Three novel protocols for protein purification by Free-Flow-Electrophoresis

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Introduction

Many clinically and scientifically relevant proteins undergo posttranslational modification (PTM) stemming for example from chemical adducts (glycosylation, phosphorylation, etc.) or mRNA splicing. These protein isoforms can differ from each other in their activity and therefore need to be closely studied. For therapeutic and clinical applications, it may be beneficial and/or necessary to separate isoforms from each other ahead of clinical application. **Monoclonal antibodies (mABs)** are a good example of such proteins, often bearing alternative and closely related PTMs that make the mAB isoforms challenging to separate, but where separation can be important for characterization and ultimately for commercial production.

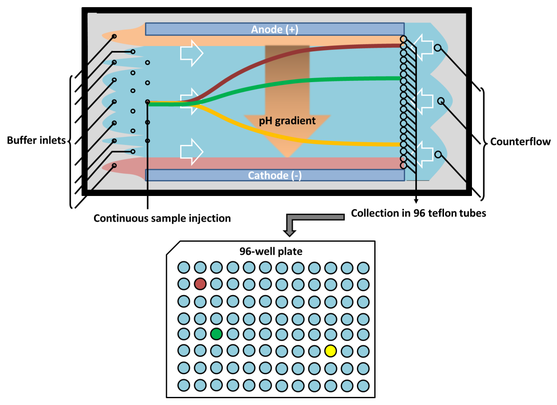
Recent developments in **capillary electrophoresis (CE)** and especially imaged capillary isoelectric focusing (iCIEF) have emerged as powerful charge based separation techniques within the biopharmaceutical industry. CE techniques offer raid and high-resolution separation. However, the very small volume of input sample per separation limits CE as a strictly analytical tool, which is not very useful for purifying sufficient amounts of isoforms for further identification and characterization.

The matrix-free separation of protein isoforms by Free Flow Electrophoresis (FFE) is an ideal tool for **preparative separation of protein isoforms**. FFE's unique attributes include fast separation, high sample-throughput and ability to recover sample and not merely analyze them. Here we present three complementary approaches for matrix-free separation of protein isoforms by Free Flow Electrophoresis (FFE).

IEF-FFE

The first technique relies on a continuous separation, utilizing a pH-gradient generated by commercial ampholytes, which was used for preparative separation of monoclonal antibodies up to 100 mg with a throughput of 3 mg/h and a resolution of < 0.02 Δ-pH.

Principle of IEF Free Flow Electrophoresis



Separation of mAB by FFE-IEF

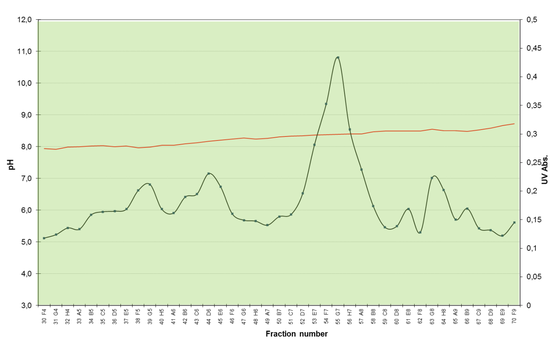


Figure 1: IEF-FFE separation of crude mAB sample on pH with an ultraflat pH-gradient from 8 to 8.5. The fractions within the green area were applied to an IEF gel (Figure 2).

IEF-PAGE

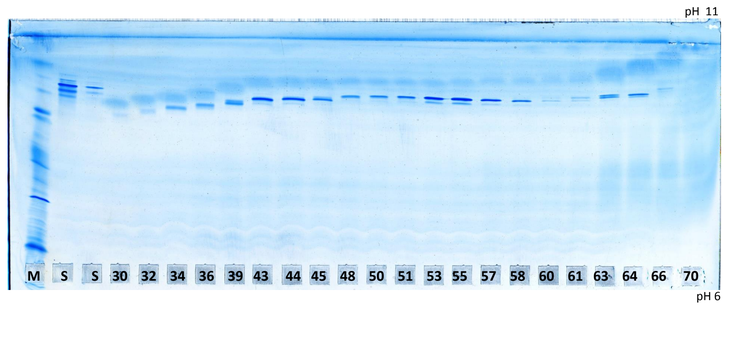


Figure 2: IEF-PAGE (pH 6-11) of the crude sample (S) and selected fractions of IEF-FFE separated mAB sample. M: Serva IEF Marker.

