TOSOH

APPLICATION NOTE

CHARACTERIZATION OF MAB AFFINITY ON FCγRIIIA-LIGAND BY ANALYTICAL AND SEMI-PREPARATIVE CHROMATOGRAPHY

INTRODUCTION

Binding between monoclonal antibodies (mAbs) and effector cells directly impacts the accompanying immune response or antibody-dependent cell cytotoxicity (ADCC). N-glycans at Asn297 of the Fc region influence this binding, as they affect the specificity of IgG to the FcγRIIIA receptor of effector cells. To monitor Fc receptor binding, a recombinant FcγRIIIa-ligand has been commercialized into TSKgel FcR-IIIA high performance affinity chromatography (AFC) columns that separate antibodies based on affinity differences to FcγRIIIa.

TSKgel FcR-IIIA-NPR has a linear loading range of 5-50 µg mAb, which is useful for analytical applications but is highly limiting if collection of elution products is necessary for further purification or analyses. This is overcome by the semi-preparative TSKgel FcR-IIIA-5PW column, allowing collection of sufficient material for improved analytical workflows in a short time.

Recombinant FcγRIIIa-ligand, with enhanced physiochemical stability, is immobilized onto a non-porous particle for analytical applications and onto a larger porous particle for semi-preparative applications. While both columns share the separation mechanism, theTSKgel FcR-IIIA-5PW achieves an increased loading capacity and elution volume.

Here, intact mAb and papain-digested mAb were used to compare the scalability of semi-preparative TSKgel FcR-IIIA-5PW and to provide a methodology to broaden from analytical to semi-preparative mode while the impact on resolution between the two different capacity columns is limited.

SPECIFICATIONS AND OPERATING CONDITIONS

Parameter	FcR-IIIA-NPR	FcR-IIIA-5PW
Ligand	Recombinant FcγIIIa receptor ligand	
Porous	No	Yes
Particle size (µm)	5	10
Dimensions	4.6 mm ID × 7.5 cm	7.8 mm ID × 7.5 cm
Volume (mL)	1.25	3.58
Max flow rate (mL/min)	1.0	1.2
Operational flow rate (mL/min)	1.0	0.25-1.0
Pressure limit (MPa)	9.0	1.0

Table 1

Table 1 indicates the specifications and operating conditions of analytical and semi-preparative TSKgel FcR-IIIA columns.

MATERIAL AND METHODS

TSKgel FcR-IIIA Conditions

Columns: TSKgel FcR-IIIA-5PW, 10 µm, 7.8 mm ID × 7.5 cm (P/N 0023532)
TSKgel FcR-IIIA-NPR, 5 µm, 4.6 mm ID × 7.5 cm (0023513)

Mobile phase: A: 50 mmol/L citrate/NaOH, pH 6.0
B: 50 mmol/L citrate/NaOH, pH 4.0

Method: Equilibrate: 5 CV mobile phase A
Wash: 4 CV 25% mobile phase B
Elution for TSKgel FcR-IIIA-5PW: linear gradient 25-90% B over 14 CV; hold 4 CV at 90% B and 100% B
Elution for TSKgel FcR-IIIA-NPR: linear gradient 0-100% B over 15 min

Flow rate: TSKgel FcR-IIIA-5PW:
Equilibration, load, and wash steps: 0.5 mL/min
Elution and hold steps: 0.25 mL/min
TSKgel FcR-IIIA-NPR: 0.5 mL/min

Instrument: ÄKTA™ avant 25 FPLC

Detection: UV @ 280 nm

Temperature: ambient

Sample: protein A-purified trastuzumab (Herceptin® biosimilar)

Load: 2.7 mg (TSKgel FcR-IIIA-5PW)
54 µg (TSKgel FcR-IIIA-NPR)

Separation of trastuzumab on the analytical and semi-preparative columns (Fig. 1) illustrates comparable peak profiles and ease of scaling using the same mobile phase and flow rate.

SEPARATION OF TRASTUZUMAB BASED ON ITS GLYCOFORM PATTERN ON ANALYTICAL (A) AND SEMI-PREPARATIVE (B) TSKgel FcR-IIIA

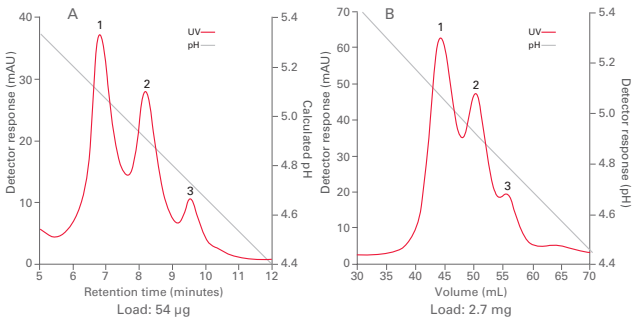


Figure 1

Figure 2 (panel A) illustrates fraction collection from the semi-preparative TSKgel FcR-IIIA-5PW column run and purity analysis for each peak. For semi-preparative peaks from 5 mg load (left panel), the fractions in the boxed area were pooled, buffer-exchanged, concentrated in spin concentrators, and subjected to the analytical TSKgel FcR-IIIA-NPR column for purity assessment. The analytical FcR runs (panels B-D) show that each pooled fraction contained predominantly homogeneous peak material and only minor amounts of other peaks were present.

