SUPPLEMENTARY INFORMATION

Linker engineering in anti-TAG-72 antibody fragments optimizes biophysical properties, serum half-life, and high-specificity tumor imaging

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Running Title: *Engineering scF$_V$ Linkers for Cancer Imaging*

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Items included in this document:

SUPPLIEMENTARY TABLE 1

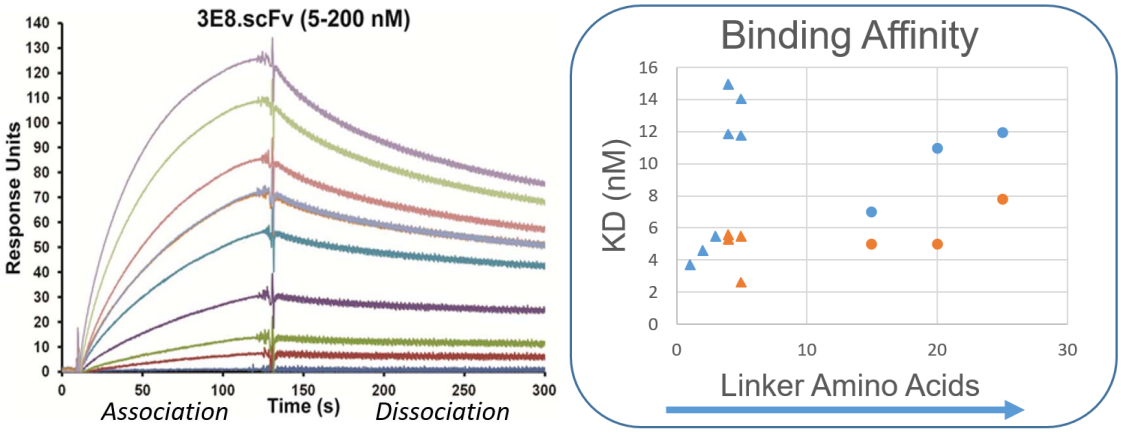
Table of Antibody Template Protein, Template DNA, and Primer DNA Sequences

