Supplementary material

Characterization of AEBSF-Antibody Modifications for a Protease Inhibitor Supplementation Strategy

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1. Analysis of the bNAb samples using a middle-up LC-MS technique

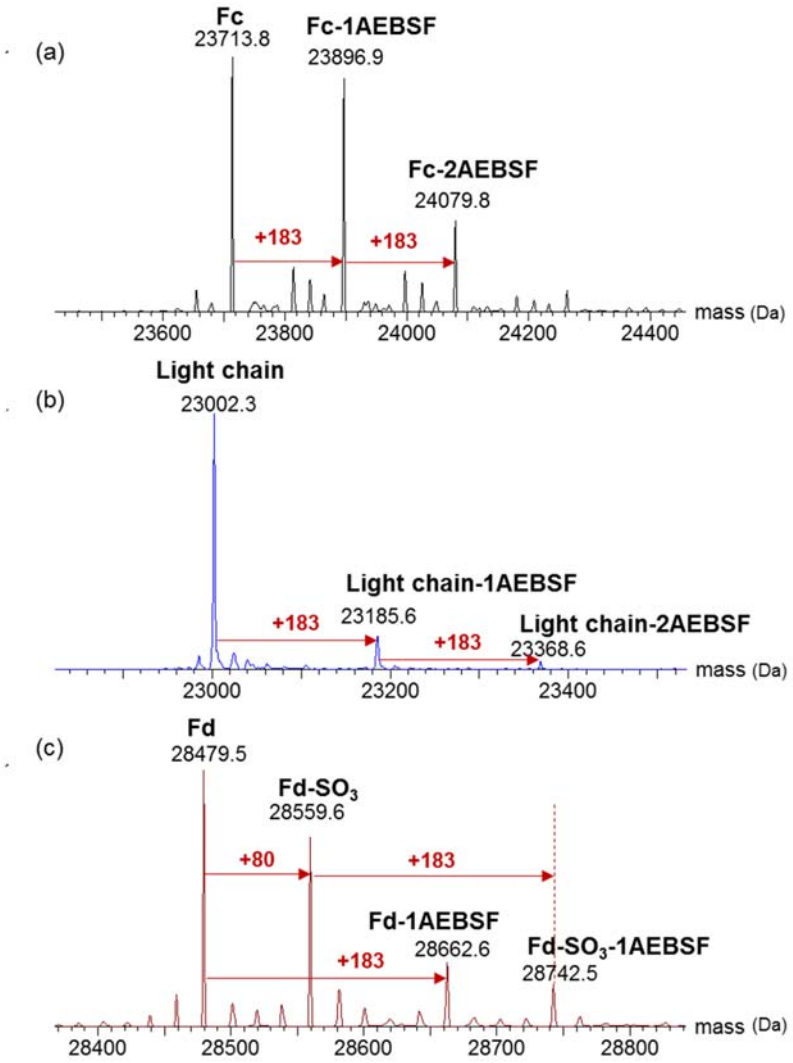


Figure S1. Deconvoluted mass spectra of the (a) Fc/2 at 5.2 min, (b) light chain at 7.3 min and (b) Fd at 7.0 min for day-3 positive control of bNAb.

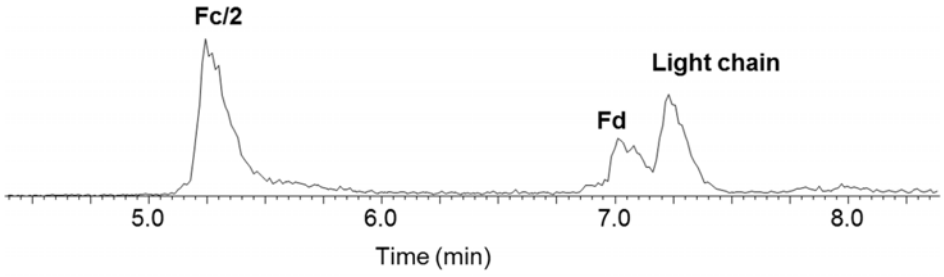


Figure S2. A typical TIC of the deglycosylated, IdeS digested and reduced bNAb test article supplemented with AEBSF during the cell culture stage.

