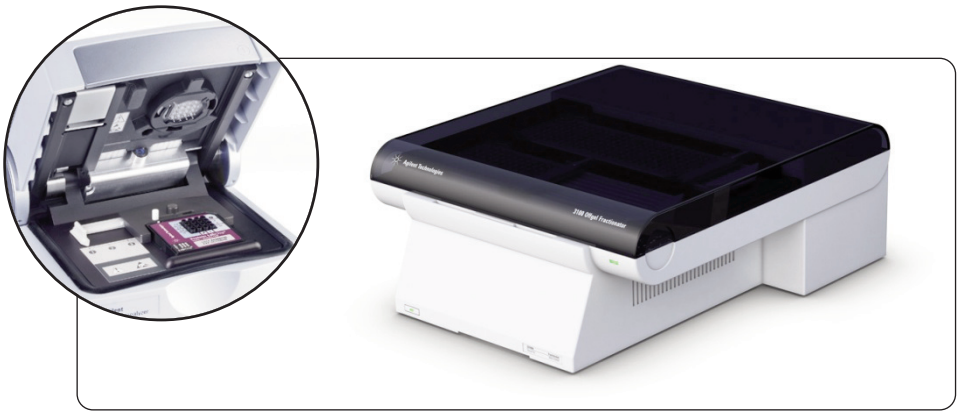
Monitoring antibody charge variants using a combination of Agilent 3100 OFFGEL Fractionation by isoelectric point and high sensitivity protein detection with the Agilent 2100 Bioanalyzer

Application Note

Drug Development



Author

Suresh Babu C.V.

Agilent Technologies

Bangalore, India

Abstract

During production and purification processes, antibodies can exhibit changes in charge heterogeneity. These changes may not only impact their stability but also their activity and cause immunologically adverse reactions. Hence, the analysis of charge heterogeneity in monoclonal antibody (mAb) preparations is a prime quality control step in the biopharmaceutical industry.

This Application Note describes the analysis of monoclonal antibodies by a two-dimensional separation based on protein charge and molecular weight. In the first dimension, Agilent 3100 OFFGEL Fractionation, an isoelectric focusing technique delivering high pI resolution and protein fractions in solution, is used. In the second dimension, OFFGEL fractions are separated by size and detected with highly sensitive laser-induced fluorescense using the Agilent 2100 Bioanalyzer High Sensitivity Protein 250 Assay.

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Introduction

Monoclonal antibodies (mAb's) have gained much attention as therapeutics due to their specificity for a single antigenic determinant. Various mAb's are approved by the Food & Drug Administration (FDA) and used for clinical therapy[1]. To use an mAb as a drug substance, biological safety, quality, purity and performance characteristics of the mAb must be assessed during manufacturing[2]. Therefore, the main objectives towards manufacturing Ab-based pharmaceuticals are production and purification of high amounts of antibody with good yields.

During biosynthesis, mAb's can undergo various modifications that lead to charge heterogeneity. These provide a great challenge for the complete characterization of the mAb. Combinations of charge and size analysis provide an excellent assessment of antibody purity. Traditionally, slab-gel-based isoelectric focusing (IEF) or capillary electrophoresis (CE) methods are used to monitor the charge profiles of antibodies. However, although they provide good resolution, gel-based or CE-based IEF offer little opportunity to introduce a second, quantitative dimension of analysis.

OFFGEL electrophoresis is an alternative technique to slab-gel based IEF in modern protein analysis[3]. It fractionates peptides or proteins based on immobilized pH gradients. However, in contrast to gel based techniques, samples are recovered in solution for downstream applications such as size analysis or liquid chromatography-mass spectrometry (LC/MS). It is flexible in resolution and in the amount of sample that can be loaded (analytical to semi-preparative sample amounts). IEF by OFFGEL fractionation of proteins or peptides can achieve a resolution of 0.1 pH. Isoelectric point (pI) values obtained from OFFGEL runs serve as additional support for MS analysis and can be used to search for post translational modifications of proteins and peptides.

For size analysis of pI fractions, the Agilent 2100 Bioanalyzer is the tool of choice. The High Sensitivity Protein 250 (HSP-250) assay provides size separation and highly sensitive detection of proteins in the picogram range.

This study compares the two-dimensional application of OFFGEL and Bioanalyzer for monitoring the charge heterogeneity of mAb's under native conditions and after addition of a detergent (Tween-20). The study outlines the methodology difference between denatured and nondenatured conditions.

Materials and Methods

Tween-20 was obtained from Sigma (MO, USA). Purified monoclonal antibody (1 mg/ml) was from Stratagene (CA, USA).