Name	**Sequence**
3E8 V_L-205C-V_H (AA)	MKYLLPTAAAGLLLLAAQPAMAA HHHHHHGSSGGGENLYFQ GSSG *DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQQKPGQPPKLLIY* *WASTRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYSYPLTFGGGTKVEIK* **LSADDAKKDAAKKDDAKKDDAKKDL** *QVQLVQSGAEVKKPGASVKVSCKASGYTFTDHAIHWVRQAPGQRLEWMGYFSPGN* *DDFKYSQKFQGRVTITADKSASTAYMELSSLRSEDTAVYYCARSWIMQYWGQGTLVTVSS*
3E8 V_L-205C-V_H (DNA)	CATATGAAATATCTGTTACCTACTGCTGCTGCGGGCCTGCTATTATTAGCGGCACAACCAG CAATGGCGGCGCATCATCATCATCATCATGGGTCCTCGGGCGGTGGCGAAAATCTGTATT TTCAGGGTAGCAGCGGC*GATATTGTGATGACCCAGAGCCCGGATAGTTTGGCCGTTAGCC* *TGGGCGAACGTGCGACGATTAATTGCAAGAGCAGCCAGAGCGTGCTTTACAGCAGCAACA* *ATAAGAATTACCTGGCGTGGTATCAGCAAAAACCCGGCCAGCCGCCGAAACTTTTGATTTAT* *TGGGCGAGCACCCGTGAAAGCGGCGTGCCGGATCGTTTCTCGGGCTCAGGCAGCGGGACC* *GATTTTACGCTGACCATCAGCAGCCTTCAGGCGGAGGATGTCGCGGTGTACTACTGCCAGC* *AGTATTACAGCTATCCGTTGACCTTTGGGGGAGGCACCAAAGTGGAGATCAAA***CTGAGCG** **CGGATGATGCTAAGAAAGATGCGGCGAAGAAGGACGATGCGAAAAAAGACGACGCAAAA** **AAGGATCTG***CAGGTGCAGCTGGTGCAGTCGGGTGCGGAAGTGAAGAAACCTGGGGCGTCG* *GTGAAAGTGAGCTGCAAAGCGAGCGGCTATACCTTTACCGATCATGCGATTCATTGGGTGCG* *TCAAGCGCCAGGCCAGCGTCTGGAATGGATGGGCTATTTTTCCCCAGGCAACGATGATTTCA* *AGTATTCCCAGAAGTTCCAAGGGCGCGTGACCATTACCGCCGATAAAAGCGCAAGCACCGCG* *TATATGGAGCTGTCCAGCCTGCGTAGCGAAGATACAGCGGTTTACTATTGCGCACGGAGCTG* *GATTATGCAATACTGGGGCCAGGGCACCCTGGTGACCGTGAGCAGC*TAAGGATCC
3E8 V_H-G_4S-V_L (AA)	MKYLLPTAAAGLLLLAAQPAMAA HHHHHHGSSGGGENLYFQ GSSG *QVQLVQSGAEVKKPGASVKVSCKASGYTFTDHAIHWVRQAPGQRLEWMGYFSPGN* *DDFKYSQKFQGRVTITADKSSSTAYMELSSLRSEDTAVYYCARSWIMQYWGQGTLVTVSS* **GGGGS** *DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQQKPGQPPKLLIY* *WASTRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYSYPLTFGGGTKVEIK*
3E8 V_H-G_4S-V_L (DNA)	CATATGAAATACTTGCTGCCAACGGCAGCTGCCGGACTACTCCTATTAGCCGCTCAACCTG CTATGGCCGCACATCACCATCACCATCACGGAAGCAGTGGAGGCGGTGAGAACCTATACT TCCAGGGAAGTTCCGGC*CAAGTACAACTAGTACAGTCGGGGGCGGAAGTCAAGAAACCG* *GGTGCAAGCGTGAAGGTGAGTTGTAAAGCATCTGGTTATACATTCACAGACCATGCGATA* *CATTGGGTCAGACAAGCACCGGGGCAACGTCTGGAATGGATGGGGTACTTTAGCCCTGGG* *AATGACGATTTCAAGTACTCTCAAAAATTTCAAGGCCGGGTTACAATCACCGCCGATAAATC* *ATCGTCAACAGCCTATATGGAGCTTTCGTCCTTGAGATCTGAGGATACGGCTGTTTACTAT* *TGCGCGAGATCTTGGATAATGCAGTATTGGGGACAAGGTACCCTCGTAACTGTGTCATCT***G** **GCGGAGGTGGCTCC***GACATTGTGATGACACAGAGTCCTGACTCATTAGCGGTTTCCTTGGG* *GGAACGGGCAACTATTAACTGTAAGTCCAGTCAATCGGTCCTGTACTCGTCAAATAACAAGA* *ATTATTTAGCTTGGTACCAGCAAAAGCCTGGGCAGCCGCCTAAACTTTTGATCTACTGGGCG* *AGCACTAGAGAGTCCGGAGTACCAGACCGCTTTAGTGGGTCAGGTTCTGGAACGGATTTTA* *CCCTCACTATTTCGAGCTTACAGGCGGAAGATGTAGCTGTCTATTACTGCCAGCAGTATTATA* *GCTATCCACTTACG TTCGGCGGTGGCACCAAGGTTGAAATAAAA*TAAGGATCC
V_L-V_H Forward Amplification Primer	5’ – ATTATTATTCATATGAAATATCTGTTACCTACTGC – 3’
V_L-V_H Reverse Amplification Primer	5’ – AATAATGGATCCTTAGCTGCTCACGGTCAC – 3’
V_L-V_H Forward Mutagenic Primer	5’ – GCACCAAAGTGGAGATCAAA – Mut. Region – CAGGTGCAGCTGGTACAG – 3’

