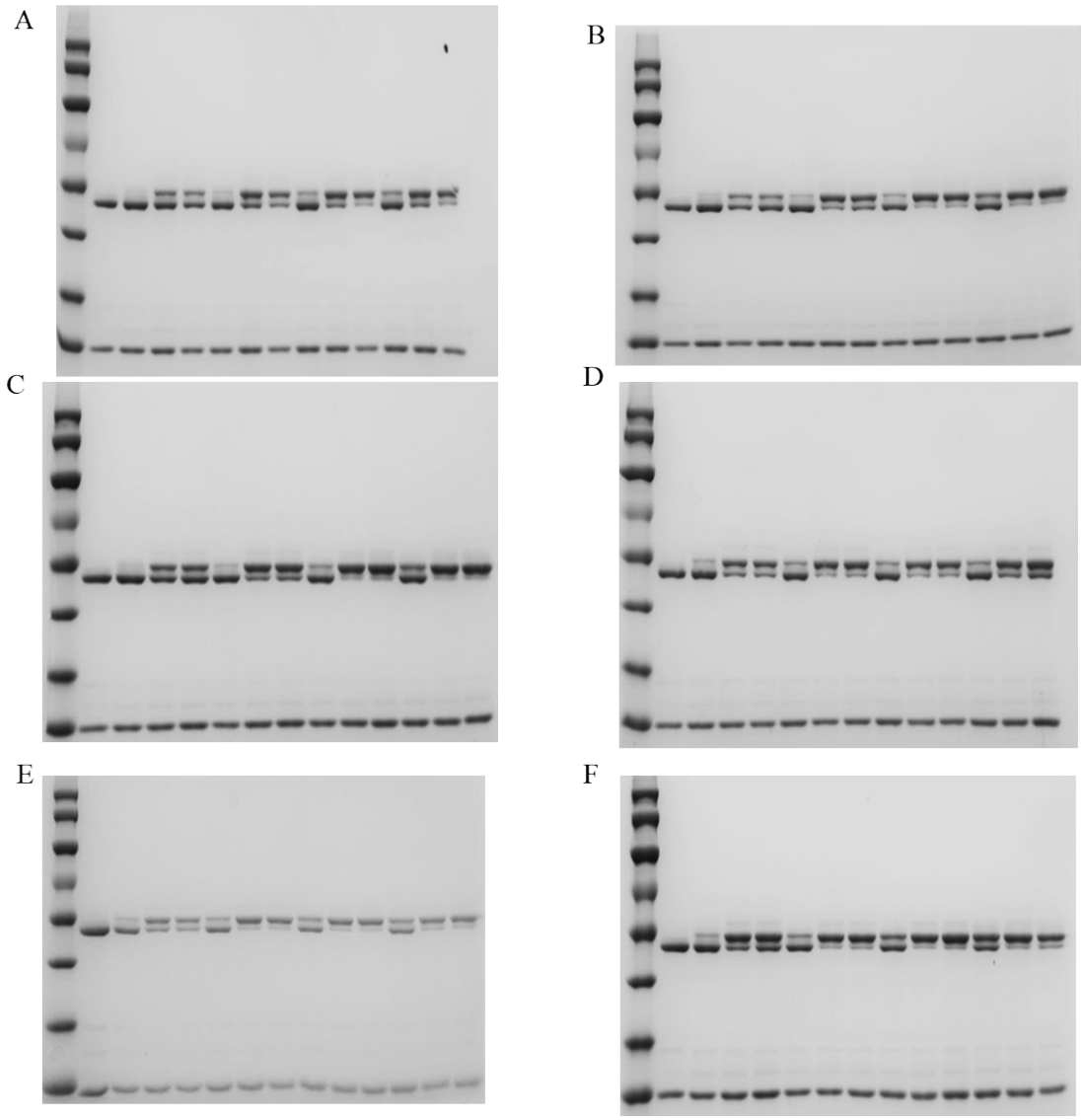


Figure S23. Transglycosylation effect of different enzymes for GN(F)-Rituximab (**9**). Reaction conditions were listed as follows: (**A**) 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 0.5 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**B**) 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**C**) 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 2 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**D**) 5 mg/ml GN(F)-Rituximab (**9**), 0.2 mg/ml enzyme, 0.5 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**E**) 5 mg/ml GN(F)-Rituximab (**9**), 0.2 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**F**) 5 mg/ml GN(F)-Rituximab (**9**), 0.2 mg/ml enzyme, 2 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. Lane 0: Marker; Lane 1: **9**; Lane 2: **9** + D165A, 15 min; Lane 3: **9** + **6b**, 15 min; Lane 4: **9** + **6c**, 15 min; Lane 5: **9** + D165A, 30 min; Lane 6: **9** + **6b**, 30 min; Lane 7: **9** + **6c**, 30 min; Lane 8: **9** + D165A, 1 h; Lane 9: **9** + **6b**, 1 h; Lane 10: **9** + **6c**, 1 h; Lane 11: **9** + D165A, 2 h; Lane 12: **9** + **6b**, 2 h; Lane 13: **9** + **6c**, 2 h.