For IEF, the Agilent 3100 OFFGEL Fractionator with a 24-well frame setup (OFFGEL High Resolution Kit, pH 3-10, Agilent p/n 5188-6425) was used with a slight deviation from the original protocol. For each OFFGEL fractionation, 100 µg of the mAb sample were dissolved in OFFGEL focusing buffer containing 5 % glycerol, and 0.25 % IPG buffer pH 3-10. Neither urea or thiourea were added to maintain native conditions because mAb's can easily disintegrate under urea/thiourea denaturing conditions (data not shown). A second data set was generated with the addition of 1% Tween-20 to enhance the solubility of the proteins. OFFGEL fractionation was performed using the default method for protein samples and 24 fractions (OG24PR01). The recovered fractions were subjected to Bioanalyzer protein analysis. Fractions obtained after the OFFGEL runs are directly compatible to the HSP-250 assay. OFFGEL recoveries were calculated by relating the mAb quantity in all the OFFGEL fractions to the concentration measured in non-fractionated sample before OFFGEL fractionation (referred to as sample load).

Protein analysis was done on the Agilent 2100 Bioanalyzer with the Agilent High Sensitivity Protein 250 kit (Agilent Technologies GmbH, Waldbronn, Germany). Protein labeling and on-chip sample analysis was performed as described in the High Sensitivity Protein 250 Kit Guide[4]. All samples were run under nonreducing conditions.

Results and Discussion

In this study, to assess the influence of protein structure on pI fractionation, mAb samples were fractionated by pI using OFFGEL electrophoresis under native conditions with the addition of Tween-20. The recovered OFFGEL fractions were analyzed with the Bioanalyzer HSP-250 assay. Tween-20 was added to the OFFGEL buffer system to enhance the solubility of the protein during focusing, because mAb's are known to disintegrate easily under urea and thiourea buffer conditions (data not shown). The main motivation for separating mAb under native conditions is the direct compatibility with downstream LC/MS analysis for identification of the fractionated charge variants[5].

Figures 1A and 2A show Bioanalyzer gel-like images for OFFGEL fractions and the initial sample load obtained under native conditions and after addition of Tween-20, respectively. Various mAb charge variants are well separated by OFFGEL fractionation and subsequently detected with the HSP-250 assay. Charge variants from the main mAb product are all found towards the anode and are therefore exhibiting acidic pI values. Figures 1B and 2B show corresponding electropherogram overlays for the OFFGEL fractions of the main product and the acidic charge variants. The initial sample load is shown as well. The difference in focusing pattern between the two conditions applied is clearly visualized by the Bioanalyzer protein assay. With the addition of Tween-20, more main product (fraction 9-11) and charge variants (fraction 1-5) are observed as indicated by the higher peak intensity values of the corresponding electropherograms (Figure 2). In terms of molecular weight, charge variants differ from the main products by a maximum of just 4 kDa and represent only 10% of the total mAb preparation. Detection of such variants is heavily dependant upon the initial sample fractionation by pI, because their overall contribution and difference in molecular weight do not allow direct detection with the Bioanalyzer (see sample load, Figures 1 and 2).

