Characterizing Glycan-Induced Structural Changes and Binding Interfaces for IgG1-C1q Complex using Hydroxyl Radical Protein Footprinting

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HRPF Introduction

The Higher Order Structure (HOS) of a protein plays a critical role in a drugs stability, safety and biological function. Incorrect HOS or incorrect protein interactions are linked to adverse drug reactions which can result in further sickness or death. Advance techniques are required to robustly address the HOS of proteins. One such method is Hydroxyl Radical Protein Footprinting (HRPF). The Fox® Protein Footprinting System is a novel Hydroxyl Radical Protein Footprinting (HRPF) method that uses a proprietary flash oxidation lamp to generate hydroxyl radicals (•OH) that irreversibly modify solvent exposed amino acid side chains. As solvent accessibility changes, the •OH modification concordantly changes.

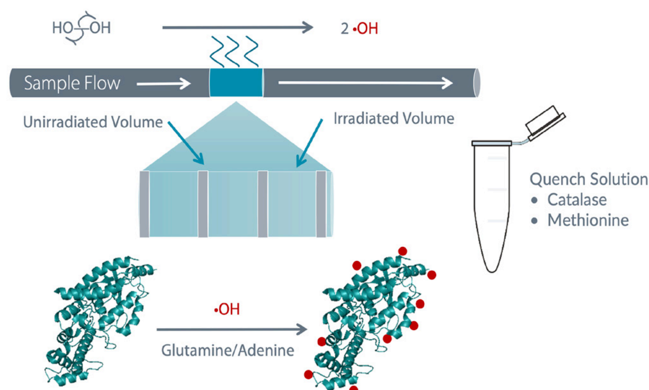


Figure 1: Schematic of an HRPF method, fast photochemical oxidation of proteins (FPOP). With FPOP, protein is mixed with hydrogen peroxide and flown passed a pulsing light source which photolyzes the hydrogen peroxide into two •OH and modifies solvent exposed amino acids. Following labeling, the sample is deposited into a quench solution of catalase and methionine.

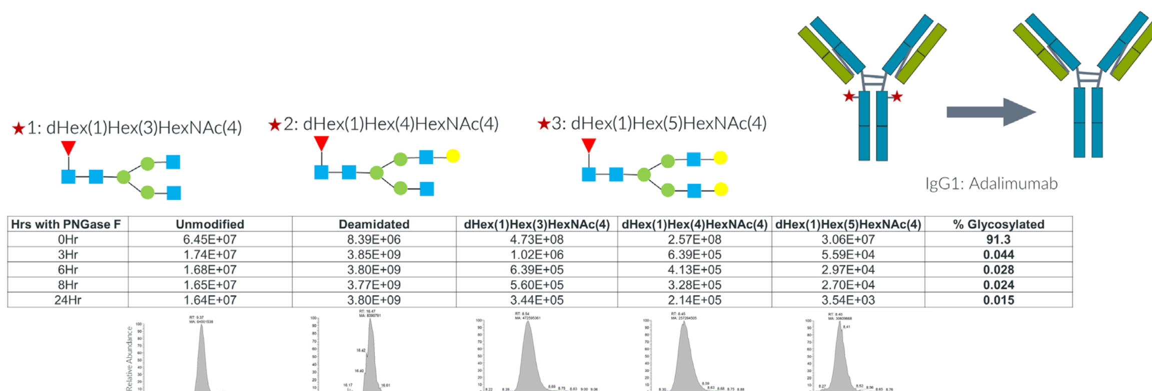
C1Q & IgG1 Introduction

The complement system is an essential part of our immune system that enhances the ability to clear pathogens and damaged cells, promotes inflammation, and attacks the pathogen's cell membrane. It consists of a series of proteins that work together in a cascade-like manner. The complement system can be activated via three pathways: the alternative pathway, the lectin pathway, and the classical pathway. The Classical pathway is initiated when the globular head of C1q which is apart of the C1 complex binds to antibodies that are attached to a pathogen. Once C1q's globular head binds to the antibody, it'll undergo a conformation change which will activate the C1 complex causing the continued cascade of enzymatic reactions ultimately leading to the lysis of the pathogen or infected cell.

C1q can bind to IgG and IgM. IgG is the most common type of antibody in blood circulation and is involved in response to most types of infections. There are four subtypes of IgG with IgG1 being the most common and it also has the highest binding affinity for C1q.