Re-electrophoresis

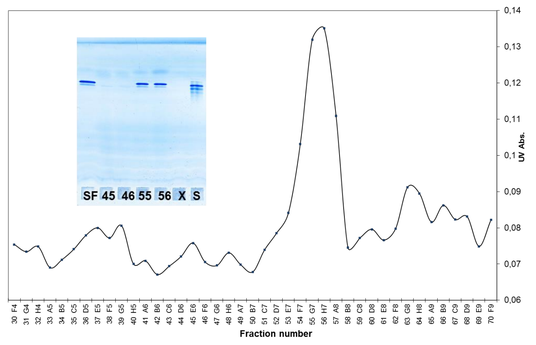


Figure 3: FFE-Re-electrophoresis was performed with fraction 56 to verify the stability of the separation process.

Material and Methods

Protein throughput:	2,5 mg/h (max. 3 mg/h)	Standard buffer flow rate:	33 ml/h	Temperature:	5 °C
Voltage:	2900 V	Transit/Separation time of sample:	20 minutes	Resolution:	0.02 Δ-pH, 0.04 Δ-pI
Field strength:	490 V/cm	pH-gradient:	8-9	IEF-gel:	Serva Focus gel pH 6-11

ProLytes™-FFE

The second technique, matching handling, resolution and throughput of the IEF-FFE, makes use of a proprietary mixture of acids and bases to generate the pH-gradient needed for separation. Because no ampholytes encounter the samples, clinical application of the separated isoforms is possible.

Separation of mAB by FFE-ProLytes

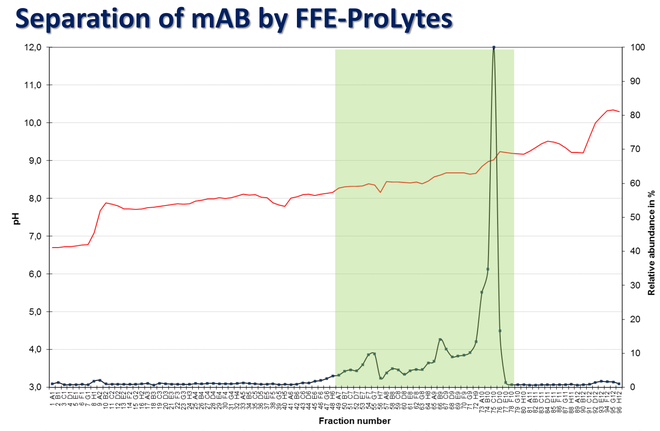


Figure 4: Relative abundance (black line) of the separated mAB sample and pH profile (red line). The fractions within the green area were applied to an IEF gel (Figure 5).

IEF-PAGE

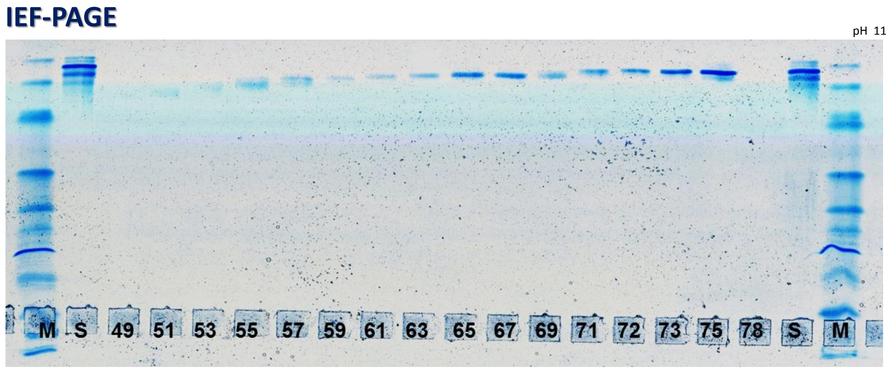


Figure 5: IEF-PAGE (pH 6-11) of the crude sample (S) and selected fractions of IZE FFE separated mAB sample. M: Serva IEF Marker.

