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Light exacerbates local effects induced by pH unfolding in monoclonal antibodies

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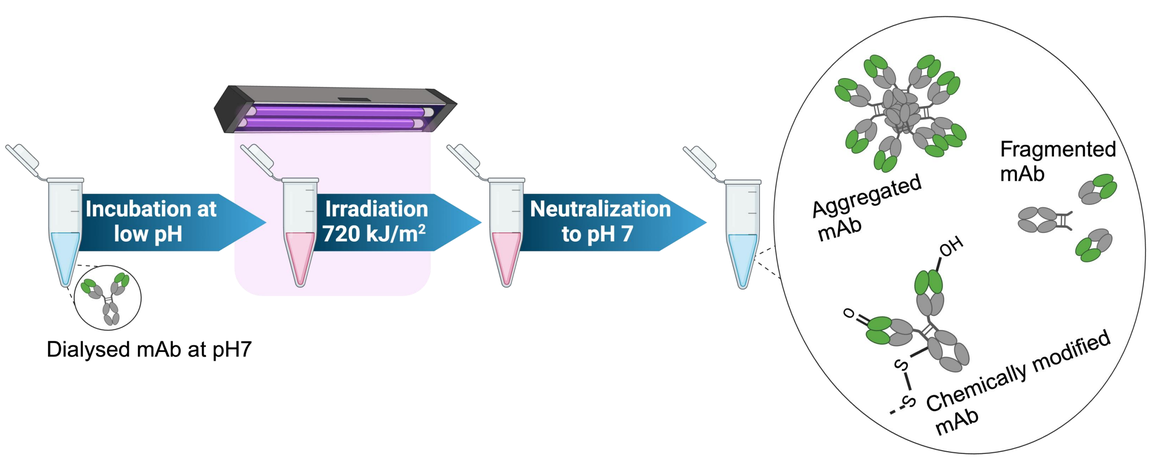
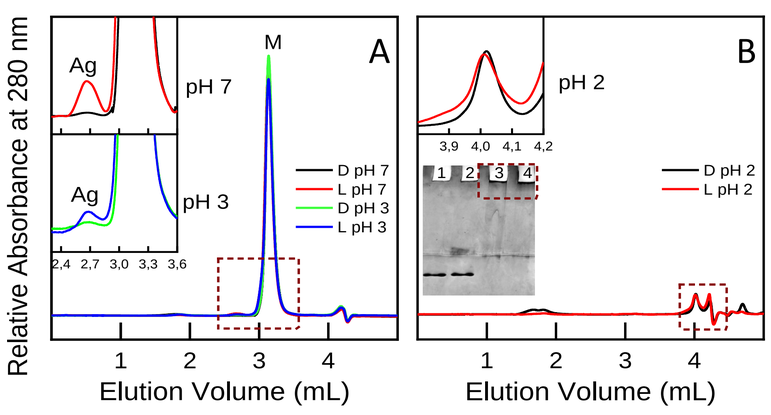
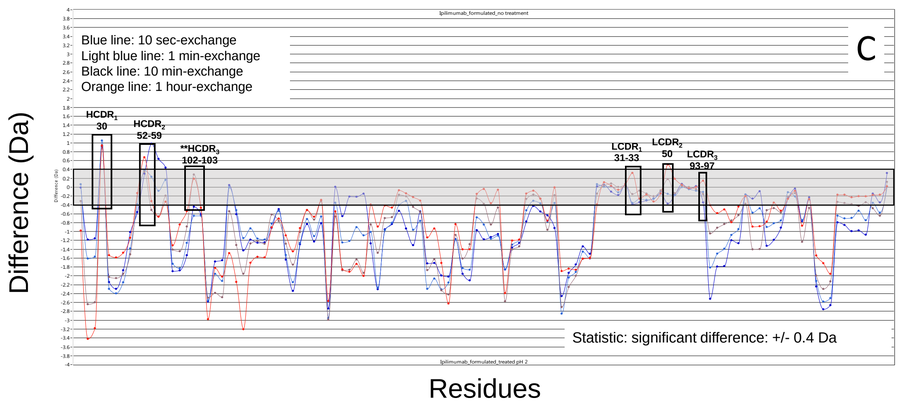


