HDX-MS reveals aggregation-prone regions in mAbs exposed to stress

Philipp Trolese[1] , Bendetta Fongaro[1], Vittoria Scapin[1], Andrea Pierangelini[1], Giorgia Miolo[1] and Patrizia Polverino de Laureto[1]

1 Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Italy

Introduction

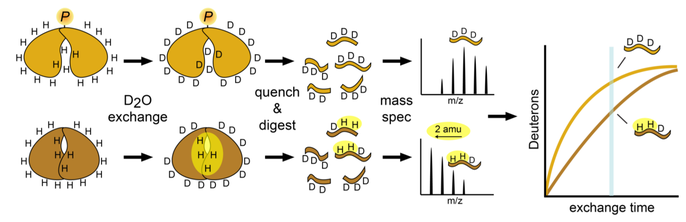
Monoclonal antibodies (mAbs) are crucial therapeutic proteins. Their large size and complex structure pose significant pharmaceutical challenges, particularly regarding stability and solubility. Structurally, mAbs consist of two light (LC) and two heavy (HC) chains, held together by disulfide bonds, and are divided into two Fab (antigen-binding) regions and one Fc (constant) region[1].
Throughout development, mAbs encounter stressors at every stage, including purification, shipping, storage, and administration. A major concern is protein aggregation, as aggregated antibodies can trigger immune responses. Identifying specific regions prone to unfolding and aggregation under pharmaceutically relevant conditions is critical. Understanding the unfolding and aggregation mechanism is essential for optimizing mAb stability and to develop strategies to reduce this form of degradation. Hence, we exposed mAbs to three different stressors: 1) low pH, 2) light, since we have previously seen they can cause aggregation[2], and 3) treatment with guanidinium to generate partially unfolded species, potentially prone to aggregation. The resulting conformations were studied by Hydrogen-Deuterium Exchange Mass Spectrometry (HDX-MS) that provides detailed insights into conformational changes and aggregation-prone regions at local resolution.

Materials

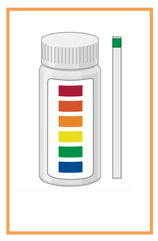
- Ipilimumab (Yervoy®): humanized IgG antibody that blocks CTLA-4
- Bevacizumab (Oyavas®): humanized IgG antibody binding and neutralizing VEGF-A
- Both antibody formulations were provided by a hospital pharmacy (Azienda Ulss 3 Serenissima, Mestre, Italy) as fresh daily residues after patient treatments.

