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Monoclonal Antibody Fragment Separation and Characterization Using Size Exclusion Chromatography Coupled with Mass Spectrometry

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INTRODUCTION

Monoclonal antibodies (MAbs) have increasingly become a major part of protein therapeutics. Monoclonal antibody fragments (such as Fab and F(ab')$_2$) offer advantages over using intact MAbs, such as reducing nonspecific antigen binding from Fc. Size exclusion chromatography (SEC) is widely used in protein analysis. Aggregates, monomers and degradation products of monoclonal antibodies are able to be separated on size exclusion columns based on their molecular weights under native conditions. In general, protein native buffer conditions, such as salts at neutral pH, are not mass spectrometry friendly. In this study we investigated antibody fragments such as heavy and light chains, Fab/Fc and F(ab')$_2$ using SEC separation. MAb fragments were also analyzed by online mass spectrometry using volatile mobile phases. The effect of different percentages of TFA, formic acid and acetonitrile in the mobile phases on the antibody fragments separation was also explored.

EXPERIMENTAL

SEC Column: Zenix™ SEC-300 (3 µm, 300 Å, 4.6 x 300 mm)
HPLC System: Agilent 1200 HPLC with binary pump
Mass Spectrometer: Waters Q-Tof Ultima
Source Temperature: 80 °C
Capillary Voltage: 4.44 kV
Detection: UV 280 nm
Scan Range: 350 - 3000 amu
Desolvation Temperature: 150 °C

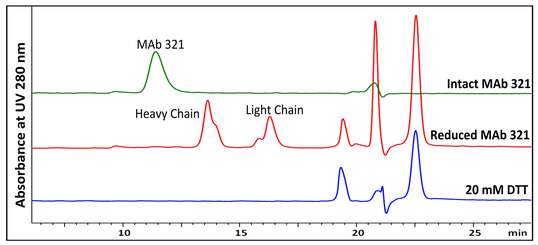
Dithiothreitol (DTT) reduction: MAb 321 was diluted to 1 mg/mL with 150 mM phosphate buffer, pH 7.0. Antibodies were reduced with a final concentration of 20 mM DTT and incubated at 65°C for 15 minutes.

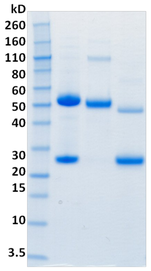
Papain digestion: MAb 321 (1 mg/mL) was incubated in 100 mM Tris-HCl, pH 7.6, 2 mM EDTA and 5 mM Cysteine. The digestion was started by adding 1 mg/mL papain. The papain/MAb ratio was at 1:100. The digestion mixture was incubated for 2, 3, 3.5 and 4 hours at 37°C.

Pepsin digestion: MAb 321 was incubated at a final concentration of 1 mg/mL in 20 mM sodium acetate, pH 4.0 with a pepsin to MAb 321 ratio of 1:40. The digestion was carried out at 37 °C for 15.5 hours. The reaction was stopped by adding 2 M TRIS to increase the pH to 8.0.

ANALYSIS OF HEAVY AND LIGHT CHAINS ON ZENIX™ SEC-300

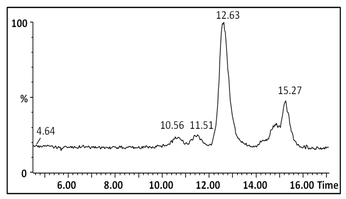
Reduced MAb 321 heavy and light chain separation on Zenix™ SEC-300, 4.6 x 300 mm. Mobile phase was 0.1% TFA, 0.1% formic acid with 20% acetonitrile. Flow rate was 0.2 mL/min. UV detection was set at 280 nm. 5 µg of intact MAb 321 and 20 µg of DTT reduced MAb 321 were injected. Peak 1 and Peak 2 were collected and dried using a speed vac before being re-dissolved in SDS-PAGE gel sample buffer and ran on a gel.