V_L-V_H Reverse Mutagenic Primer	5’ – CTGTACCAGCTGCACCTG - Mut. Region - TTTGATCTCCACTTTGG TGC – 3’
V_H-V_L Forward Amplification Primer	5’ – ATTATTATTCATATGAAATACTTGCTGCCAAC – 3’
V_H-V_L Reverse Amplification Primer	5’ – AATTATGGATCCTTATTTTATTTCAACCTTG – 3’
V_H-V_L Forward Mutagenic Primer	5’–CTC GTA ACT GTA TCA TCT – Mut. Region - GAC ATT GTA ATG ACA CAG–3’
V_H-V_L Reverse Mutagenic Primer	5’–CTG TGT CAT TAC AAT GTC – Mut. Region - AGA TGA TAC AGT TAC GAG–3’

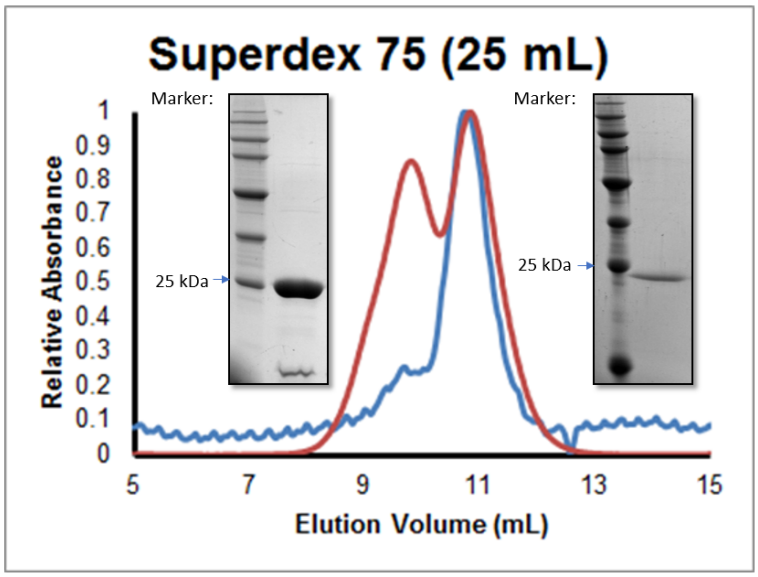
Supplementary Table 1: The table above shows the protein and DNA sequences of the V_L-V_H and V_H-V_L templates (from Yoon et al. 2006) used to create the original 17-member tagged library. Variable light and heavy domains are *italicized* and the linker is shown in **bold**. In addition, amplification primer sequence as well as general mutagenic sequences for mutagenic primers are shown.

FIGURE S1



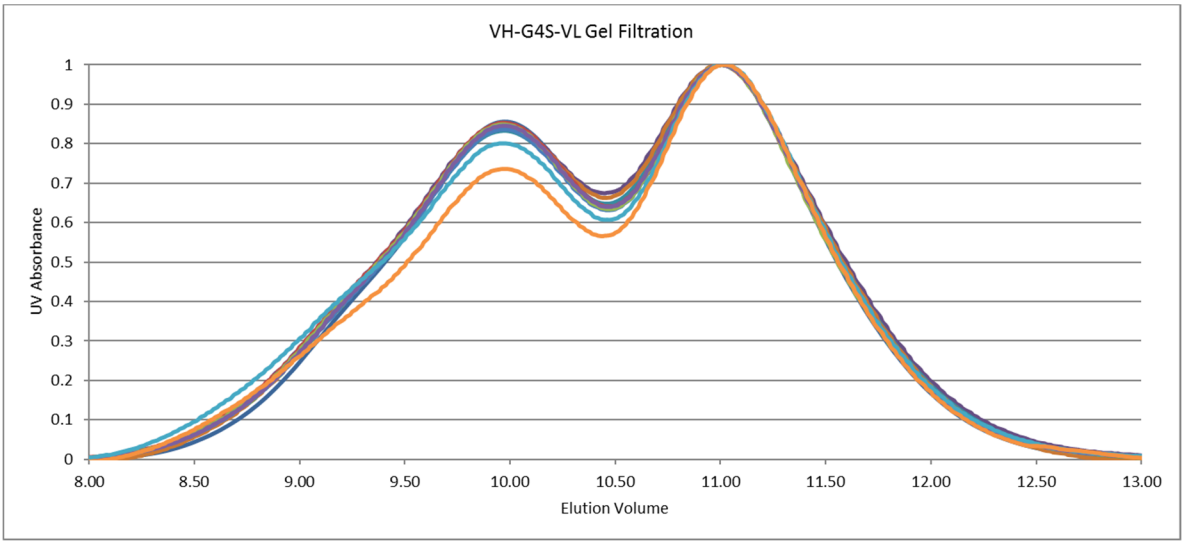
Supplementary Figure 1: Binding affinity of 3E8 library members was determined by SPR. (A) Representative association and dissociation curves of 3E8 V_L-205C-V_H construct produced by BIACORE software. Concentrations of antibodies were 0 nM, 5 nM, 10 nM, 25 nM, 50 nM, 75 nM (in duplicate), 100 nM, 150 nM, and 200 nM. (B) Correlative scatter plot showing calculated K_D in relation to linker length in both V_L-V_H orientation (blue) and V_H-V_L orientation (orange). Constructs with majority monomeric species are shown as a circle, constructs with majority dimeric species are shown as a triangle.

