Conformational Epitope Mapping by Cross-link Mass Spectrometry: Analysis of Ipilimumab, Nivolumab and Pembrolizumab

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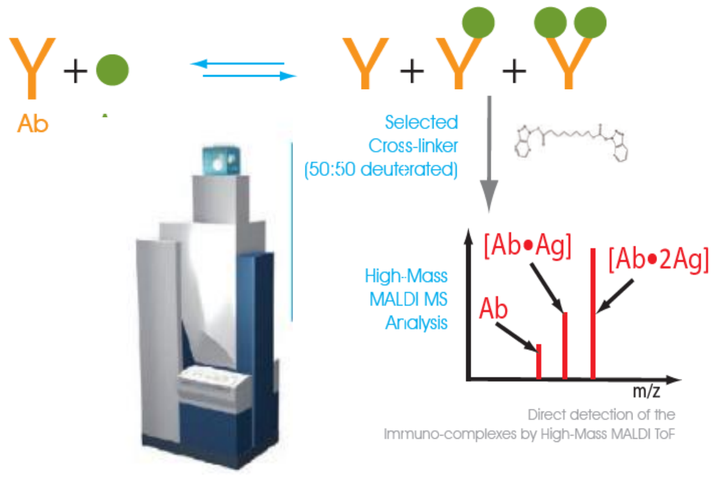
Introduction

Epitope mapping is one of the key structural analysis during the development of new therapeutic monoclonal antibodies. The characterization of the molecular interface between an antibody and its target antigen is critical, not only for patent purpose but also for understanding the mechanism of action of the drug itself. If linear epitope are relatively easy to resolve, conformational epitopes are still challenging to characterize, mainly due to the resolution required for such analysis. We are presenting the use of chemical cross-linking and high resolution mass spectrometry for conformational epitope mapping of Ipimumab, Nivolumab and Pembrolizumab monoclonal antibodies.

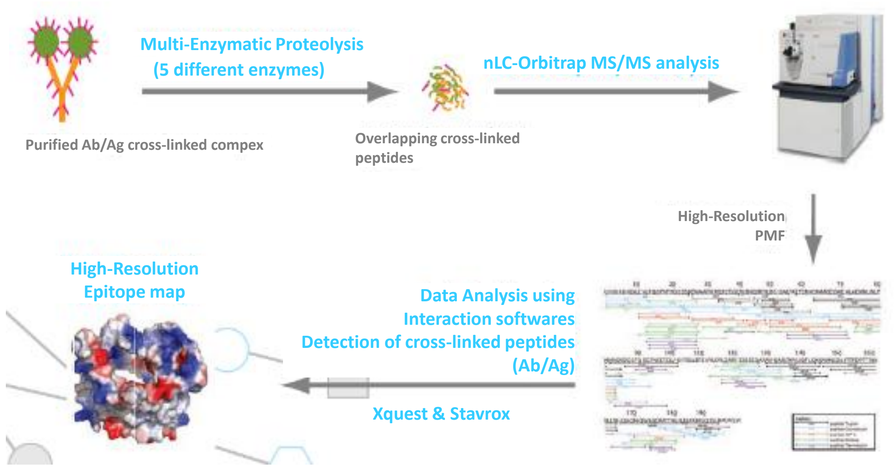
Method

In a first step, each samples were tested for aggregation and non-covalent multimers by chemical cross-linking and High-Mass MALDI ToF MS. Then the cross-linking conditions were optimized for each complex: Ipilimumab/CTL4; Nivolumab /PD-1 and Pembrolizumab/PD-1. After optimization, each cross-linked complexes were subjected to proteolysis using five different enzymes. The cross-linked peptides generated were analysis using nLC/orbitrap MS/MS analysis. The data generated were analyzed by Xquest and Stavrox softwares in order to detect the cross-linked between peptides of the antibody and peptides of the antigen.

Workflow



High-Mass MALDI ToF MS analysis of the intact protein complex Ab/Ag. After mixing the antibody and the antigen, the sample was submitted to cross-linking. After incubation, the cross-linked complexes were analyzed by High-Mass MALDI in order to detect cross-linked Ab/Ag complexes. The high-mass MALDI is used to optimize the cross-linking of the Ab/Ag complexes before proteolysis.