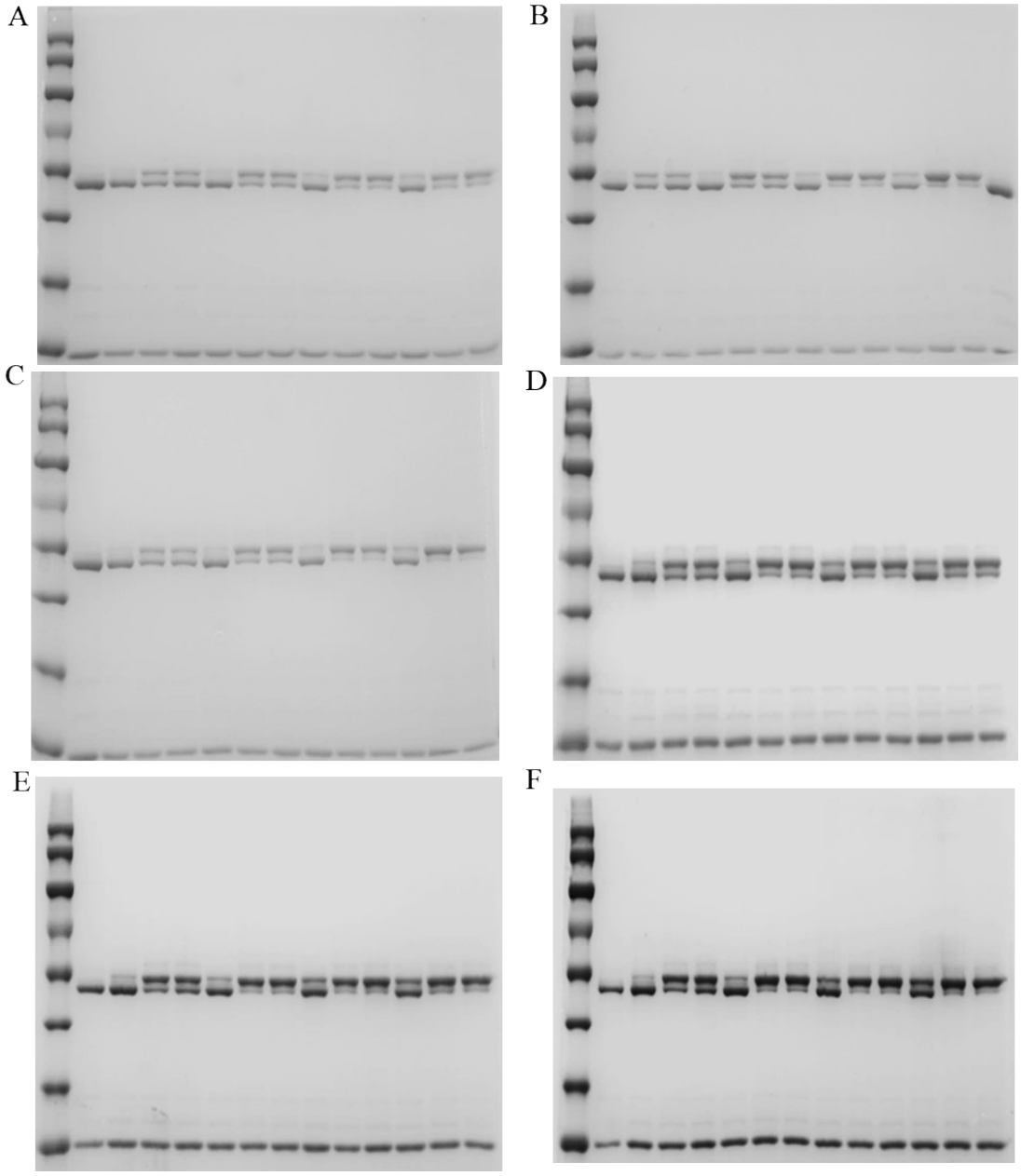


Figure S24. Transglycosylation effect of different enzymes for GN(F)-Rituximab (**9**). Reaction conditions were listed as follows: (**A**) 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 0.5 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**B**) 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**C**) 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 2 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**D**) 5 mg/ml GN(F)-Rituximab (**9**), 0.2 mg/ml enzyme, 0.5 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**E**) 5 mg/ml GN(F)-Rituximab (**9**), 0.2 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**F**) 5 mg/ml GN(F)-Rituximab (**9**), 0.2 mg/ml enzyme, 2 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. Lane 0: Marker; Lane 1: **9**; Lane 2: **9** + D165A, 15 min; Lane 3: **9** + **6b**, 15 min; Lane 4: **9** + **6c**, 15 min; Lane 5: **9** + D165A, 30 min; Lane 6: **9** + **6b**, 30 min; Lane 7: **9** + **6c**, 30 min; Lane 8: **9** + D165A, 1 h; Lane 9: **9** + **6b**, 1 h; Lane 10: **9** + **6c**, 1 h; Lane 11: **9** + D165A, 2 h; Lane 12: **9** + **6b**, 2 h; Lane 13: **9** + **6c**, 2 h.