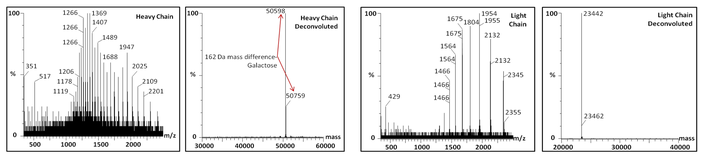


1. Protein marker
2. Reduced Mab321
3. Heavy chain
4. Light chain

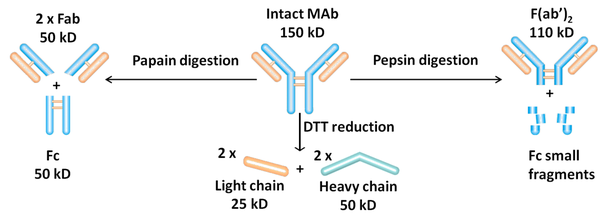
SEC-MS of HEAVY AND LIGHT CHAINS ON ZENIX™ SEC-300



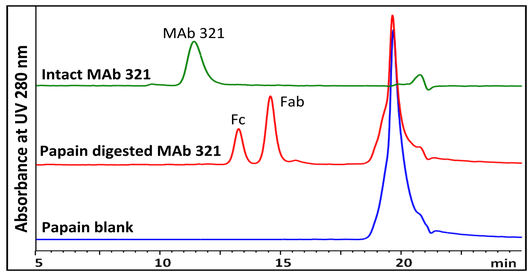
Online MS analysis of heavy and light chain from SEC separation of reduced MAb 321. A 4.6 x 300 mm Zenix™ SEC-300 column was used to separate the heavy and light chains. Flow rate was 0.2 mL/min with 0.02% TFA, 1% formic acid and 20% acetonitrile as the mobile phase. Samples were directly introduced to online Q-TOF after SEC. Deconvoluted mass spectra of peaks were shown as in heavy chain and light chain.

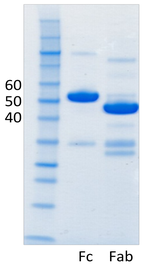


Monoclonal Antibody Fragments



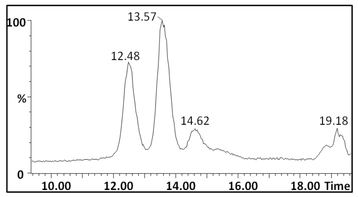
ANALYSIS OF Fab/Fc ON ZENIX™ SEC-300



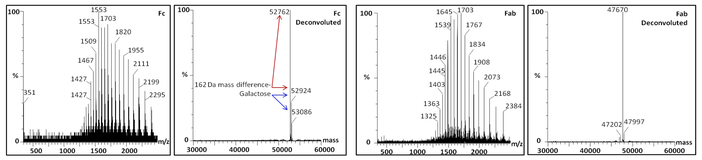


Fab/Fc separation on Zenix™ SEC-300, 4.6 x 300 mm. Mobile phase was 0.1% TFA, 0.1% formic acid with 20% acetonitrile. Flow rate was 0.2 mL/min. 5 µg of intact MAb 321 and 5 µg of papain digested MAb 321 were injected. On the right is the 4-12% Bis-Tris gel image of collected Fc and Fab fractions separated on a Zenix™ SEC-300, 7.8 x 300 mm column at 0.5 mL/min.

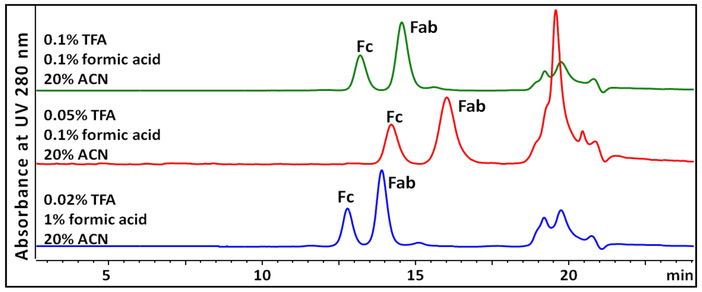
SEC-MS of Fab/Fc ON ZENIX™ SEC-300



Online MS analysis of Fc and Fab from SEC separation of papain digested MAb 321. A 4.6 x 300 mm Zenix™ SEC-300 column was used to separate the Fc and Fab fragments. Flow rate was 0.2 mL/min with 0.02% TFA, 1% formic acid and 20% acetonitrile as the mobile phase. Samples were directly introduced to online Q-TOF after SEC. Deconvoluted mass spectra of peaks were shown as in Fc and Fab fragments.