IgG has a single N-linked glycosylation site at asparagine 297 on each of the CH2 domains in the Fc region of the heavy chain. This glycosylation site is highly conserved in all four IgG subclasses. This glycan makes roughly 70 noncovalent interactions which stabilize the glycan and the Fc domain as well as how the Fab domain binds the antigen.

Previous studies have found IgG hexamerization is very important before C1q binds to the antibody. Further studies discovered the glycan on IgG is important for hexamer assembly which then results in C1q binding. However, there is still limited information in the full antibody characterization in native solution state conditions on exactly how the full antibody changes in structure following the removal of the glycan as well as how glycosylation effects C1q binding location and structure change.



Hrs with PNGase F	Unmodified	Deamidated	dHex(1)Hex(3)HexNAc(4)	dHex(1)Hex(4)HexNAc(4)	dHex(1)Hex(5)HexNAc(4)	% Glycosylated
0Hr	6.45E+07	8.39E+06	4.73E+08	2.57E+08	3.06E+07	**91.3**
3Hr	1.74E+07	3.85E+09	1.02E+06	6.39E+05	5.59E+04	**0.044**
6Hr	1.68E+07	3.80E+09	6.39E+05	4.13E+05	2.97E+04	**0.028**
8Hr	1.65E+07	3.77E+09	5.60E+05	3.28E+05	2.70E+04	**0.024**
24Hr	1.64E+07	3.80E+09	3.44E+05	2.14E+05	3.54E+03	**0.015**

*Figure 2: Removing IgG1's glycan. Removed the glycan in native conditions of PBS and 37*C using PNGase F. We incubated at multiple time points, stopped the reaction by adding a denaturing buffer, digested with trypsin and then identified the peptide with the glycan. We searched for the peptides unmodified mass, deamidated mass, and the mass with the addition of the three most common glycans observed on this IgG1 (Adalimumab). After 3 hours we observed the majority of the glycans were removed with minor decrease with additional times.*

Fox® Protein Footprinting System: HRPF labeling Method

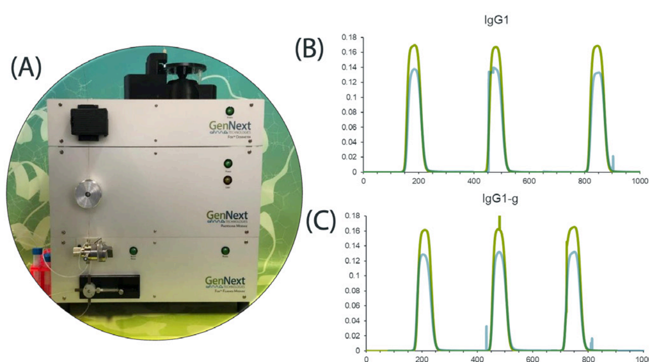


Figure 3: Next we performed HRPF using (A) The Fox® System. All samples were incubated for six hours at 37 °C half with PNGase and half without. A single sample is 12 µL of IgG at 1.25 µM and about 3.5 minutes of labeling time. All samples and controls were acquired in triplicates. (B) The dose response plot for IgG1 with the glycan (green control and blue sample). (C) The dose response plot for IgG1 without the glycan. The dose response plots monitore the effective •OH concentration. This ensures we get the same change in absorbance/ •OH concentration between the conditions so any change in oxidation is a direct result from a change in interaction or structure. Following labeling the samples were digested with trypsin and were identified using Thermo's Vanquish Neo and Exploris 240. The data was processed using GenNext's FoxWare Software.

IgG1 ± Glycan HRPF Peptide Level Analysis

Glycosylation is known to stabilize the CH2 domain, however exactly how the structure changes has contrasting observations. Two x-ray crystallographic studies of de-glycosylated Fc showed the CH2 domain collapsing into a closed conformation while two other studies saw the opposite where the CH2 domain forms an open conformation. It is possible both take place, and the observed structures is highly dependent on the crystal packing environment. This points towards the importance of studying the antibody in native solution state conditions like HRPF. Not only does HRPF observe the more open flexible Fc domain but also provides information on the intact hinge region becoming protected and varying changes within the Fab region which is very important to keep in mind due to varying antigen binding following changes in glycosylation.