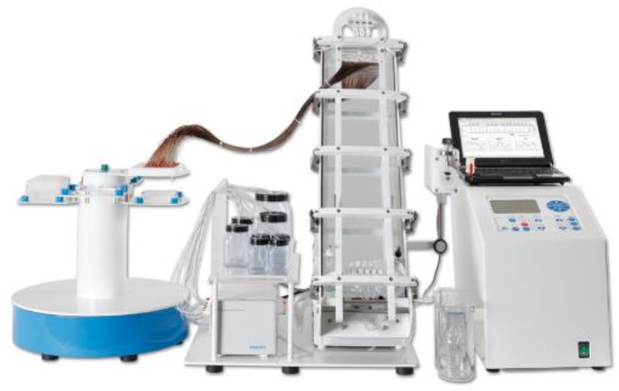
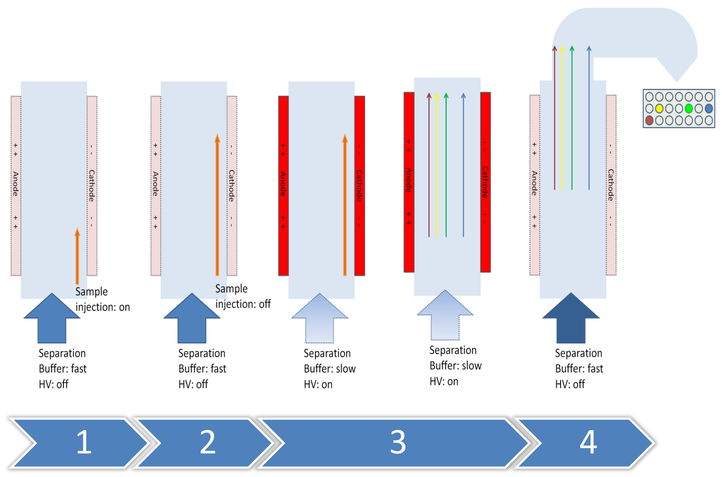


Figure 6: FFE unit with separation chamber, media pumps, power supply and collection robot. The cooling unit is not shown.

IZE-FFE

The third technique (IZE-FFE) relies on differences of electromigration in a stepwise pH-gradient, formed by the use of different acid and base containing buffers over the width of the separation chamber. Because the proteins are not separated at their isoelectric point, this technique minimizes protein-protein interactions and its short interval time of 10 minutes and throughput of up to 100 µg protein per interval, allows for rapid analysis or preparation of samples by continuous collection of fractions. Direct MS measurements are possible, due to the lack of ampholytes and polymers.