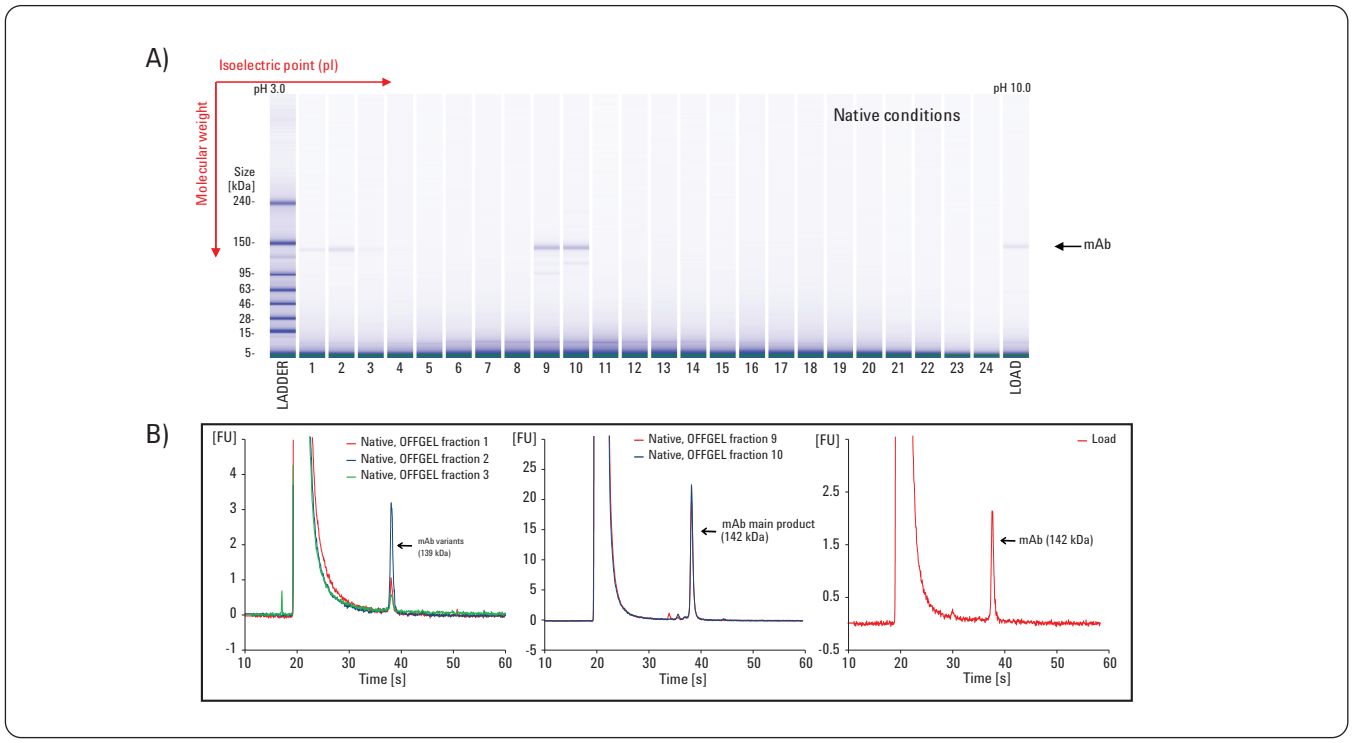

Figure 1

Native protein OFFGEL fractionation followed by Agilent 2100 Bioanalyzer analysis with the Agilent High Sensitivity Protein 250 Assay.

(A) Gel-like image of OFFGEL fractions 1-24 from pH 3 to 10, the initial sample load, and the HSP-250 protein ladder for molecular weight information. **(B)** Bioanalyzer electropherogram overlays of OFFGEL fractions 1 to 3, 9 and 10, and the initial sample load. The molecular weight of the antibody isoforms is indicated as determined by the Bioanalyzer.