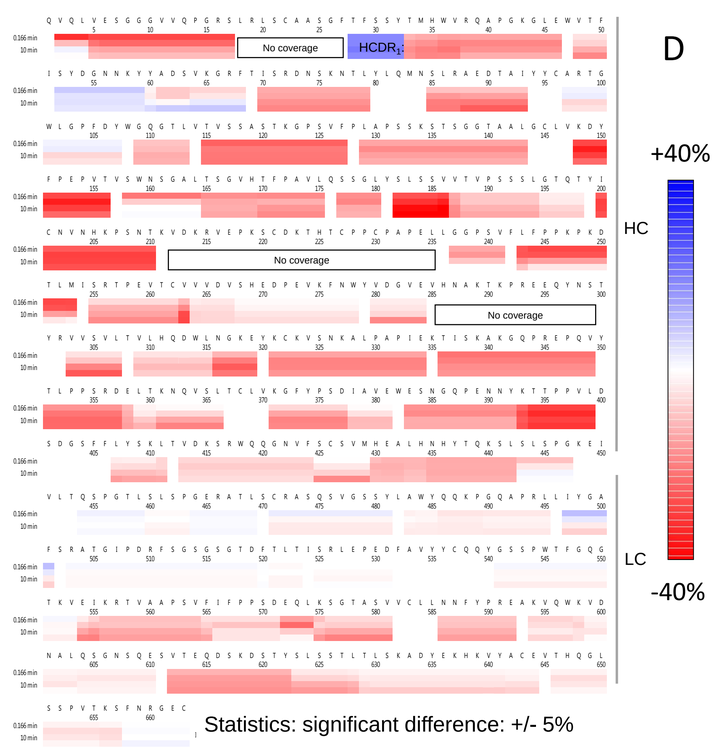
Monoclonal antibodies (mAbs) are an essential class of proteins with several therapeutic applications. Throughout their production, storage, and administration, these proteins encounter various stressors. Viral inactivation, obtained by low pH exposure, is a key step in their downstream process. It represents a critical stress factor, which can result in protein unfolding, chemical and physical modifications. Additionally, exposure to light during this process can further affect the structural integrity of mAbs.

Here, we have studied the nature of the Ipilimumab modifications occurring due to the simultaneous exposure to pH and light. Ipilimumab was first incubated at pH 2 or 3 for 30 min, then neutralized to pH 7 and analyzed by several biophysical techniques. The pH 3 induced denaturation appears reversible, and a moderate aggregation was detected. On the contrary, the pH 2-exposed samples underwent extensive aggregation with formation of insoluble aggregates and fragmentation. These events are particularly critical since they can reduce the availability of the therapeutic protein and increase the occurrence of immune reactions. Moreover, the exposure to light could chemically modify Ipilimumab generating new species distinct from the formulated one amplifying the mentioned effects. Detailed conformational studies on the structural modifications of mAbs may improve the chemical-physical knowledge of these effective drugs and suggest new production strategies for more stable products.

