Optimizing sample preparation and nLC-MS/MS for mAB characterization

Rodrigo Soares Caldeira Brant[1], Kelly Machado Cavalcanti[1], Thiago Bousquet Bandini[1], Hulyana Brum[1], Anna Erika Vieira de Araujo[2], Iaralice Medeiros de Souza[2], MIchel Batista[1]

[1.] ICC, Instituto Carlos Chagas - Fiocruz-PR, Curitiba, Paraná;
[2.] BM, Biomanguinhos - Fiocruz-RJ, Rio de Janeiro, Rio de Janeiro;

Monoclonal antibodies (mAB) are highly effective drugs for treatment of cancer and other diseases. Production and development of mAB, both originator and biosimilar, require physicochemical characterization of Critical Quality Attributes, including post-translational-modifications, disulfide bond and amino acid primary sequence mapping. In this study we applied design of experiments (DoE) to improve both sample preparation and nLC-MS/MS analysis by decreasing protein digestion time and artificial amino acid modifications, increasing protein sequence coverage and analysis throughput. We aimed to develop an optimized nano LC method with in-house produced columns capable of characterizing CQA of mAB.

Nivolumab, Pembrolizumab and Trastuzumab mAb were used in this study. For Peptide mapping DoE, a full factorial 2^2 or 2^4 with or without central point were used. For protein digestion protocol, different parameters were compared: time of digestion (1 and 18 h), digestion buffer composition (ammonium bicarbonate, Tris-HCl and the addition of calcium chloride), denaturation agent (8 M urea or 8 M guanidine), digestion pH and temperature. For disulfide bond mapping, a 12 run Plackett Burman design followed by a 2^3 center composite design were used to create a response surface area for the best proteases and digestion time combination.

Different chromatographic conditions were tested, varying gradient and sample injected volume for mAB peptide mapping. The nLC-MS/MS was carried out on an Ultimate 3000 coupled with an Orbitrap Exploris 120. Peptides were sequenced using Biopharma Finder 5.1. Data analysis was conducted on Microsoft excel and Jamovi.

Decreasing trypsin digestion time, from 18 h to 1 h, reduced asparagine deamidation by over 30 times on, both, Pembrolizumab and Nivolumab, with no prejudice to peptide identification and protein sequence coverage. The use of 50 mM Tris-HCl pH 8.0 as a digestion buffer instead of ammonium bicarbonate had no impact on the results of peptide mapping of Nivolumab. For trypsin digested mAB, the gradient of 120 min for separation of protein digest showed no advantage over 60 min gradient. GluC digestion was evaluated aiming to increase protein sequence coverage. The use of ammonium acetate pH 4.5 as digestion buffer greatly reduced the rate of Asn deamidation, and protein coverage for Nivolumabe and Pembrolizumab was of over 90%.

Disulfide bond mapping was more efficient with the combination of pepsin and trypsin than Lys-C and trypsin or Pepsin and Glu-C for the three tested mAB. Lower times of pepsin digestion (under 20 minutes), followed by a 135-minute digestion with trypsin generates more di-peptides (containing disulfide bonds) that are identified by LC-MS/MS and decreases the abundance of free cysteine. The use of lo bind tubes during sample preparation for disulfide bond mapping can impact the identification of bonds.

In conclusion, we were able to achieve 100% protein coverage by using two complementary proteases, Glu-C and trypsin. Chemical modification of Asn during sample preparation was reduced for both enzymes. Total protocol time for trypsin, from sample preparation to LC-MS/MS analysis, was improved from over 24 hours to around 4 hours. A protocol capable of detection of all 16 disulfide bonds for the three mAb was achieved with a quick digestion of pepsin followed by trypsin with high reproducibility. A response surface area of digestion time with trypsin and pepsin

was described.

Agradecimentos: The author's thank Technological Platforms Network FIOCRUZ and Bioscience and Biotechnology Graduate Program of Carlos Chagas Institute.