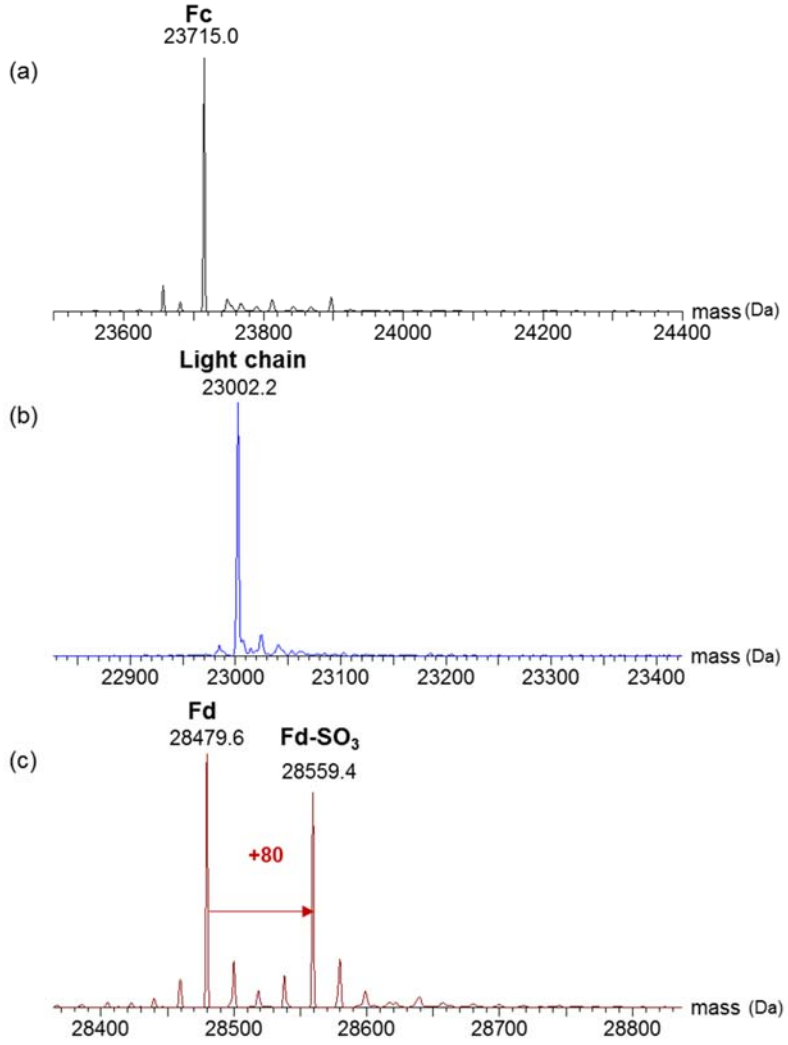


Figure S3. Representative deconvoluted mass spectra of the (a) Fc/2 at 5.2 min, (b) light chain at 7.2 min and (c) Fd at 7.0 min for bNAb test article, supplemented with AEBSF during the cell culture stage.

2. Mass accuracy for the bNAb samples using a middle-up LC-MS technique

Table S1. Theoretical and measured average masses (mass error < 1 Da) of the Fc/2 and light chain subunit in one of the test article lots and day-3 positive control of bNAb from Figure S1 and S3. (ND: not detected)

Subunit	# of AEBSF adducts		0	1	2
Fc	Theoretical mass (Da)		23714.5	23897.5	24080.6
	Measured mass (Da)	Test article Lot#1	23715.0	ND	ND
		Day-3 positive control	23713.8	23896.9	24079.8
Light chain	Theoretical mass (Da)		23002.3	23185.3	23368.4
	Measured mass (Da)	Test article Lot#1	23002.2	ND	ND
		Day-3 positive control	23002.3	23185.6	23368.6

Table S2. Theoretical and measured average masses (mass accuracy < 1 Da) of the Fd subunit in one of the test article lots and day-3 positive control of bNAb from Figure S1 and S3. (ND: not detected)

# of sulfation groups		0	1	0	1
# of AEBSF adducts		0	0	1	1
Theoretical mass (Da)		28479.9	28559.9	28662.9	28742.9
Measured mass (Da)	Test article lot#1	28479.6	28559.4	ND	ND
	Day-3 positive control	28479.5	28559.6	28662.6	28472.5