AGGREGATION







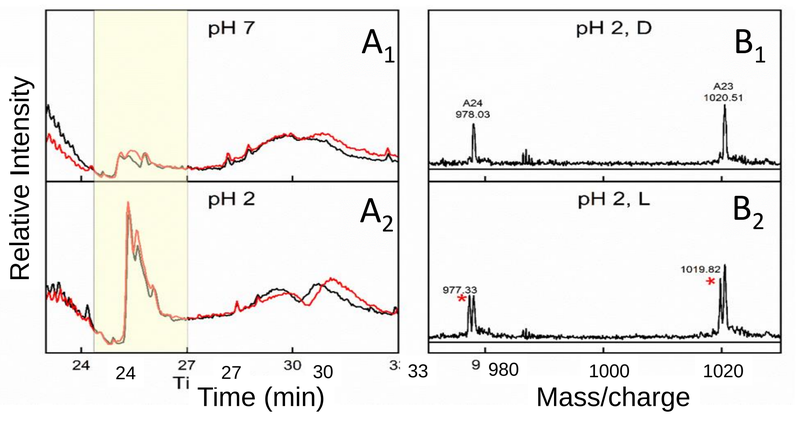
SEC chromatograms of Ipilimumab at pH 7 and 3 **(A)**, and pH 2 **(B)**, after light exposure (L) and in the dark (D). In B, a native gel of dark (1, 3) and irradiated (2, 4) samples at pH 3 (1, 2) and pH 2 (3, 4) is shown.

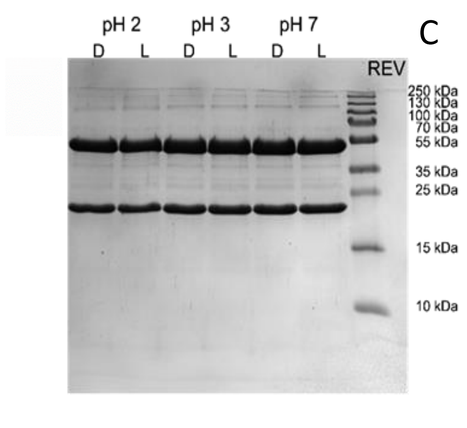
The samples exposed to pH 3 exhibit a SEC profile similar to the control (pH 7). The pH 2-exposed samples underwent degradation, formation of insoluble aggregates, visible in native-PAGE, with loss of the monomeric species. Light exacerbates aggregation in samples at pH 3 and 7, no differences were detected for pH 2.

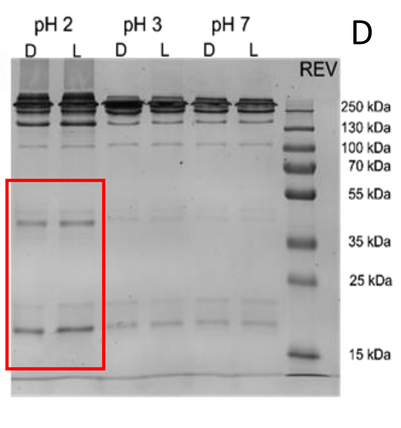
The conformational state of Ipilimumab was investigated by hydrogen/deuterium exchange mass spectrometry (HDX-MS) **(C,D)**. In **(C)**, each point represents the differences in the deuterium uptake between pH 7 (control) and pH 2 for a specific peptide at the reported time. **(D)**, difference (%) bi-dimensional heatmap between the two states. Red to blue shift correlates to an increased deuterium uptake. In both C and D, deuterium uptake data of control (Ipilimumab pH 7) were subtracted from the corresponding data obtained after treatment at pH 2.

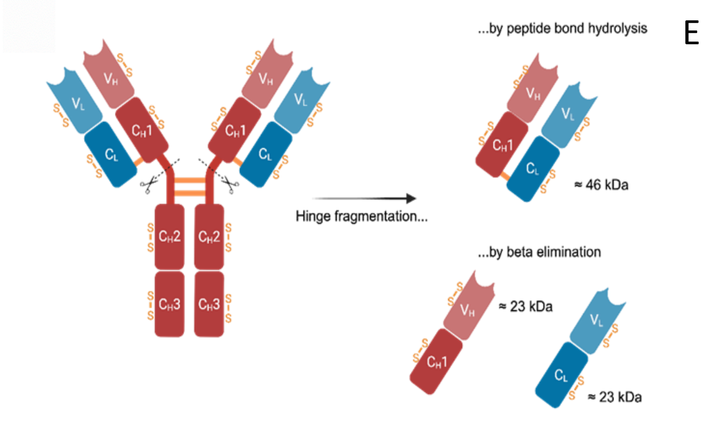
HDX-MS experiments reveal that after exposure at pH 2 and back-titration to pH 7, the deuterium uptake increases compared to the control at pH 7, suggesting an overall protein unfolding. On the contrary, the CDR regions exhibit an apparent lower deuterium uptake, suggesting the involvement of these segments in the aggregation process, with a more pronounced effect in the heavy chain.