FIGURE S2



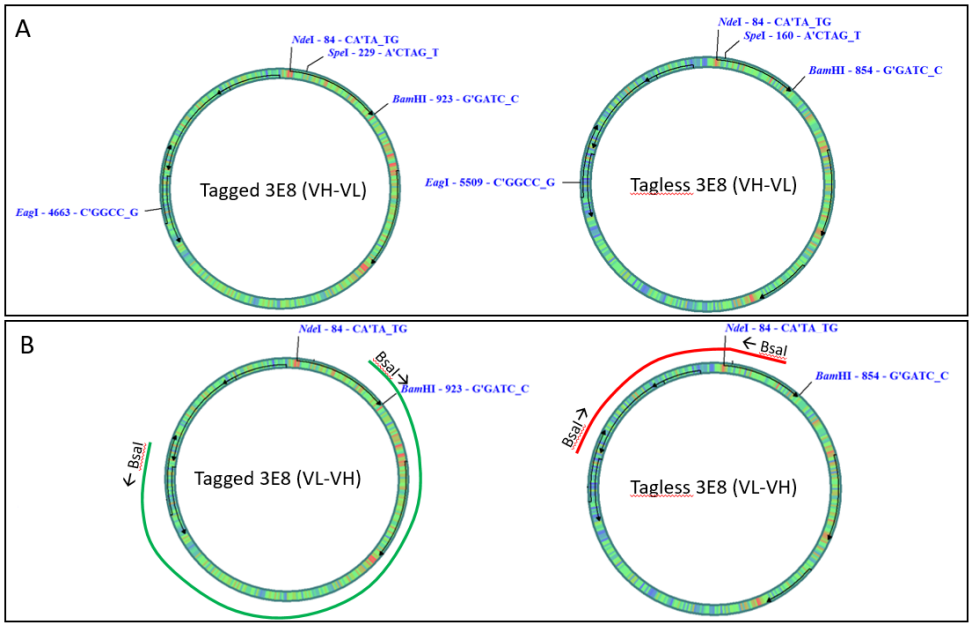
Supplementary Figure 2: Comparison of Ni-NTA IMAC purification with Protein L purification. SDS-PAGE gel shows purified protein yields of 3E8.G$_4$S are increased with Protein L (left) than that of IMAC purification (right). The gel filtration chromatograph shows substantial differences in oligomeric states between methods of purification. Protein L purification (red) produces two peaks corresponding to dimer and higher order oligomer trimer and tetramers. IMAC purification (blue) produces a nearly homogeneous dimer peak.