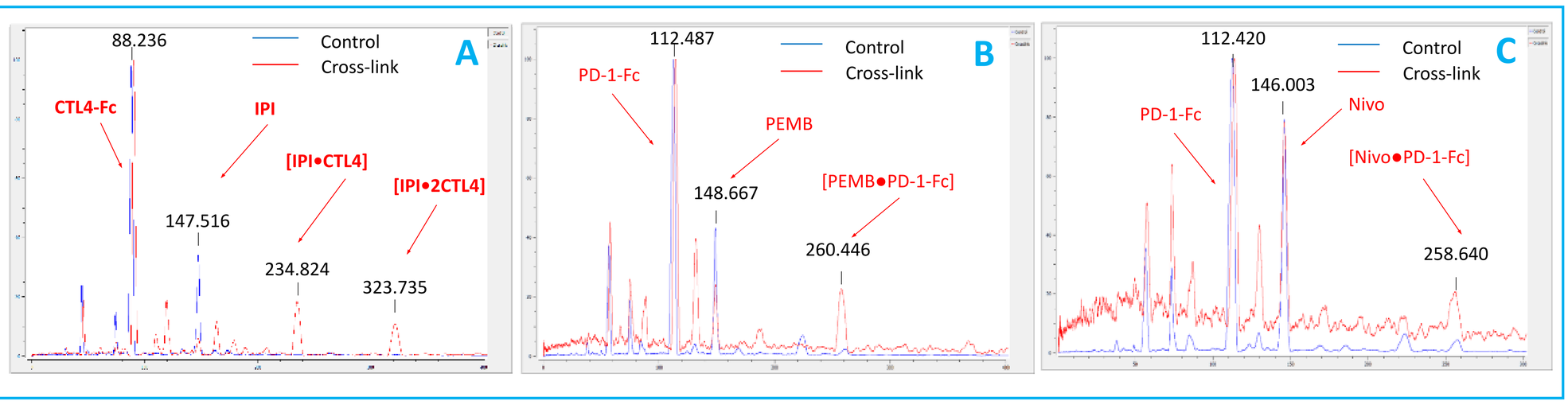


After High-Mass MALDI, the cross-linked Ab/Ag complexes were proteolyzed using five different enzymes to ensure maximum antigen sequence coverage (PMF). The peptides generated were analyzed using nLC and orbitrap MS/MS. Data were processed using dedicated softwares in order to identify inter-protein cross-linked peptides.

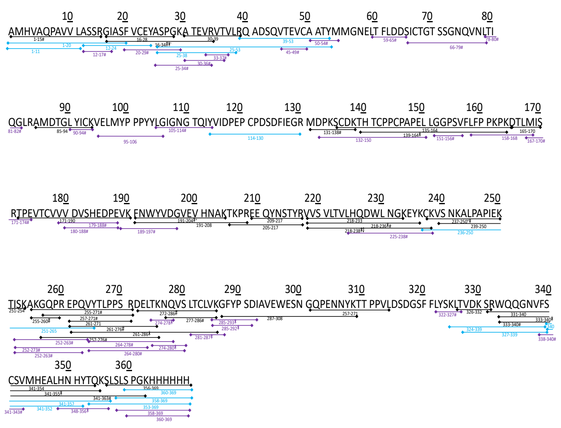
Results

High-Mass MALDI Analysis of the intact Antibody/Antigen complexes

High-Mass MALDI MS analysis of the antibody/antigen protein complexes Ipilimumab/CTL4 (A), Pembrolizumab/PD-1 (B) and Nivolumab/PD-1 (C). For each sample, antibody/antigen mixtures were prepared and analyzed by High-Mass MALDI ToF mass spectrometry (blue traces) (HM4, CovalX). Then each sample was cross-linked (K200, CovalX) and incubated 180 minutes before High-Mass MALDI analysis (red traces). For each sample, after cross-linking, the non-covalent complexes [antibody●antigen] were detected in the high-mass range.

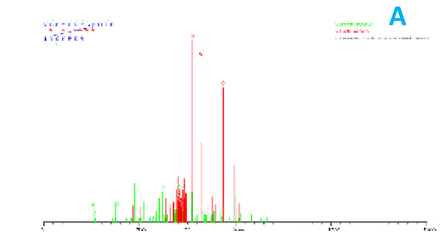


Peptide Mass Fingerprint (PMF)



Peptide Mass Fingerprint of the protein complex Ipilimumab/CTL4. After High-Mass MALDI analysis, the cross-link sample was proteolyzed using five different enzymes (Trypsin, Chymotryptin, ASP-N, Elastase, Thermolysin). The peptides generated were analyzed by nLC-Orbitrap MS/MS. X-quest and Stavrox softwares were used to identified the cross-linked peptides. 89% of the sequence of CTL4 was covered by the different peptides identified. For PD-1 antigen, 88% of the sequence was covered by the peptides identified.