FRAGMENTATION









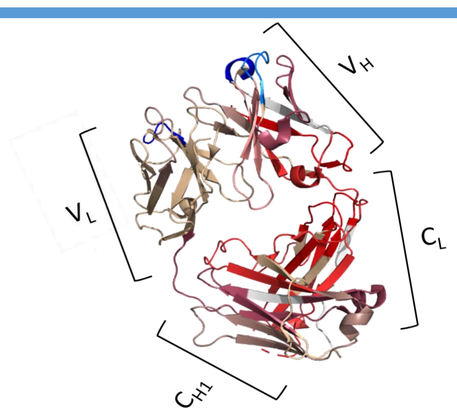
LC-MS/MS profiles (TIC) of Ipilimumab at pH 7 (A_1) and pH 2 (A_2) before (black line) and after (red line) light exposure. The region containing the difference in the profiles between pH 2 and pH 7 is highlighted in yellow and the corresponding mass spectra (m/z) before **(B_1)** and after **(B_2)** light exposure at pH 2 are reported. Stars indicate the m/z signals belonging to the species formed after irradiation.

The LC-MS/MS profile reveals the formation of the isolated Light Chain (LC) fragment when Ipilimumab is exposed to pH 2. Light-induced species correspond to LC fragment containing a Δ mass of -18 Da, which is compatible with the loss of a water molecule following the formation of an isopeptide bond.

SDS-PAGE of Ipilimumab in reducing (**C**) and non-reducing conditions (**D**). The protein samples incubated at pH 2 and 3, exposed to light (L), kept in the dark (D) and neutralized to pH 7 (REV) were analyzed.

In C, in addition to the two characteristic bands, at ~ 50 kDa (heavy chain) and at ~ 25 kDa (light chain), high molecular weight bands are detectable indicating protein aggregation. In D, the presence of aggregates as well as fragments in the samples exposed at pH 2 was detected. Specifically, two fragment species produced two intense bands at 46 and 23 kDa, respectively. Mass spectrometry analysis indicated the fragments correspond to a species containing the $VHCH_1VLCL$ domain (46 kDa) and a mixture of $VHCH_1$ and VLCL (23 kDa). The latter species could be formed by a cleavage between Ser220 and Cys221 (**E**). The formation of these species implies that the light chain could dissociate from the heavy chain by a mechanism that is independent of the reduction of the disulfide bridge.

CONCLUSIONS



- The biophysical characterization of stressed Ipilimumab reveals that pH variation is a considerable risk factor, with irreversible unfolding and fragmentation observed at pH 2.
- The threshold for Ipilimumab denaturation lies between pH 2 and 3 and is correlated with the loss of the protein structural cooperativity, which is the most critical factor determining the protein refolding.
- Light exacerbates some local and global effects making pH-induced exposed residues more vulnerable to structural and chemical changes.
- HDX-MS complements traditional biophysical techniques providing protein structural information at molecular level. This technique is suitable for studying proteins in their native states, making it an indispensable tool for investigating the complexities of protein interactions and conformation in response to changes in the surrounding environment. Here, this technique allowed the study of protein folding revealing some specific regions in Ipilimumab at pH 2 to be more (highlighted in blue) or less (highlighted in red) compact compared to the control at pH 7.
- Our findings underscore the critical role of pH optimization in preserving the structural integrity of proteins such as Ipilimumab and indicate that precautions to real-life light exposure during the sterilization process of mAbs should be considered to avoid loss of the therapeutic activity and to increase the yield of production.

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