FRACTION COLLECTION FROM THE SEMI-PREPARATIVE COLUMN RUN AND PURITY ANALYSIS FOR EACH PEAK

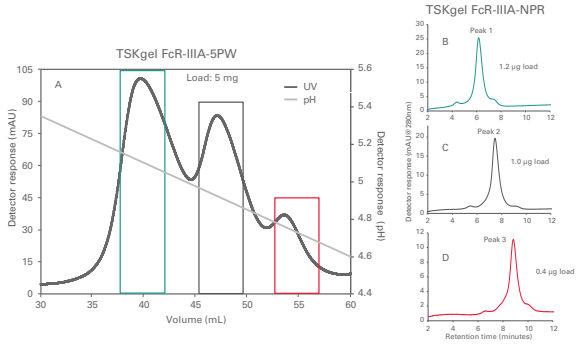


Figure 2

LINEARITY WITH TOTAL PEAK AREA

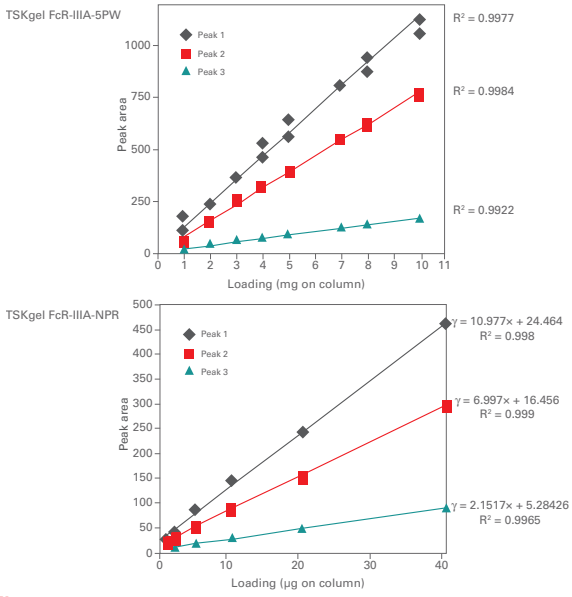


Figure 3

Figure 3 shows linearity of peaks 1, 2, and 3 for different loads on the FcR-IIIA-5PW (upper figure) and FcR-IIIA-NPR (lower figure) columns. A wide range of loading capacity was confirmed: 2-50 µg on the NPR and 1-5 mg on the 5PW column. In larger loads up to 10 mg, peaks started to blend together (not shown). Thus, for the semi-preparative column, the recommended load range was determined as 0.5 mg to 5 mg of mAb.

To demonstrate binding specificity of the TSKgel FcR-IIIA columns to the Fc region of antibodies, papain (Figure 4; papain cleavage site after His-228 in trastuzumab) was used to digest intact IgG to its Fab and Fc fractions and the reaction mix was loaded onto the semi-preparative and analytical TSKgel FcR-IIIA columns (Figure 5). Intact mAb and the Fc fragment both bound onto the FcR-IIIA columns and eluted as typical three peaks.

Intact mAb and the Fc fragment have comparable peak profiles between the analytical and semi-preparative formats, as demonstrated in Figure 5. Tighter binding of the Fc fragment relative to the intact mAb was observed on both columns, potentially due to the removal of steric hindrance of the Fab portions. Approximately 50× more material was loaded (2.7 mg vs 54 µg) on the semi-preparative TSKgel FcR-IIIA-5PW, allowing greater elution volumes and fraction collection. The purity of the three peaks eluted from the TSKgel FcR-IIIA-5PW column are confirmed by the peak profile and different retention times when applied to the analytical TSKgel FcR-IIIA-NPR column. Each pooled fraction analyzed on the analytical column are predominantly homogeneous. As reported, the fractions are linked to different glycan patterns, which can be confirmed by subjecting them to more detailed glycan analysis.

SCHEMATIC ILLUSTRATION OF MAB FRACTIONATION BY PAPAIN DIGESTION

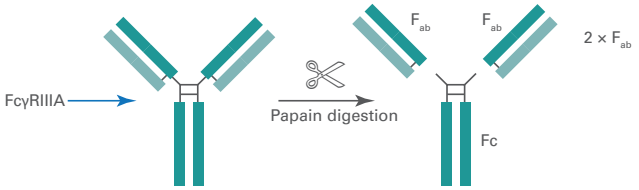
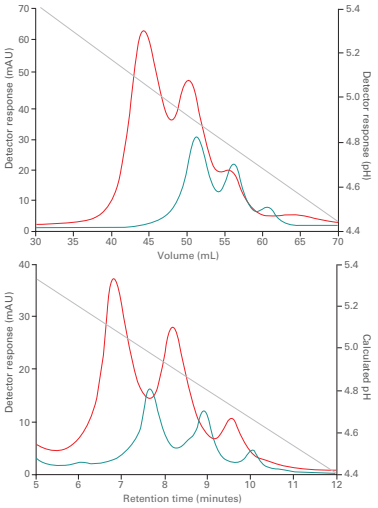


Figure 4

ELUTION PROFILES OF TSKgel FcR-IIIA COLUMNS FOR INTACT MAB AND THE PAPAIN-RELEASED FC-FRACTION



TSKgel FcR-IIIA-5PW		
Peak #	pH at Peak Max (Detector)	
	Intact mAb	Fc Fragment
1	5.07	4.88
2	4.92	4.77
3	4.79	4.66

TSKgel FcR-IIIA-NPR		
Peak #	pH at Peak Max (Calculated)	
	Intact mAb	Fc Fragment
1	5.09	5.00
2	4.90	4.84
3	4.73	4.65

Figure 5

CONCLUSION

In summary, selectivity of intact mAb and papain-digested mAb can be maintained, even at high load, with use of the semi-preparative TSKgel FcR-IIIA-5PW column. The ability to collect more material per injection allows for more detailed ADCC activity estimation of specific mAb glycoforms or mAb modalities related to its Fc domain. These modalities can be separately studied through other orthogonal methods.