Principle of IZE Free Flow Electrophoresis



Separation of mAB by FFE-IZE

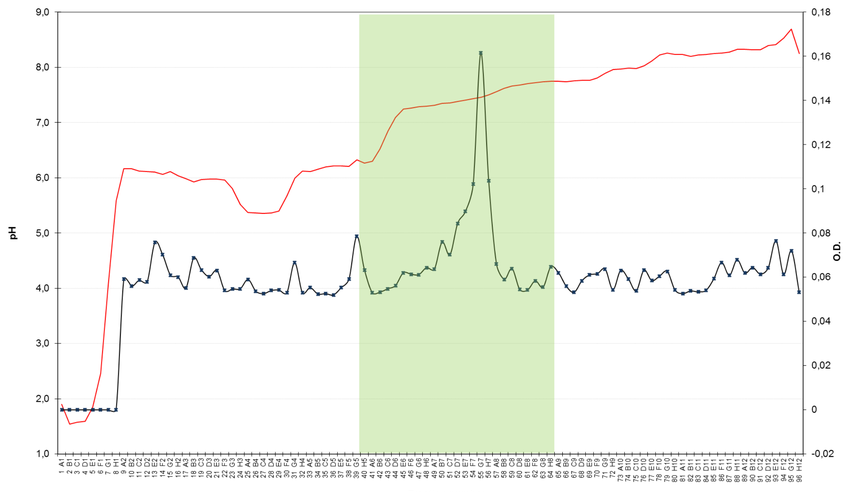


Figure 7: UV-profile (black line) of the separated mAB sample and pH profile (red line). The fractions within the green area were applied to the IEF gel (Figure 8).

IEF-PAGE

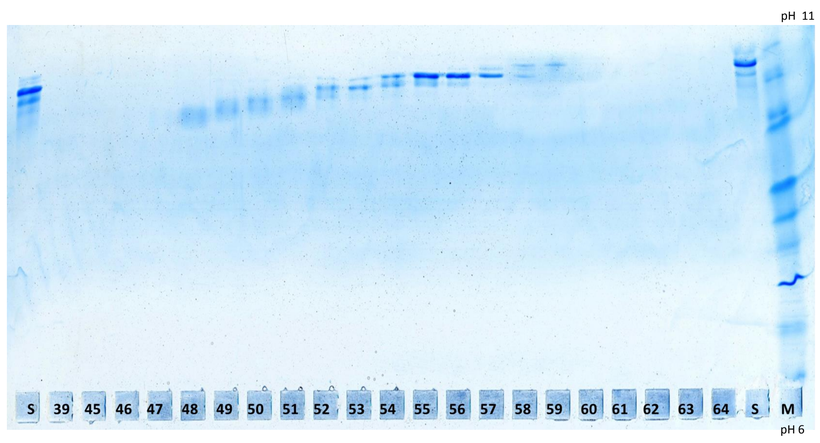


Figure 8: IEF-PAGE of the crude sample (S) and selected fractions of IZE FFE separated mAB sample. M: Serva IEF Marker.

Material and Methods

Protein throughput:	60 µg/interval (max. 100 µg/interval)	Interval/Separation time :	10 minutes (min. 4 minutes)
Voltage:	1400 V (max. 1800 V)	Temperature:	10 °C (min. 5 °C)
Current:	75 mA	IEF-gels:	Serva Focus gel pH 6-11

Conclusion

Protein isoform separation using FFE:
- **Entirely liquid** methods (no gels, no matrix)
- **Native conditions** maintain structure and function of separated isoforms
- **Quick and easy** to set up and operate
- **High resolution** separations
- Scale **from analytical to milligram** preparation ability
- One instrument supports **many different separation needs**:
  - Flexible and adaptable methods
  - From simple to highly complex samples
  - Specific protein targets (and their unique characteristics)
  - From basic research to production-scale throughput

IEF-FFE:
- The method of choice for
  - Characterizing complex mixtures
  - Screening preparations of unknown isoelectric composition
- Uses ampholyte cocktails
  - Commercially available in a variety of defined pH ranges, from broad to narrow
- Unique continuous run method (not batch) can allow high separation capacity of up to 100 mg of mAB

ProLyte-FFE:
- Same workflow as IEF-FFE
- Unique benefits versus IEF-FFE
  - No dependency on commercial ampholytes
  - Clinical application possible (no ampholytes)
  - Easy removal of separation buffer
  - Custom pH-gradients possible

IZE-FFE:
- The method of choice for
  - Achieving exquisitely fine resolution
  - Separating targets with known isoelectric properties
- Unique benefits versus IEF
  - Easily tuned for precise pH ranges using same reagents
  - Reduces precipitation concern: protein is never held at its isoelectric point
  - Reduced chemical complexity of separation media
  - Improved compatibility with downstream methods
  - Reduced cost per run

The Antibody samples for this study were supplied by Biogen, RTP, North Carolina, USA.