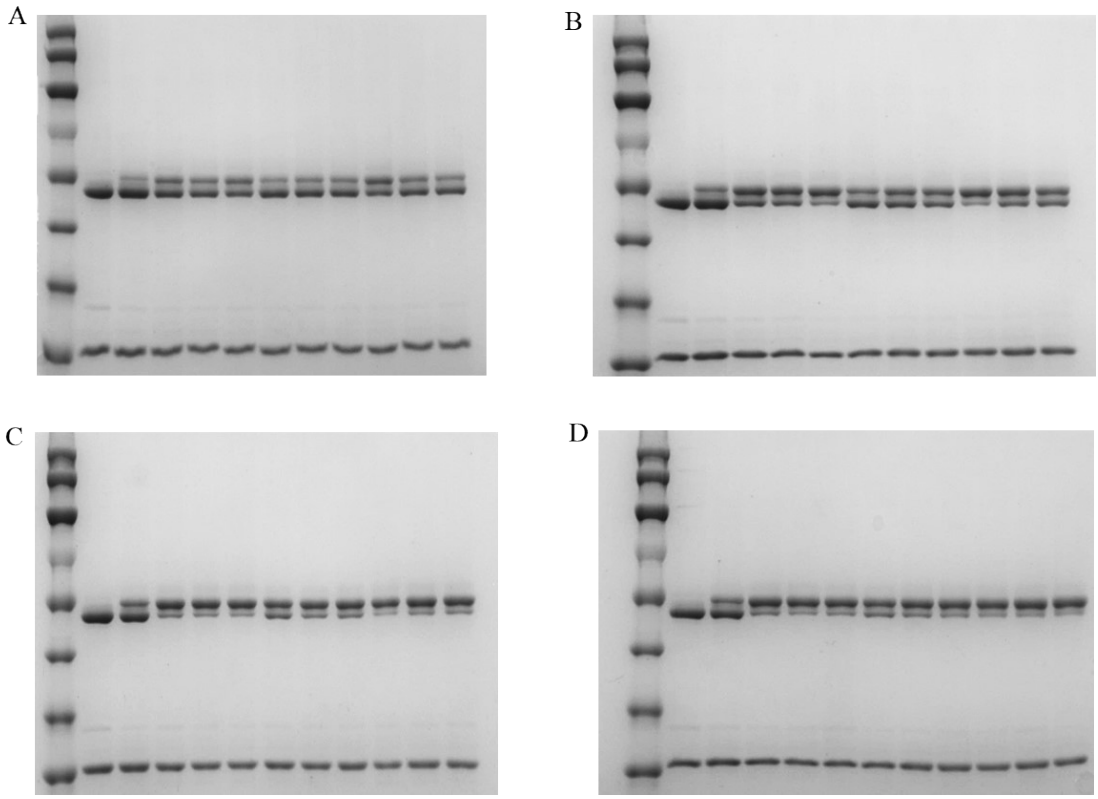


Figure S25. Transglycosylation effect of different enzymes for GN(F)-Rituximab (**9**). Reaction conditions: 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**A**) 15 min; (**B**) 30 min; (**C**) 1 h; (**D**) 2 h; Lane 0: Marker; Lane 1: **9**; Lane 2: **9** + D165A; Lane 3: **9** + **7a**; Lane 4: **9** + **7c**; Lane 5: **9** + **7b**; Lane 6: **9** + **5a**; Lane 7: **9** + **5b**; Lane 8: **9** + **5c**; Lane 9: **9** + **6a**; Lane 10: **9** + **6b**; Lane 11: **9** + **6c**.