FIGURE S3



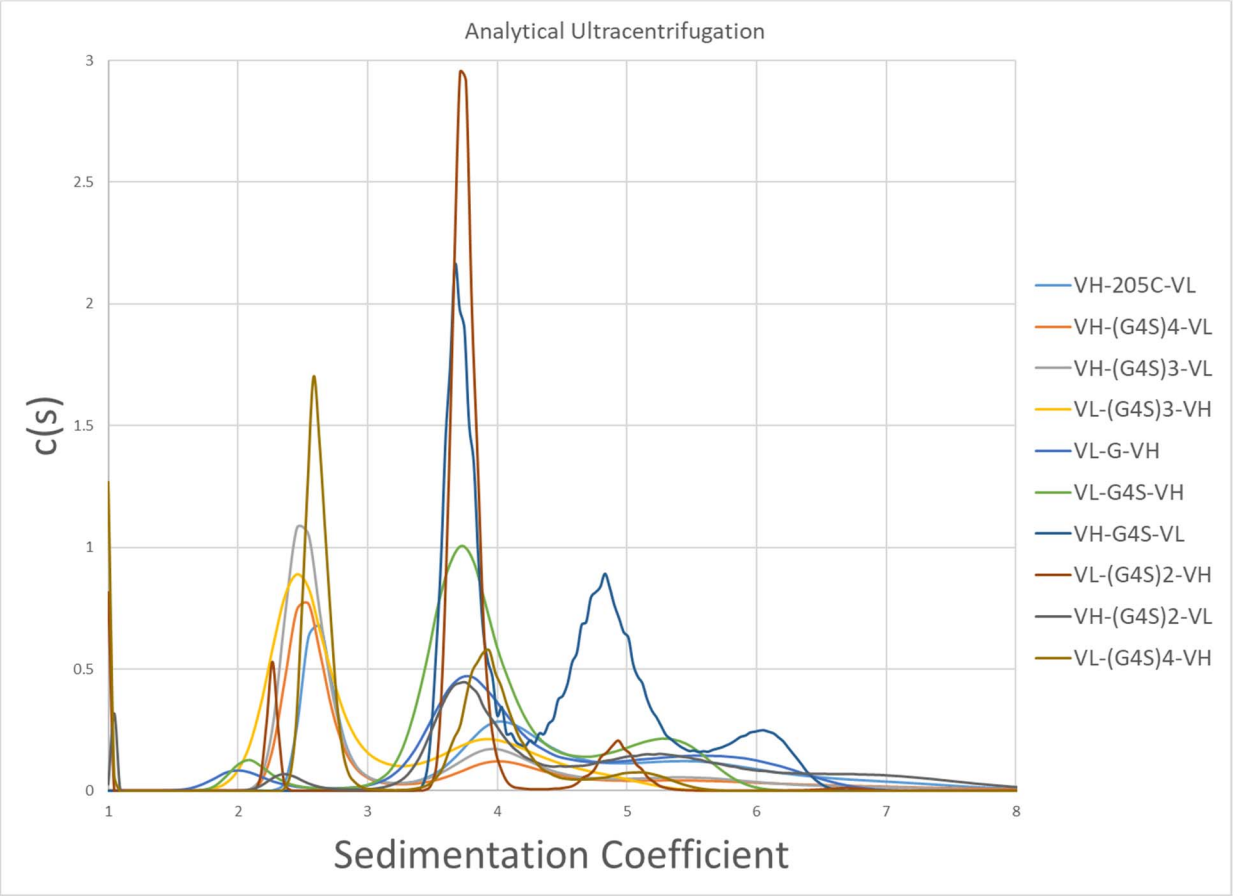
Supplementary Figure 3: The reproducibility of the oligomeric states of 3E8.G$_4$S (V_H-G$_4$S-V_L) was tested to determine if the distribution was dependent on the specific linker or varied with conditions. Our results showed a small fluctuation of trimer and tetrameter relative to the dimer species but overall the ratio fell within an average of ten percent**.**

FIGURE S4



Supplementary Figure 4: Cloning schematic of tagless linker library. (A) Tagless library members with V_L-V_H orientation were cloned using SpeI and EagI restriction sites. SpeI is located within the 3E8 gene between the TEV site and variable linker region. The small digested fragment of tagless construct 3E8.G_4S was ligated to the large digested fragments from each V_H-V_L library member. (B) Mutagenic primers with flanking BsaI sites were used to PCR out a small fragment of the tagless 3E8 construct V_L-205C-V_H and a large fragment from all tagged V_H-V_L library members. BsaI sites were removed after digestion and complimentary overhangs between the two PCR fragments were ligated together.