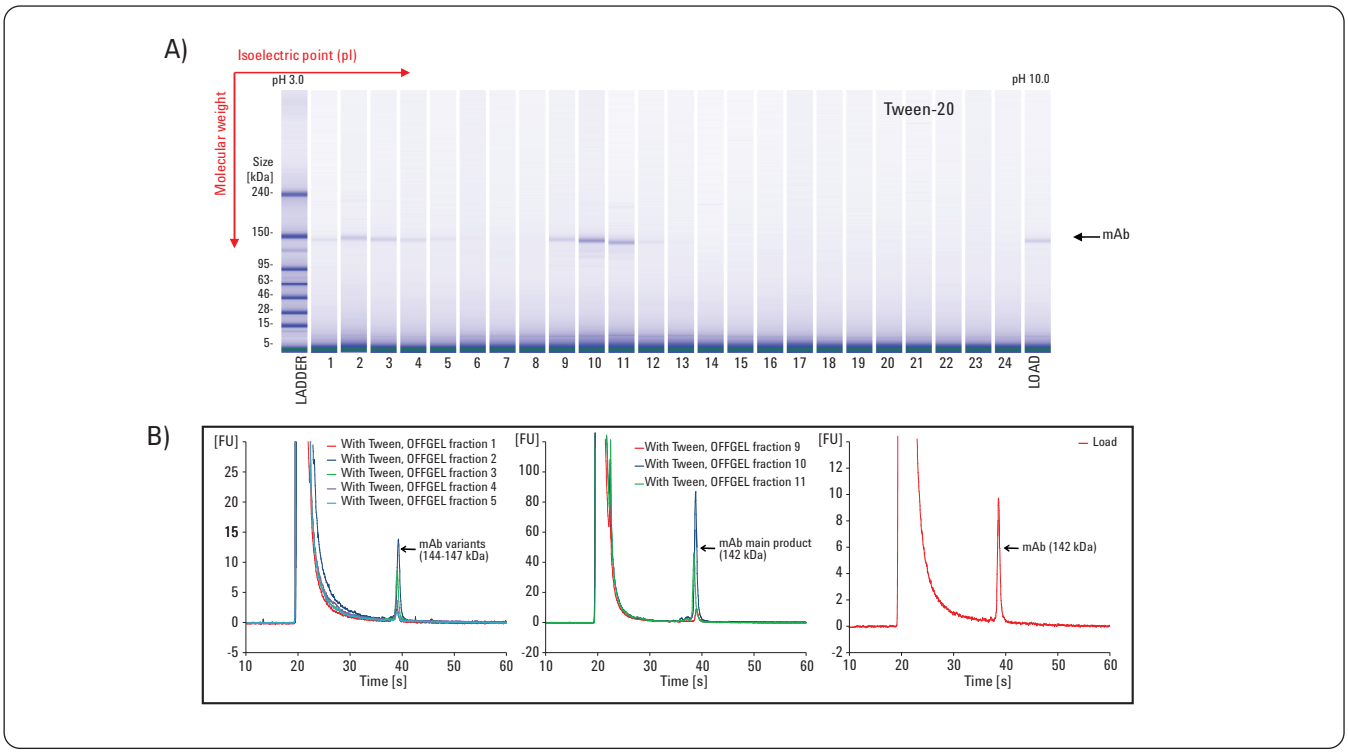

Figure 2

Protein OFFGEL fractionation with Tween-20 for enhanced sample solubility followed by Agilent 2100 Bioanalyzer analysis with the Agilent High Sensitivity Protein 250 assay.

(A) Gel-like image of OFFGEL fractions 1-24 from pH 3 to 10, the initial sample load, and the HSP-250 protein ladder for molecular weight information.

(B) Bioanalyzer electropherogram overlays of OFFGEL fractions 1 to 5, 9 to 11, and the initial sample load. The molecular weight of the antibody isoforms is indicated as determined by the Bioanalyzer.

Table 1 compares native OFFGEL electrophoresis to the second sample set containing Tween-20.

Tween-20 supports the overall solubility of proteins, therefore its addition to the OFFGEL sample fractionation enhances the overall protein recovery and the detection of additional variants of the main mAb product. For example, with the addition of Tween-20 one variant was well focused in fraction 11, whereas under native conditions this fraction did not contain a detectable amount of protein. It is important to note, however, that the addition of a detergent like Tween-20 will unfold the protein sample, exposing its side chains to the surrounding solvent, leading to multiple conformations with different pI[6]. Further experiments are thus needed to investigate the nature of the different charge variants detected between native and Tween-20 conditions.

If the availability of antibody samples is limited as it is in the present study, then the recovery of the analyte is very important. Here, OFFGEL runs including Tween-20 are the best choice as the protein recovery rates are high (> 70% in the present study). Due to diminished recovery rates under native conditions, a significantly higher amount of input material is required to improve the detection of low concentrated pI variants. In addition, other OFFGEL fractionation parameters can be optimized for improved protein recovery such as increased fractionation temperature up to 30 °C or increased glycerol concentration in the initial sample[5]. However, under both native and Tween-20 conditions, OFFGEL fractionation and Bioanalyzer molecular weight measurements demonstrate excellent resolution of variants and main product.

Native conditions	With addition of Tween-20
+ Separation of different structural variants of mAb. Major forms: fractions 9-10 Charge variants: fractions 1-3	+ Separation of different structural variants of mAb. Major forms: fractions 9-11 Charge variants: fractions 1-5
+ Fractions can directly be applied to downstream LC/MS analysis	– Need to remove Tween-20 before downstream LC/MS analysis
– Lower protein recovery (< 50%)	+ Enhanced protein recovery (>70%)
	+ Tween-20 enhances protein labeling efficiency with the HSP-250 kit

Table 1
Comparison of the overall results of this study: native OFFGEL conditions vs. the addition of a detergent, Tween-20.

Conclusion

This study demonstrates the use of OFFGEL electrophoresis in combination with the Agilent 2100 Bioanalyzer High Sensitivity Protein 250 assay for the sensitive detection and quantification of charge variants of monoclonal antibodies. The OFFGEL method offers high separation efficiency by pI for mAb's under both native conditions and conditions including a mild detergent (Tween-20) and allows recovery of pI fractions in the liquid phase. This makes the technique compatible to downstream analysis techniques such as LC/MS or microfluidics. The Agilent 2100 Bioanalyzer HSP-250 Assay accurately measures the molecular weight and concentration of antibody variants in OFFGEL fractions with high sensitivity.

References

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