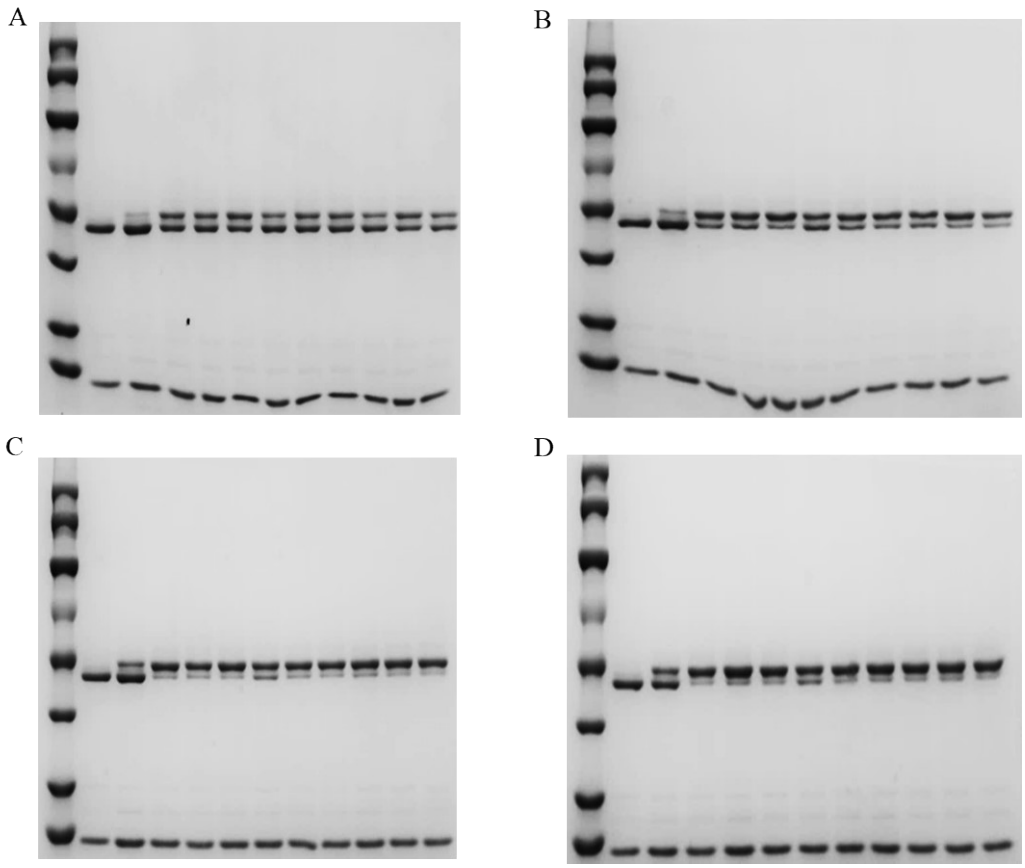


Figure S26. Transglycosylation effect of different enzymes for GN(F)-Rituximab (**9**). Reaction conditions: 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**A**) 15 min; (**B**) 30 min; (**C**) 1 h; (**D**) 2 h; Lane 0: Marker; Lane 1: **9**; Lane 2: **9** + D165A; Lane 3: **9** + **7a**; Lane 4: **9** + **7c**; Lane 5: **9** + **7b**; Lane 6: **9** + **5a**; Lane 7: **9** + **5b**; Lane 8: **9** + **5c**; Lane 9: **9** + **6a**; Lane 10: **9** + **6b**; Lane 11: **9** + **6c**.