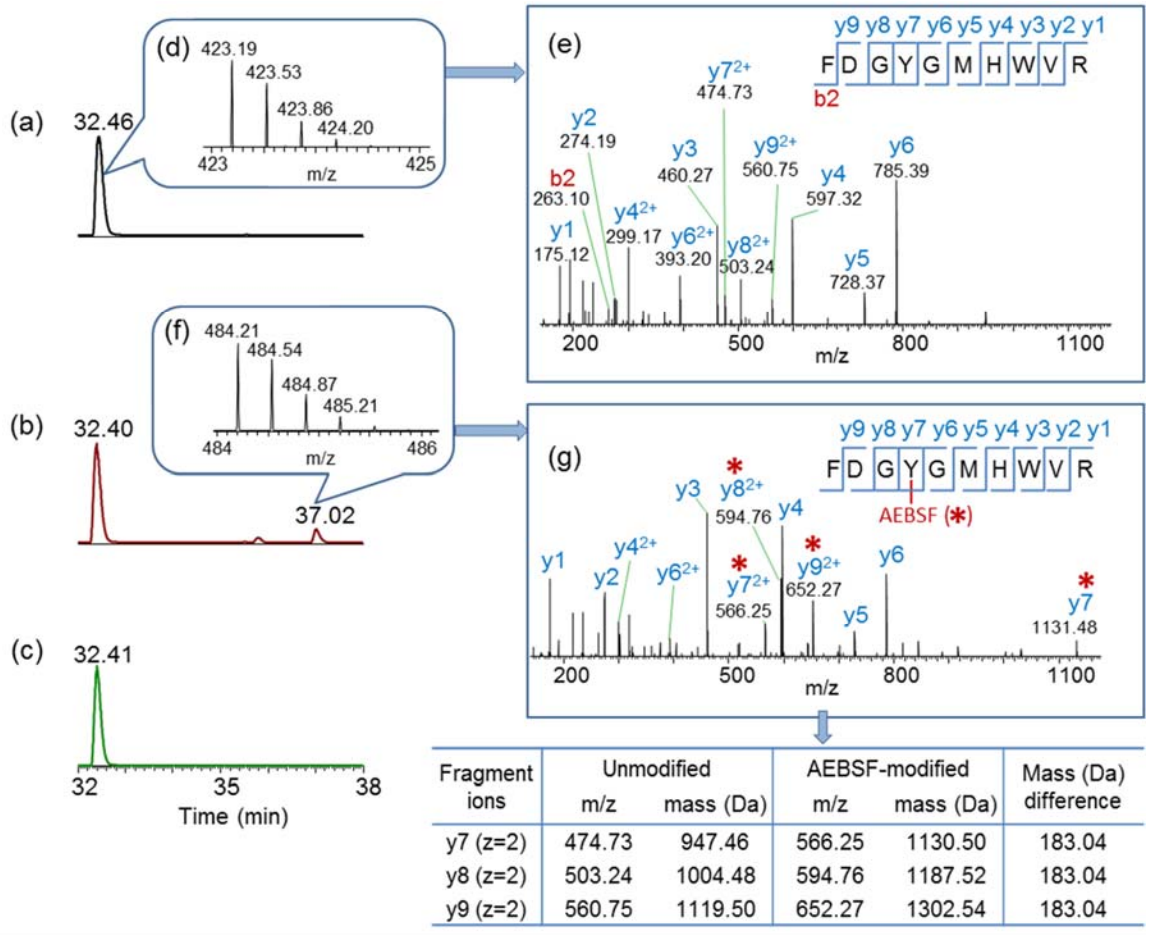
3. Characterization of AEBSF-modified amino acids in the positive control samples via peptide mapping LC-MS analysis

Table S3. Identification and relative quantification of AEBSF-modified peptides in the positive control samples.

Protein subunit	Amino acid residue #	% AEBSF-modification in positive controls	
		Day-3	Day-7
Light Chain	Lys67	1.1	5.4
	Lys161	2.9	8.0
	Lys176	2.1	4.0
	Lys191	3.4	8.3
	Lys209	1.9	6.1
	Tyr50	1.1	4.7
	Tyr177	29.7	66.5
Fd	Tyr32	1.7	9.7
	Lys57	1.2	5.2
	Lys123	1.6	6.3
	Lys161	2.4	13.3
	Lys250	27.5	31.7
Fc	Lys362	2.0	6.3
	Lys420	1.5	5.1
	Lys442	2.8	12.4
	Tyr401	5.0	18.7
	His313	1.4	3.0
	His461	7.6	16.2

4. An example of AEBSF-modified peptide identification

Unmodified peptide F[29]DGYGMHWVR (monoisotopic mass 1266.57 Da) was observed at 32.4 min in the negative control, day-7 positive control and test article. The AEBSF-modified peptide (monoisotopic mass 1449.63 Da) was well-separated from the unmodified one and was detected at 37.0 min only in the day-7 positive control with +183 Da mass shift. In addition, the fragment ion mass shifts of +183 Da were detected for y7-y9 not for y1-y6 in MS/MS spectra in two peptides, which pinpoints the modified amino acid, Y32.