After nLC-Orbitrap MS/MS analysis, the data generated were analyzed using Xquest and Stavrox software. For each proteolysis (Trypsin, Chymotrypsin, ASP-N, Elastase and Thermolysin), the data generated were tested for the presence of inter-protein cross-links. A, B and C: MS/MS data analyzed by Xquest software; example of the cross-linked peptides PD-1 (21-32) - Iplimumab_LC (49-55). D. Inter-protein cross-linked peptides of the complex CTL4/Ipilimumab after thermolysin digestion.



beta-chain B

beta_common_b_standard_plus1	114.09	-	-	-	-	-	-
beta_common_b_standard_plus2	57.55	-	-	-	-	-	-
beta_common_b_standard_plus3	38.70	-	-	-	-	-	-
beta_xlink_b_standard_plus1	-	1725.80	1782.83	1853.86	2000.93	2087.96	2244.06
beta_xlink_b_standard_plus2	-	863.41	891.92	927.44	1000.97	1044.49	1122.54
beta_xlink_b_standard_plus3	-	575.94	594.95	618.63	667.65	696.66	748.69
AA	I	Y	G	A	F	S	R
beta_common_y_standard_plus1	-	-	537.28	480.26	409.22	262.15	175.12
beta_common_y_standard_plus2	-	-	269.14	240.63	205.11	131.58	88.06
beta_common_y_standard_plus3	-	-	179.76	160.76	137.08	88.06	59.05
beta_xlink_y_standard_plus1	2292.08	2148.99	-	-	-	-	-
beta_xlink_y_standard_plus2	1131.54	1075.00	-	-	-	-	-
beta_xlink_y_standard_plus3	754.70	717.00	-	-	-	-	-