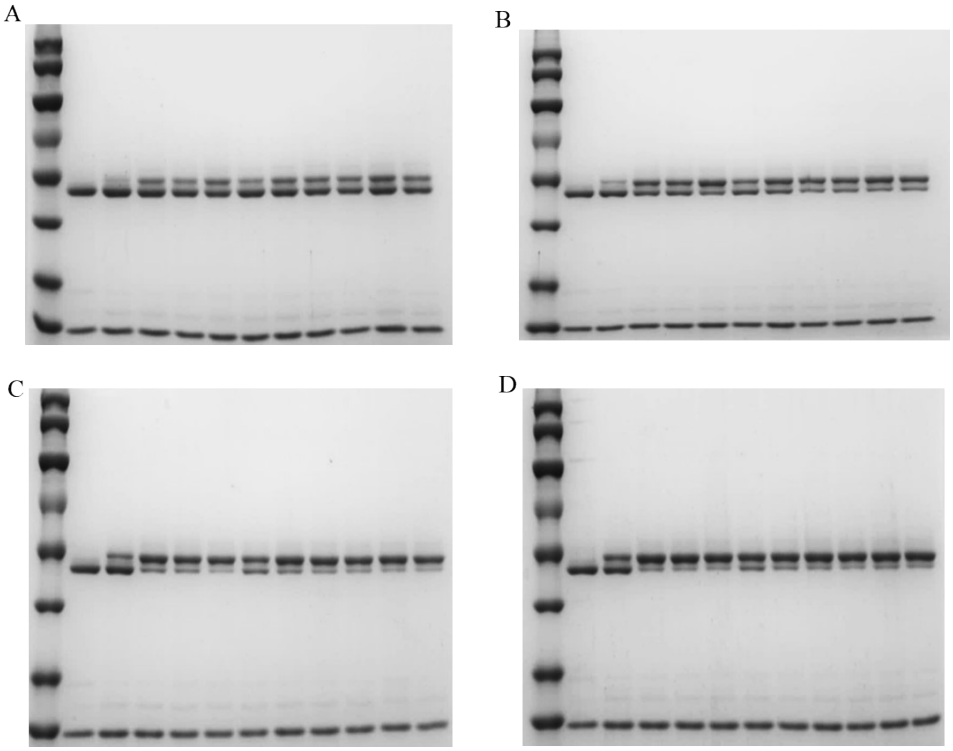


Figure S27. Transglycosylation effect of different enzymes for GN(F)-Rituximab (**9**). Reaction conditions: 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. (**A**) 15 min; (**B**) 30 min; (**C**) 1 h; (**D**) 2 h; Lane 0: Marker; Lane 1: **9**; Lane 2: **9** + D165A; Lane 3: **9** + **7a**; Lane 4: **9** + **7c**; Lane 5: **9** + **7b**; Lane 6: **9** + **5a**; Lane 7: **9** + **5b**; Lane 8: **9** + **5c**; Lane 9: **9** + **6a**; Lane 10: **9** + **6b**; Lane 11: **9** + **6c**.

