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, as they have reduced nonspecific antigen binding and reduced immunogenicity. In this study, we investigated antibody fragments such as heavy and light chains, Fab, F(ab’) and F(ab’)_2 using SEC separation. MAb fragments were also analyzed by online mass spectrometry using volatile mobile phases.

**EXPERIMENTAL**

*SEC Column: Zenix™ SEC-300 (3 µm, 300 A, 4.6 x 300 mm)*

*HPIC System: Agilent 1200 HPLC with binary pump*

*Mass Spectrometer: Waters Q-ToF Ultima*

*Scan Range: 350 - 3000 amu*

*Source Temperature: 80 °C*

*Desolvation Temperature: 150 °C*

*Capillary Voltage: 4.44 kV*

**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.

Pepsin 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 pepsin. The pepsin/MAb ratio was 1:1. The digestion mixture was incubated for 2, 3, 5, and 15 hours at 37°C.

Papain digestion: MAb 321 was incubated at a final concentration of 1 mg/mL in 20 mM sodium acetate, pH 4.0 with a papain 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 with 0.02% TFA, 1% formic acid and 20% acetonitrile. Samples were directly introduced to online Q-TOF after SEC. Deconvoluted mass spectra of peaks were shown as in Fc and Fab fragments.

**ANALYSIS OF Fab/Fc ON Zenix™ SEC-300**

Online MS analysis of Fab and Fc 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 Fab and Fc fragments.

**ANALYSIS OF F(ab')_2 ON Zenix™ SEC-300**

Online MS analysis of Fab’ from SEC separation of pepsin digested MAb 321. A 4.6 x 300 mm Zenix™ SEC-300 column was used to analyze the Fab’_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 Fab’_2 fragment.

**REFERENCES**


5. Online MS analysis of Fab’ from SEC separation of pepsin digested MAb 321. A 4.6 x 300 mm Zenix™ SEC-300 column was used to analyze the Fab’_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 Fab’_2 fragment.

**CONCLUSION**

- Zenix™ SEC-300 4.6 x 300 mm can successfully separate MAb fragments including heavy/light chains, Fab/Fc and Fab’ using 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.

**INTRODUCTION**

Monoclonal antibodies (MAbs) have increasingly become a major part of protein therapeutics. Monoclonal antibody fragments (such as Fab and Fab’_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, F(ab’) 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.

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

Online MS analysis of Fab and Fc 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 Fab and Fc fragments.

**SEC-MS of Fab’ ON Zenix™ SEC-300**

Online MS analysis of Fab’ from SEC separation of pepsin digested MAb 321. A 4.6 x 300 mm Zenix™ SEC-300 column was used to analyze the Fab’_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 Fab’_2 fragment.

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

Online MS analysis of Fab and Fc 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 Fab and Fc fragments.

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5. Online MS analysis of Fab’ from SEC separation of pepsin digested MAb 321. A 4.6 x 300 mm Zenix™ SEC-300 column was used to analyze the Fab’_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 Fab’_2 fragment.

**CONCLUSION**

- Zenix™ SEC-300 4.6 x 300mm can successfully separate MAb fragments including heavy/light chains, Fab/Fc and Fab’_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.