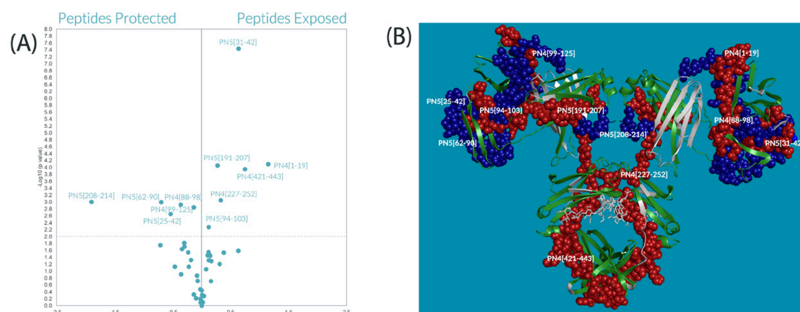
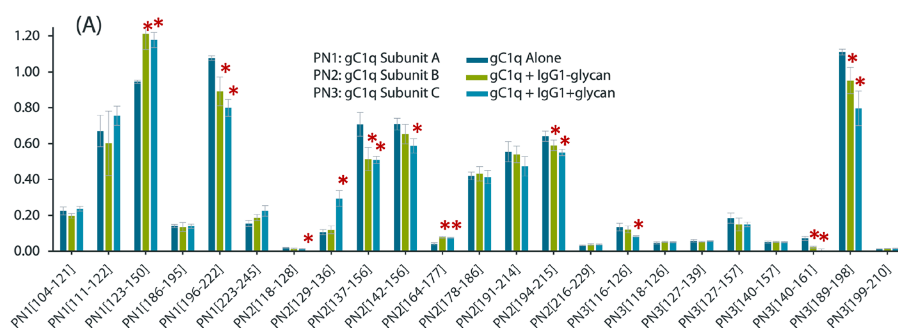


Figure 4: Peptide level HRPF Results. (A) Volcano plot comparing the Log2(Fold Change) against –Log(p-value). Dotted line represents a p-value of 0.01. From this, we can easily identify multiple regions on IgG that change in average solvent accessibility following de-glycosylation. (B) Crystal structure of an IgG decorated to show regions with a significant change in solvent accessibility following the removal of the glycan. Regions in green were detected with no significant change in oxidation. Red peptides became more exposed while blue became more protected after glycan removal.

C1Q Peptide Level



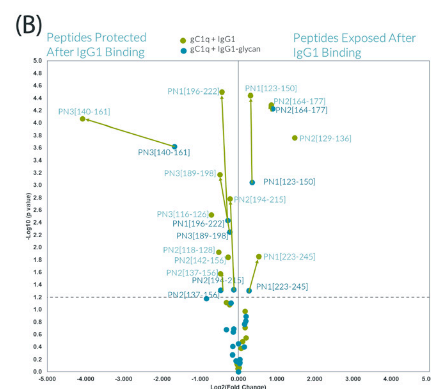
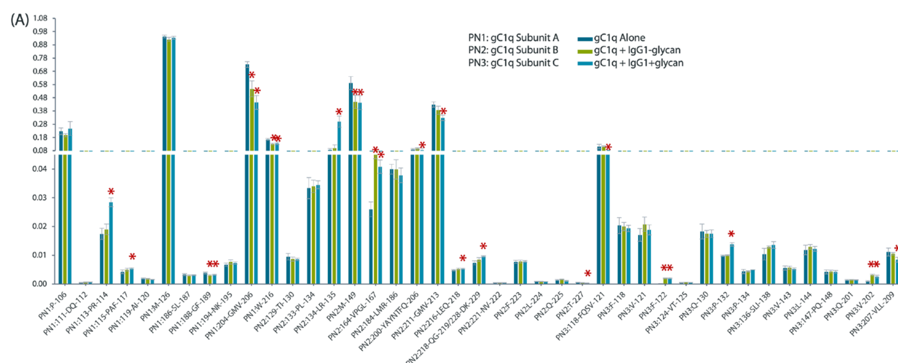


Figure 5: Peptide Level HRPF results for C1Q ± (IgG1 ± glycan). (A) Histograms to show the total magnitude of oxidation for C1Q alone (dark blue), C1Q + IgG1-glycan (green), and C1Q + IgG1 + glycan (light blue). Peptides with a significant change in oxidation (p-value > 0.05) is marked with a red astricks. (B) Volcano plot to show the fold change in oxidation and significance of change for C1Q with IgG1 + glycan (green) and C1Q with IgG1 – glycan (blue).