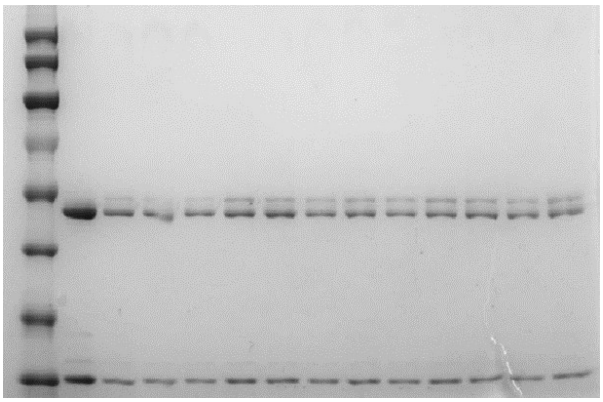


Figure S28. Transglycosylation effect of unconjugated FcBPs and enzyme for GN(F)-Rituximab (**9**). Reaction conditions were listed as follows: 5 mg/ml GN(F)-Rituximab (**9**), 0.1 mg/ml enzyme, 1.8 μM FcBPs and 1 mM SCT-ox in 50 mM PB (pH 7.4) at 30 ℃. Lane 0: Marker; Lane 1: **2**; Lane 2: FcBP3 + D165A, 15 min; Lane 3: FcBP1 + D165A, 15 min; Lane 4: FcBP2 + D165A, 15 min; Lane 5: FcBP3 + D165A, 30 min; Lane 6: FcBP1 + D165A, 30 min; Lane 7: FcBP2 + D165A, 30 min; Lane 8: FcBP3 + D165A, 1 h; Lane 9: FcBP1 + D165A, 1 h; Lane 10: FcBP2 + D165A, 1 h; Lane 11: FcBP3 + D165A, 2 h; Lane 12: FcBP1 + D165A, 2 h; Lane 13: FcBP2 + D165A, 2 h.

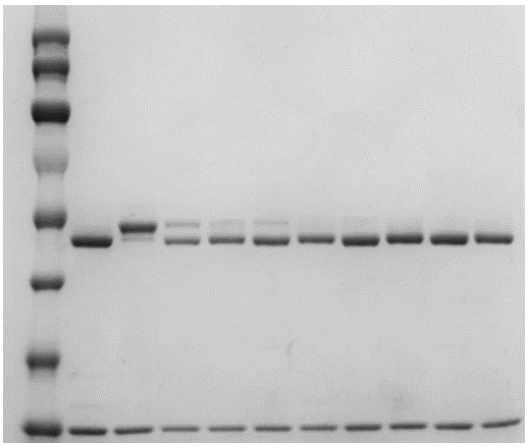


Figure S29. The glycan hydrolysis on Az-S2G2F-Rituximab (**10e**) by PNGase F or Endo S. Reaction conditions were listed as follows: 2 mg/ml Az-SCT-Rituximab, 0.1 mg/ml PNGase F or 0.01 mg/ml Endo S in 1X PBS buffer at 37 ℃. Lane 0: Marker; Lane 1: **9**; Lane 2: **10e**; Lane 3: **10e** + PNGase F, 15 min; Lane 4: **10e** + Endo S, 15 min; Lane 5: **10e** + PNGase F, 30 min; Lane 6: **10e** + Endo S, 30 min; Lane 7: **10e** + PNGase F, 1 h; Lane 8: **10e** + Endo S, 1 h; Lane 9: **10e** + PNGase F, 2 h; Lane 10: **10e** + Endo S, 2 h.