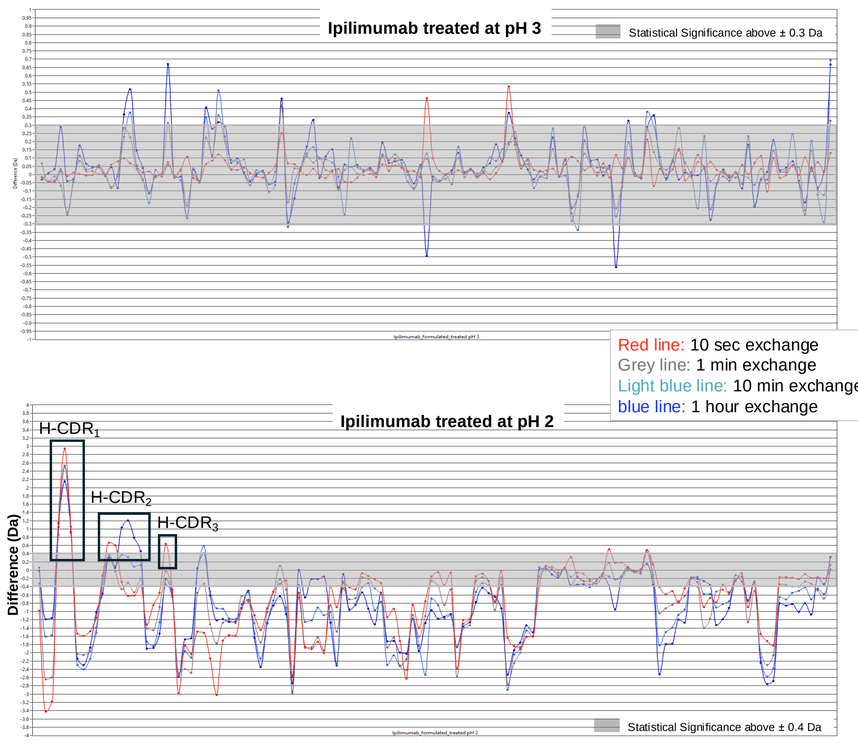
Methods

- Ipilimumab was exposed to two pharmaceutical relevant stressors: i) low pH and ii) light irradiation. i) Ipilimumab was diluted to pH 2 and 3. After 30 min incubation the antibody was titrated back to neutral pH. ii) Light radiation was generated through at a dose of 720 KJ/m^2 (200 W hours/m^2), corresponding to the amount of light the sample would take approximately upon a daily exposure. The irradiance was 360 mW/cm^2 (300–800 nm). The stress-exposed antibody was then subjected to HDX-MS, with exchange times of 10 sec, 1 min, 10 min and 1 h.
- Bevacizumab unfolding was studied by exposing the antibody for increasing time (2 min, 10 min, 45 min, 2 h and ON) to 4 M Gnd-HCl. After dilution to 1 M Gnd-HCl the antibody was let to equilibrate/refold for the same amount of unfolding time. After pulse labeling (10 sec) in deuterated buffer the deuterium uptake was measured. Dynamic light scattering was performed at every timestep to discern between refolding or aggregation events.
- Schematic diagram of HDX-MS to probe conformational changes[4]



Results





Δ > 0
Less uptake after treatment
= less exposed

Δ < 0
More uptake after treatment
= more exposed

Figure 1: Differences in the deuterium uptake between pH 7 and pH 3 (top) and pH 7 and pH 2 (bottom) for each peptide generate by peptic fragmentation at the reported time. While at pH 3, changes in deuterium uptake are scarce, after exposure to pH 2 and back titration to pH 7, the D uptake is overall increased, suggesting protein unfolding. Interestingly, the CDR regions, especially in the heavy chains, exhibit an apparent lower D uptake.

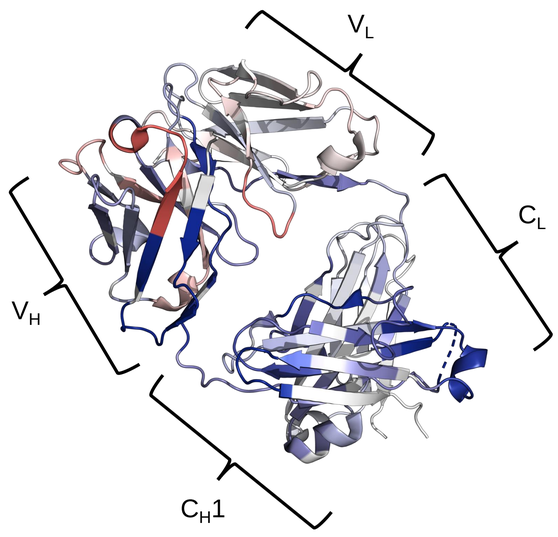


Figure 2: Crystal structure of the Fab of Ipilimumab (PDB code: 5TRU) highlighting the difference in D uptake at pH 2 after 10 sec exchange. Blue areas appear more exposed after treatment indicating general unfolding, while red regions appear less exposed after treatment, suggesting they may represent aggregation hotspots.

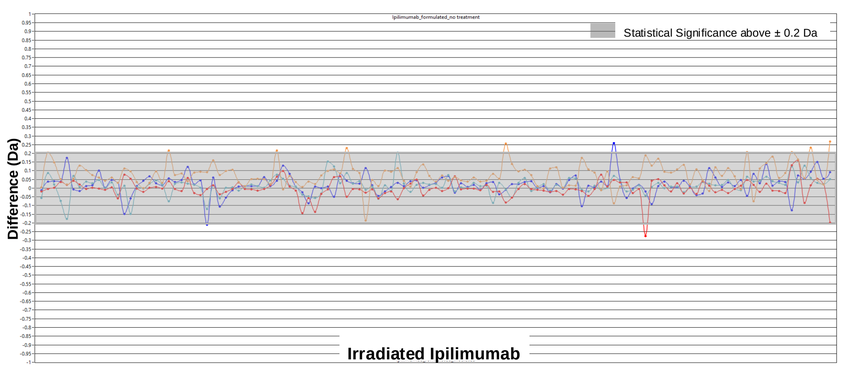


Figure 3: Difference in the deuterium uptake between the non treated and the irradiated Ipilimumab samples for each specific peptide at the reported time. No significant changes in D uptake are observed across the whole structure.