C1Q Residue Level



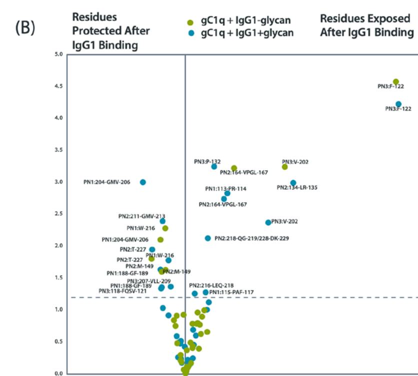


Figure 6: Residue Level HRPF results for C1Q ± (IgG1 ± glycan). (A) Histograms to show the total magnitude of oxidation for C1Q alone (dark blue), C1Q + IgG1-glycan (green), and C1Q + IgG1 + glycan (light blue). Peptides with a significant change in oxidation (p-value > 0.05) is marked with a red astricks. (B) Volcano plot to show the fold change in oxidation and significance of change for C1Q with IgG1 + glycan (blue) and C1Q with IgG1 – glycan (green).

gC1Q Coverage Map

Subunit	Start	Sequence
(A)	104	G S P G N I K D Q P R P A F S A I R R N P P M G G N V V I F D T V I T N Q E E P Y Q N H S G R F V C T V P G Y Y Y F T F
	164	Q V L S Q W E I C L S I V S S S R G Q V R R S L G F C D T T N K G L F Q V V S G G M V L Q L Q Q G D Q V W V E K D P K K
	224	G H I Y Q G S E A D S V F S G F L I F P S A
(B)	118	A T Q K I A F S A T R T I N V P L R R D Q T I R F D H V I T N M N N N Y E P R S G K F T C K V P G L Y Y F T Y H A S S R
	178	G N L C V N L M R G R E R A Q K V V T F C D Y A Y N T F Q V T T G G M V L K L E Q G E N V F L Q A T D K
(C)	116	Q K F Q S V F T V T R Q T H Q P P A P N S L I R F N A V L T N P Q G D Y D T S T G K F T C K V P G L Y Y F V Y H A S H T
	176	A N L C V L L Y R S G V K V V T F C G H T S K T N Q V N S G G V L L R

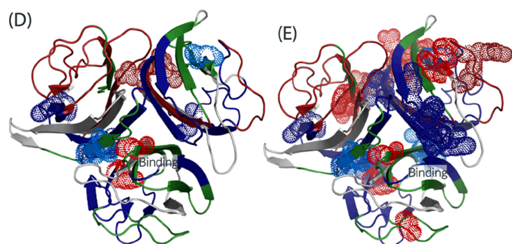


Figure 7: gC1Q coverage map for (A) subunit A (B) subunit B and (C) subunit C. Green font identifies peptide that were detected without a significant change in oxidation following peptide level analysis, red an increase in oxidation, and blue a decrease in oxidation. Residue cell's colored green were identified for residue level analysis with no change in oxidation, orange an increase in oxidation, and blue a decrease in oxidation.

Crystal structures decorated to identifies regions with a significant change in oxidation in (D) C1Q + IgG1-glycan and (E) C1Q + IgG1+glycan. The peptide backbone that is colored green are peptides identified with no significant change in oxidation following peptide level analysis, red an increase in oxidation, and blue a decrease. The residues that showed a significant change in oxidation are decorated as dot structures (red a significant increase in oxidation and blue a significant decrease in oxidation).

Conclusions

- HRPF identified glycan induced structure change for IgG1. The Fc region becomes more open, compacting around the hinge region resulting in multiple changes in the FAB region. The change in the FAB region points towards the significance of the glycan for antigen binding.
- HRPF identifies a binding pocket of IgG1 with C1Q with improved resolution using residues level analysis.
- C1Q binding decreases with de-glycosylated IgG1 but is not inhibited completely showing some similar regions of protection and exposure that are the same as IgG1 with the glycan. However, IgG1 with the glycan shows a larger fold change in oxidation.

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