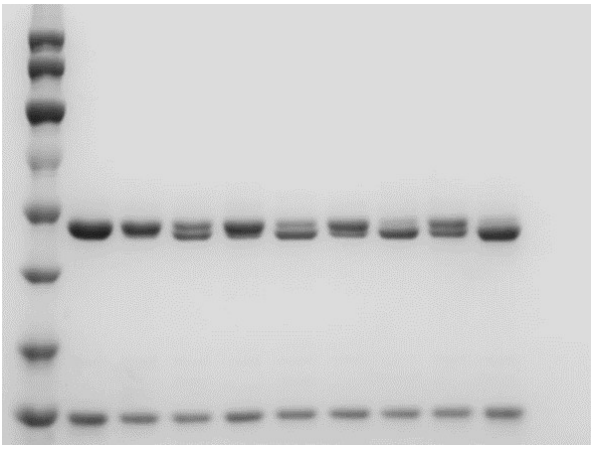


Figure S30. The hydrolysis activity of Endo F3 and FcBP2-PEG_8-MCC-F3 on WT-Rituximab (**8**). Reaction conditions were listed as follows: 5 mg/ml WT-Rituximab, 0.05 mg/ml enzymes in 50 mM PB (pH 6.5) at 37 ℃. Lane 0: Marker; Lane 1: **8**; Lane 2: **8** + Endo F3, 15 min; Lane 3: **8** + FcBP2-PEG_8-MCC-F3, 15 min; Lane 4: **8** + Endo F3, 30 min; Lane 5: **8** + FcBP2-PEG_8-MCC-F3, 30 min; Lane 6: **8** + Endo F3, 1 h; Lane 7: **8** + FcBP2-PEG_8-MCC-F3, 1 h; Lane 8: **8** + Endo F3, 2 h; Lane 9: **8** + FcBP2-PEG_8-MCC-F3, 2 h.

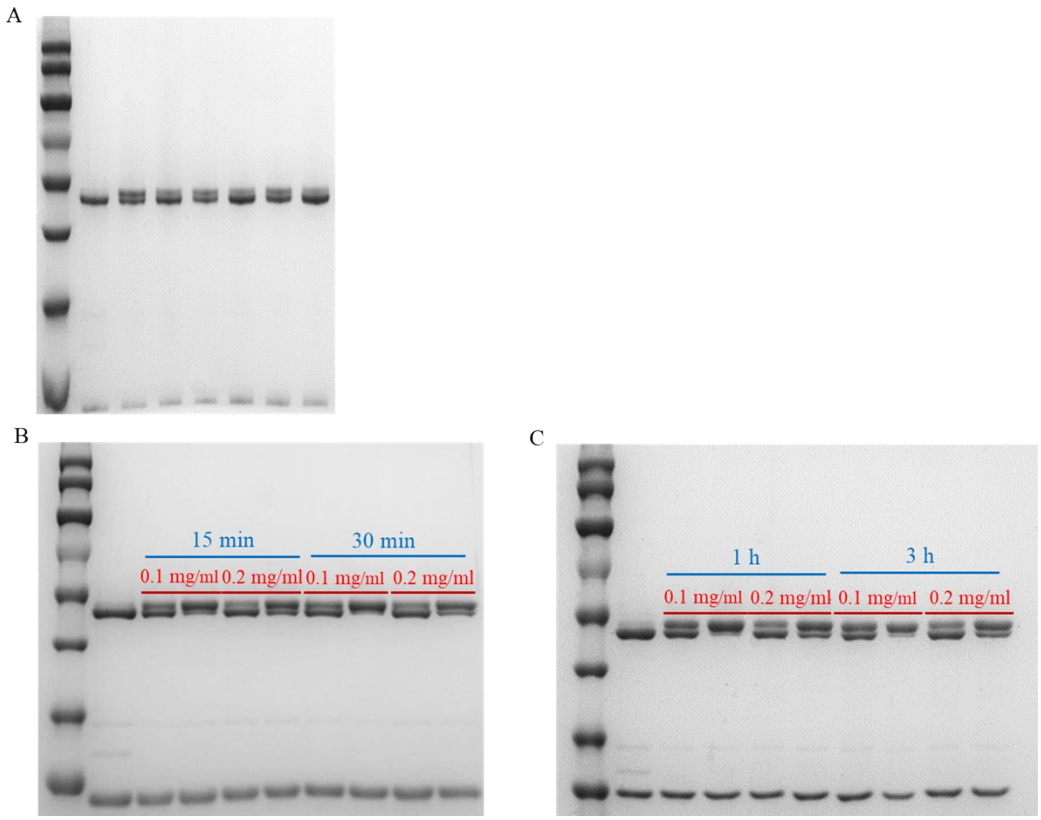


Figure 31. **(A)** 4 mg/ml WT-Rituximab (**8**) and 0.1 mg/ml or 0.2 mg/ml FcBP2 **6c** were added in 1X PBS at 37 ℃ at first. Lane 0: Marker; Lane 1: **9**; Lane 2: 0.1 mg/ml **6c** + **9**, 12h; Lane 3: 0.2 mg/ml **6c** + **9**, 12 h; Lane 4: 0.1 mg/ml **6c** + **9**, 18 h; Lane 5: 0.2 mg/ml **6c** + **9**, 18 h; Lane 6: 0.1 mg/ml **6c** + **9**, 24 h; Lane 7: 0.2 mg/ml **6c** + **9**, 24 h.