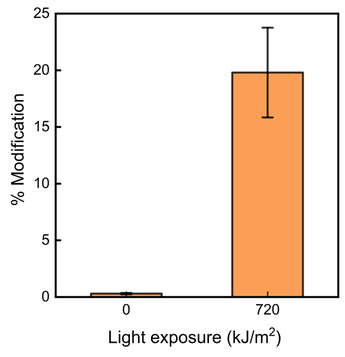
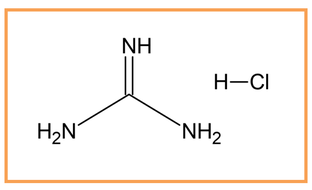
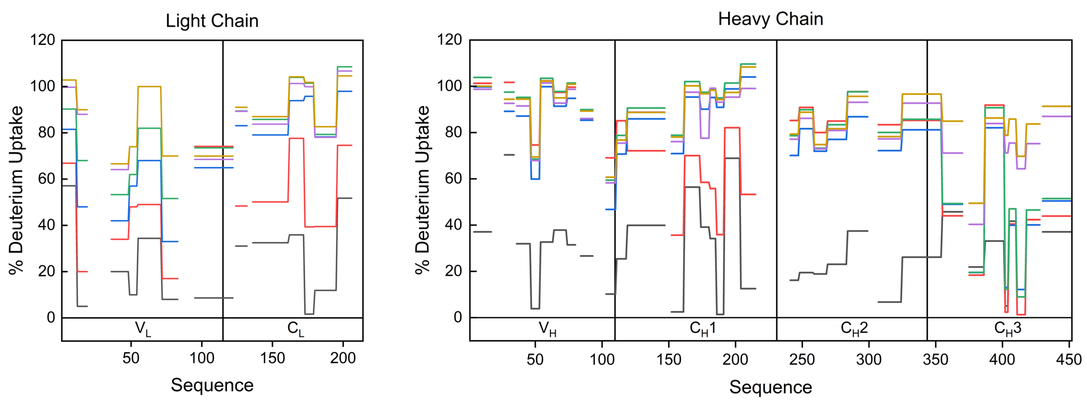


Figure 4: Percentage of light-oxidized Ipilimumab at time 0 and after light exposure. Interestingly, while no changes in structure are observed the antibody presents a high amount of oxidation.



Black line: 0 min Gnd exposure
Red line: 2 min Gnd exposure
Blue red: line: 10 min Gnd exposure
Green line: 45 min Gnd exposure
Violet line: 2 hours Gnd exposure
Yellow line: ON Gnd exposure



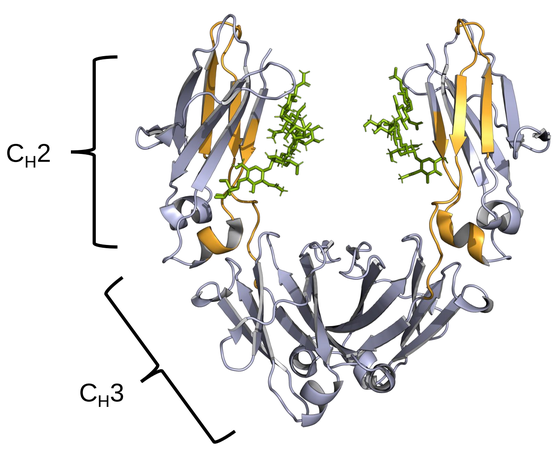


Figure 6: FC region of an antibody (PDB code: 5JII) presenting more than 99% sequence with Bevacizumab. Highlighted in green the glycosylation and in orange the peptides in the C_H2 domain that present difference in D uptake after refolding from a state where the CH3 domain was unfolded. Of note, these peptides contain 40-60% hydrophobic residues, suggesting that if exposed for enough time they could serve as aggregation hotspots.