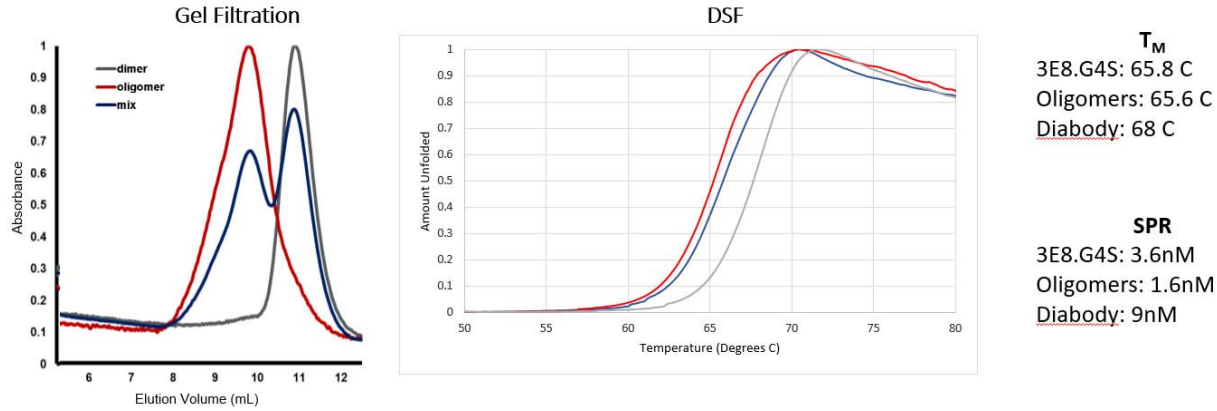
FIGURE S5



Supplementary Figure 5: Analytical ultracentrifugation (AUC) of select tagless antibodies was performed to corroborate gel filtration data. Sedfit analysis determined the peaks (left to right) to be monomer (2.5), dimer (3.75), trimer (4.8), and tetramer (6). Calculated ratios of oligomeric state were in general agreement with those determined by gel filtration.

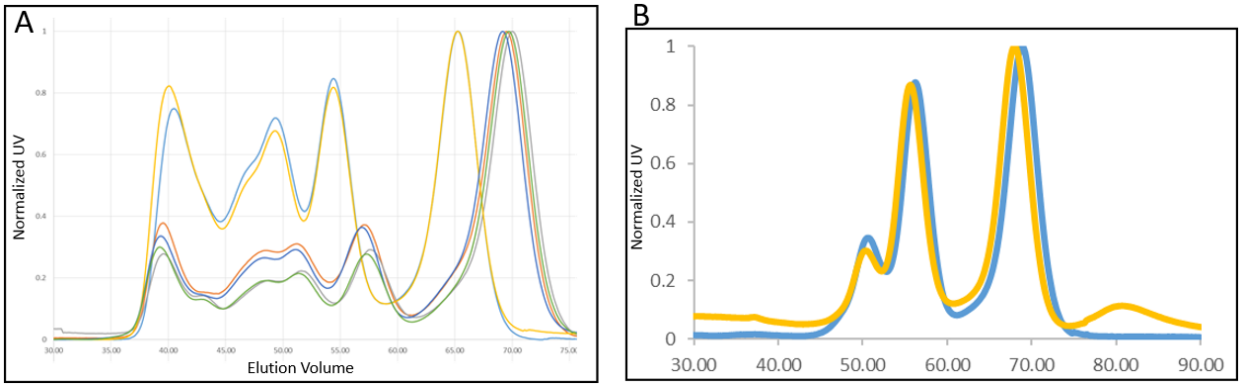
FIGURE S6

Separation and characterization of oligomeric species:



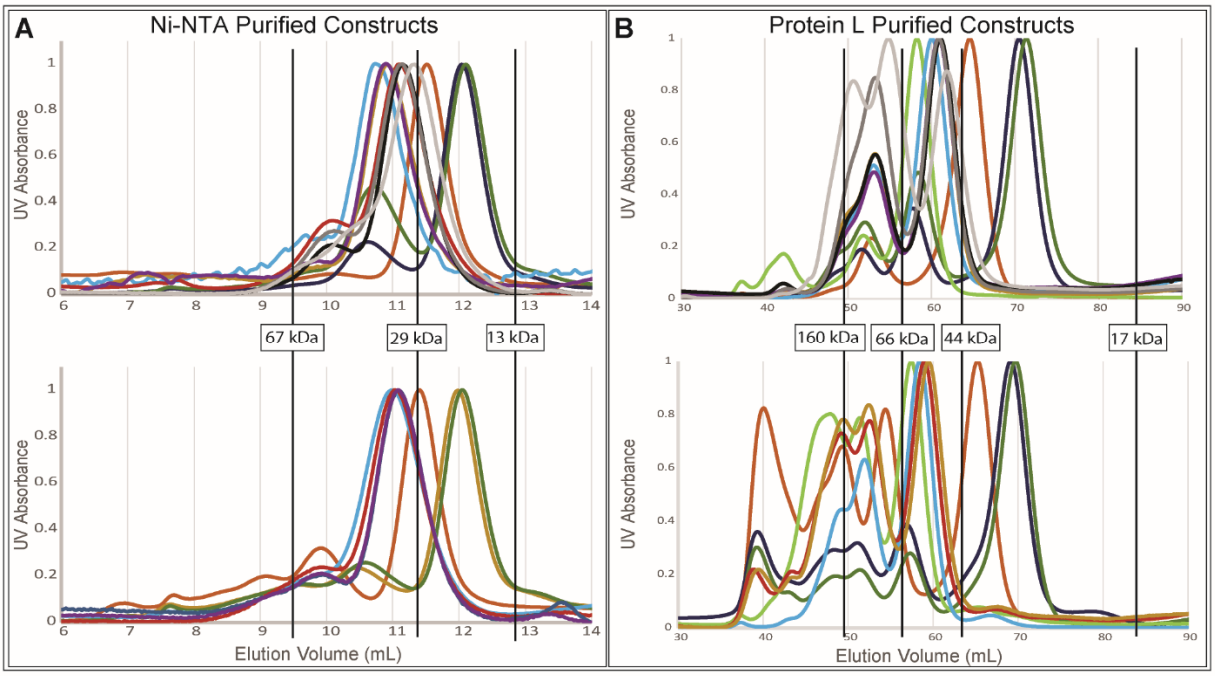
Supplementary Figure 6: Separation and characterization of 3E8.G$_4$S oligomeric states. (Left) Gel filtration chromatograph showing unfractionated 3E8.G$_4$S (blue). Dimer and oligomer peaks were collected and re-injected onto the column as a single dimer peak (grey) and oligomer peak (red). (Middle) DSF of 3E8.G$_4$S and separated dimer/oligomer samples. (Right) DSF and SPR melting temperatures and measured binding affinities of unfractionated 3E8.G$_4$S, diabody fraction, and oligomer fraction.

FIGURE S7



Supplementary Figure 7: Oligomer concentration and 6x-His tag independence. (A) Normalized gel filtration of V_H-205C-V_L, V_H-$(G_4S)_4$-V_L, and V_H-$(G_4S)_3$-V_L antibody constructs at 0.2 mg/mL and 0.8 mg/mL concentrations. (B) 6x-His tag removal of V_L-$(G_4S)_4$-V_L antibody construct

FIGURE S8



Supplementary Figure 8: Protein standards for size exclusion chromatography columns. (A) Analytical Superdex 75 column with protein standards BSA (67 kDa), carbonic anhydrase (29 kDa), and cytochrome c (13 kDa). Gly-Ser linker antibody constructs elute more slowly than expected, probably due to non-spherical shape or interactions with the resin. Protein size was further validated with (B) prep grade Superdex 75 column with protein standards IgG (160 kDa), albumin (66 kDa), ovalbumin (44 kDa), and myoglobulin (17 kDa).