After 36 h, a half of solution was supplied with 1 mM CT-ox, while the left solution supplied with 1 mM CT-ox and 0.1 mg/ml **6c** for transglycosylation evaluation (30 ℃). **(B)** 15 min and 30 min; **(C)** 1 h and 3 h. Lane 0: Marker; Lane 1: **9**; Lane 2: 0.1 mg/ml **6c** + CT-ox; Lane 3: 0.1 mg/ml **6c** + CT-ox + 0.1mg/ml **6c**; Lane 4: 0.2 mg/ml **6c** + CT-ox; Lane 5: 0.2 mg/ml **6c** + CT-ox + 0.1 mg/ml **6c**; Lane 6: 0.1 mg/ml **6c** + CT-ox; Lane 7: 0.1 mg/ml **6c** + CT-ox+ 0.1 mg/ml **6c**; Lane 8: 0.2 mg/ml **6c** + CT-ox; Lane 9: 0.2 mg/ml **6c** + CT-ox + 0.1 mg/ml **6c**.

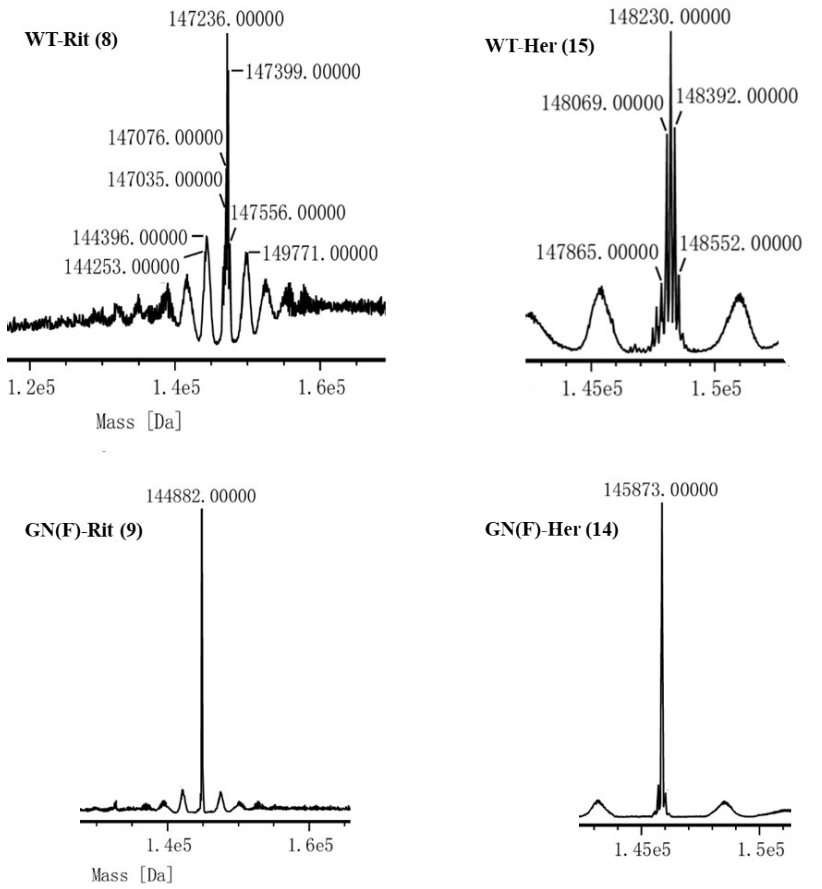


Figure S32. LC-MS profiles of native antibodies, GN (F)-Rituximab and GN (F)-Herceptin.