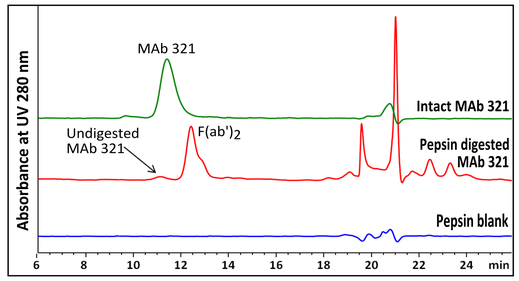


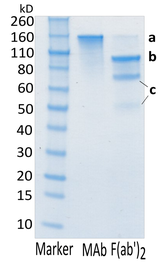
EFFECT OF TFA AND FORMIC ACID CONCENTRATION ON Fab/Fc SEPARATION USING ZENIX™ SEC-300



Fab and Fc separations on Zenix™ SEC-300, 4.6 x 300 mm with different mobile phases; varying the concentrations of TFA and formic acid. Flow rate was 0.2 mL/min, 5 µg of papain digested MAb 321 was injected for all three runs.

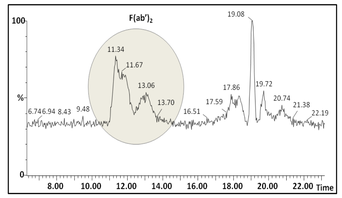
ANALYSIS OF F(ab')$_2$ ON ZENIX™ SEC-300



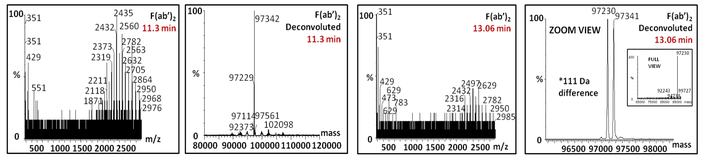


F(ab')$_2$ separation on Zenix™ SEC-300, 4.6 x 300 mm. Mobile phase was 0.1% TFA, 0.1% formic acid with 20% acetonitrile. Flow rate was 0.2 mL/min. 5 µg of intact MAb 321 and 15 µg of pepsin digested MAb 321 were injected. On the right is the 4-12% Bis-Tris gel image. 5 µg of each sample were loaded. Band (a) is undigested MAb, band (b) is F(ab')$_2$, and bands (c) are smaller fragments from the digestion.

SEC-MS of F(ab')$_2$ ON ZENIX™ SEC-300



Online MS analysis of F(ab')$_2$ from SEC separation of pepsin digested MAb 321. A 4.6 x 300 mm Zenix™ SEC-300 column was used to analyzie the F (ab')$_2$ fragment. Flow rate was 0.2 mL/min with 0.02% TFA, 1% formic acid and 20% acetonitrile as the mobile phase. Samples were directly introduced to online Q-TOF after SEC. Deconvoluted mass spectra of peaks were shown as F(ab')$_2$ fragment.



CONCLUSION

- Zenix™ SEC-300 4.6 x 300mm can successfully separate MAb fragments including heavy/light chains, Fab/Fc and F(ab')$_2$ from their reaction mixture, respectively.
- Organic mobile phases containing a very low concentration of TFA or no TFA in formic acid and acetonitrile are suitable for the fragment's separation and SEC-MS online analysis.
- Baseline separation of Fab and Fc fragments can be achieved with low sample loading of 0.1 µg of papain digested MAb.

REFERENCES

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