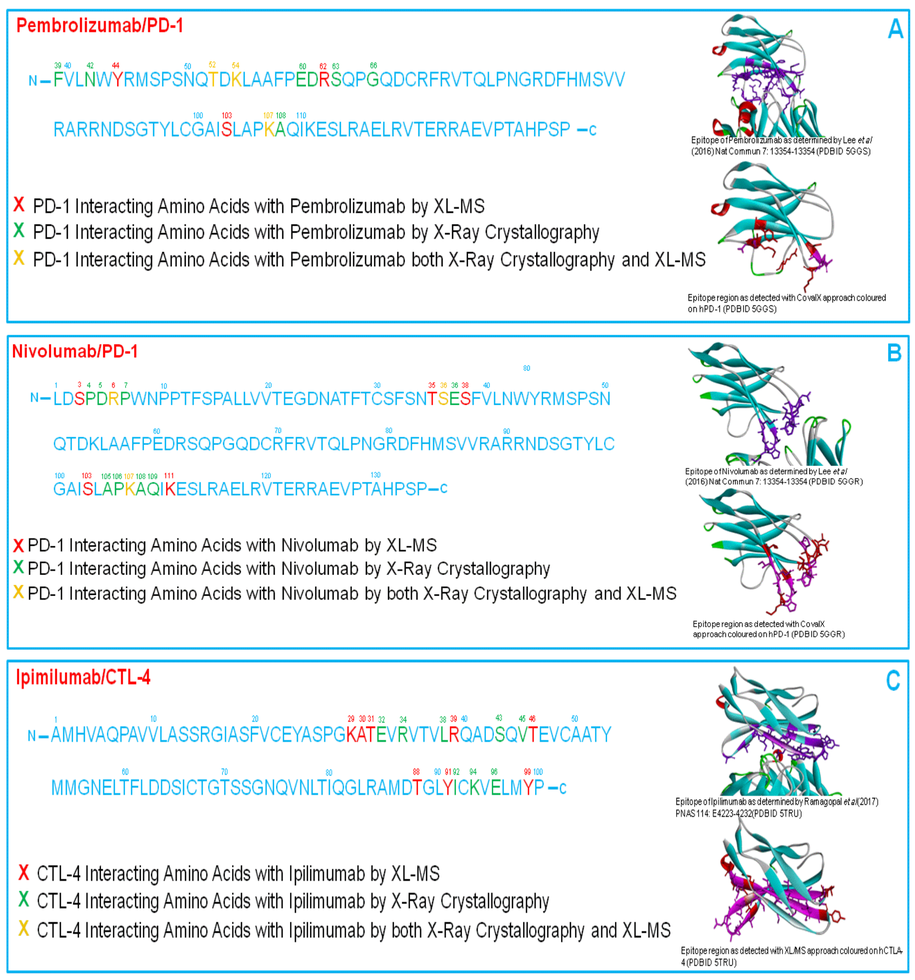
alpha-chain C

alpha_common_b_standard_plus1	106.61	268.11	368.15	553.21	603.25	716.30	887.35	964.36	-	-	-	-
alpha_common_b_standard_plus2	54.04	134.56	184.58	277.11	302.13	358.65	444.17	482.68	-	-	-	-
alpha_common_b_standard_plus3	36.69	89.37	123.39	184.74	201.42	239.43	296.78	322.79	-	-	-	-
alpha_xlink_b_standard_plus1	-	-	-	-	-	-	-	-	1042.84	1091.87	1115.88	1244.06
alpha_xlink_b_standard_plus2	-	-	-	-	-	-	-	-	971.87	1007.48	1058.84	1122.54
alpha_xlink_b_standard_plus3	-	-	-	-	-	-	-	-	648.33	672.66	705.58	748.69
AA	V	C	E	Y	A	S	P	G	K	A	T	F
alpha_common_y_standard_plus1	-	-	-	-	-	-	-	-	-	328.15	249.11	148.06
alpha_common_y_standard_plus2	-	-	-	-	-	-	-	-	-	164.58	125.06	74.53
alpha_common_y_standard_plus3	-	-	-	-	-	-	-	-	-	110.39	83.71	50.03
alpha_xlink_y_standard_plus1	2292.08	2183.04	2092.00	1873.93	1710.87	1639.83	1552.80	1455.75	1398.73	-	-	-
alpha_xlink_y_standard_plus2	1131.54	1082.01	1001.99	937.47	855.94	820.42	776.90	728.38	699.87	-	-	-
alpha_xlink_y_standard_plus3	754.70	721.67	668.33	625.32	570.64	547.28	518.27	485.92	466.91	-	-	-

D

Sequence	Protein1	Protein2	Sequence Proteine 1	Sequence Proteine 2	XLType	nAA1	nAA2	Identified on xquest	Identified on StavroX
YICKVELMYPPPY-SRATGIPDRF-a9-b4	db|CTLA-4|Ag_CTLA-4	Ipilimumab_LC	91-103	54-63	inter-protein xl	99	57	YES	YES
VCEYASPGKATEVRVTVLRQADSQVTEVCAATY-RLSCAASGFTF-a26-b10	db|CTLA-4|Ag_CTLA-4	Ipilimumab_HC	21-53	19-29	inter-protein xl	46	28	YES	YES
TIQGLRAXDTGL-SSYTXHWVRQAPGKGL-a10-b4	db|CTLA-4|Ag_CTLA-4	Ipilimumab_HC	79-90	30-45	inter-protein xl	88	33	YES	YES
RAXDTGLYICKVEL-GSSPIW-a8-b3	db|CTLA-4|Ag_CTLA-4	Ipilimumab_LC	84-97	93-97	inter-protein xl	91	95	YES	YES
RAMDTGLY-GAFSRATGIPDRF-a5-b5	db|CTLA-4|Ag_CTLA-4	Ipilimumab_LC	84-91	51-63	inter-protein xl	88	55	YES	YES
ASPGKATEVRVTVLRQADSQVTEVCAATY-SCRASQSVGSSY-a7-b7	db|CTLA-4|Ag_CTLA-4	Ipilimumab_LC	25-53	22-33	inter-protein xl	31	28	YES	YES
ASPGKATEVRVTVLRQADSQVTEVCAATY-SCRASQSVGSSY-a15-b7	db|CTLA-4|Ag_CTLA-4	Ipilimumab_LC	25-53	22-33	inter-protein xl	39	28	YES	YES

Comparison with X-ray Crystallography Data



Comparison of the epitope mapping results obtained by cross-linking mass spectrometry (XL-MS) and X-ray crystallography for the antibody/antigen complexes Pembrolizumab/PD-1 (A), Nivolumab/PD-1 (B) and Ipimilumab/CTL-4 (C). A red amino acid corresponds to a cross-linked amino-acid of the antigen with the antibody. A green amino acid corresponds to an X-ray crystallography interacting amino acid as described in the literature[1,2]. A yellow amino acid corresponds to both cross-linked and X-ray crystallography interacting amino acids between the antibodies and the antigens. For Pembrolizumab/PD-1 and Nivolumab/PD-1, X-ray data are based on PDB ID 5GGS. PD-1 sequence numbering corresponds to PD-1/FC recombinant protein from RnD systems 1086-pd. For Ipilimumab/CTL-4, X-ray data are based on PDB ID 1I85 and 1I8L. CTL-4 sequence numbering is corresponding to CTL-4/FC recombinant protein from R&D systems 325-ct-cf.

[1] Ramagopal et al. PNAS, E4223-E432, May 2017. [2] Lee et al, Doi: 10.1038/ncomms13354.