Fragment ions	Unmodified		AEBSF-modified		Mass (Da) difference
	m/z	mass (Da)	m/z	mass (Da)	
y7 (z=2)	474.73	947.46	566.25	1130.50	183.04
y8 (z=2)	503.24	1004.48	594.76	1187.52	183.04
y9 (z=2)	560.75	1119.50	652.27	1302.54	183.04

Figure S4. The XICs, MS and MS/MS spectra of the unmodified and AEBSF-modified peptide F[29]DGYGMHWVR. The XIC (extracted m/z ranges: 363.0-365.0, 423.0-425.0, 484.0-486.0, 634.0-636.0) of the unmodified peptide (m/z 423.19, z=3 and m/z 634.39, z=2) was detected at 32.4 min for (a) the negative control, (b) the day-7 positive control and (c) test article (y-axis: relative abundance, normalized to the highest peak in the selected time range), and the AEBSF-modified peptide (m/z 363.41, z=4 and m/z 484.21, z=3) was only detected in the 7-day positive control. The unmodified peptide with (d) precursor ion m/z 423.19 (z =3) and the AEBSF-modified peptide with (f) precursor ion m/z 484.21 (z=3) shows (g) similar y1-6 ions but different y7-y9 ions respectively. (*: AEBSF modified fragment ions; y-axis of MS is relative abundance, normalized to the highest peak in each spectrum.)

5. PyMOL review of the bNAb

PyMOL™ software was used for visualizing the 3D structure of the Fab region of the bNAb. 3-D-crystallographic analysis of the bNAb (Protein Databank sequence entry: 5dt1) was reviewed using PyMOL™ Molecular Graphic System, v. 1.8.2.3.

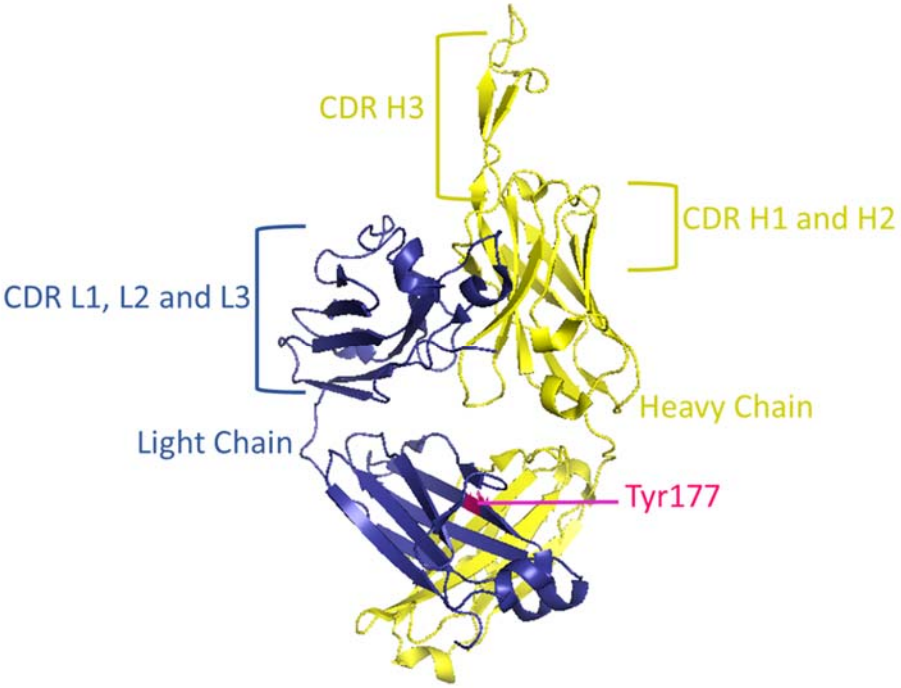


Figure S5. PYMOL review of the antigen-binding fragment (Fab) of the bNAb. CDR regions are labeled, Tyr177 is in pink color, and the unlabeled Lys250 is below the green ribbon diagram (not shown) [12].