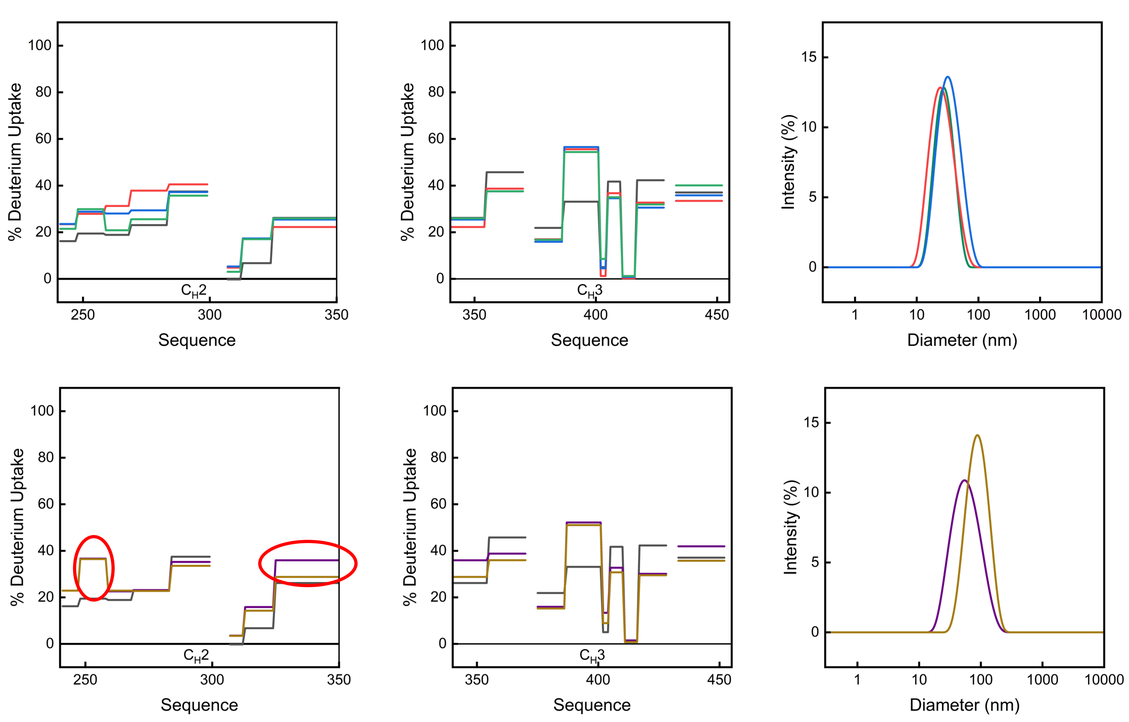


Figure 5: HDX-MS measurements tracking Bevacizumab unfolding after incubation in 4 M Gnd for increasing time (top). Interestingly, some regions in the V_L domain unfold gradually while other regions (e.g., the C_H2 domain) seem to follow an On/Off mechanism. The latter seems to be the least stable. The C_H3 domain seems quite stable unfolding only after 2 hour and ON incubation in 4 M Gnd.
HDX and DLS measurements of samples unfolded and refolded for 2 min, 10 min and 45 min (center). Most unfolded regions in the C_H2 domain seem fully recovered leaving the mAb in a partial unfolded state with a hydrodynamic radius of about 30 nm.
HDX and DLS measurements of the refolded samples where the C_H3 domain was unfolded after 2 h and ON incubation (bottom). The C_H3 domain seems to correctly refold, while the C_H2 domain presents differences in D uptake that were not present previously. Interestingly, this samples are accompanied by a hydrodynamic radius of 60-90 nm, suggesting aggregation.

Conclusions

- Incubating the protein at pH 3 before titrating it back to pH 7 does not seem to severely affect protein structure. On the other hand, bringing the protein as low as pH 2 before the back titration causes overall unfolding of the protein. Interestingly, the CDR regions of the heavy chain experience less uptake after the treatment, suggesting they may function as aggregation hotspots.
- While exposure to light at a dose of 720 kJ/m^2 does increase the percentage of chemically modified antibody, it does not seem to affect protein structure.
- Unfolding experiments in Gnd confirm that the C_H2 domain is the least stable. Interestingly, unfolding of the C_H2 alone does not cause aggregation when refolding the sample, as seen in DLS experiments. Only the samples where also the C_H3 domain was unfolded after 2 hours and ON incubation led to aggregation of the samples when trying to refold them from 4 M Gnd. Probably, refolding of the C_H3 domain in concomitance with the C_H2 domain, changes the refolding pattern of the latter possibly exposing aggregation hotspots.
- This technique proves to be highly effective in providing detailed insights into protein conformational changes, though further analysis is needed to fully understand the underlying mechanisms of mAb instability and aggregation.

References

1. Chiu, M. L., Goulet, D. R., Teplyakov, A. & Gilliland, G. L. Antibody Structure and Function: The Basis for Engineering Therapeutics. Antibodies (2019).
2. Rizzotto, E. et al. Light exacerbates local and global effects induced by pH unfolding of Ipilimumab. Eur. J. Pharm. Biopharm. (2024).
3. Ozohanics, O. & Ambrus, A. Hydrogen-Deuterium Exchange Mass Spectrometry: A Novel Structural Biology Approach to Structure, Dynamics and Interactions of Proteins and Their Complexes. Life (2020).
4. https://www.underbakkelab.org/techniques

Acknowledgments

This project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking (JU) under grant agreement N° 101007939 (RealHOPE).
This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA. Content of this study reflects only the author's view and the JU is not responsible for any use that may be made of the information it contains.

Let's connect!