

Nonradioactive Analytical Methods for Quantifying the Modification Ratio of Antibody Fragment Chelator Conjugates with Deferoxamine and NODAGA



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Abstract

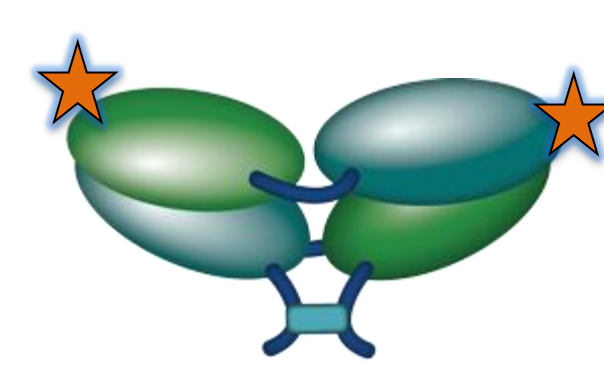
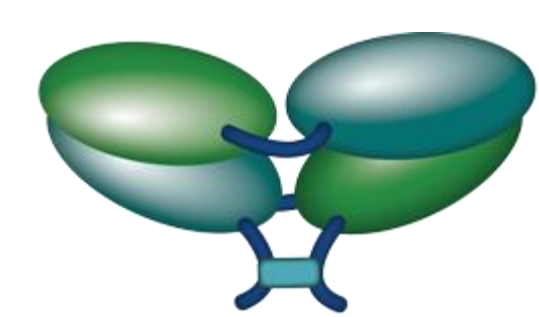
Objective: A primary challenge in the synthesis and cGMP production of antibody conjugates is the control of the modification ratio, such as the drug/antibody ratio (DAR) or chelator/antibody ratio (CAR). While radio-ITLC can be used to estimate the CAR of antibody-chelator conjugates (ACCs), development of a nonradioactive analytical method eliminates hurdles associated with implementing radioactive assays in analytical laboratories and enables such assays to be performed with standard laboratory equipment. Here, we describe the development of colorimetric assays for CAR quantification of ACCs synthesized using deferoxamine (Df) and NODAGA chelators.

Methods: Antibodies or antibody fragments (minibodies, cys-diabodies) were conjugated with lysine-reactive chelators (Df-isothiocyanate, NODAGA-NHS) or cysteine-reactive chelators (Df-maleimide, NODAGA-maleimide). Df quantification was performed by spectroscopic detection of ferrioxamine complex formation, while NODAGA quantification was performed using an indicator dye, arsenazo III, to spectroscopically determine the concentration of free metal ions following chelation by the ACC. The accuracy of each colorimetric assay was established by comparison to the CAR determination by mass spectrometry (LC/MS). Additionally, the chemical and functional properties of the chelated proteins were evaluated by IEF, SDS-PAGE, SE-HPLC, and ELISA.

Results: The average CAR determined by the respective colorimetric assay was consistent with the value determined by mass spectrometry. Mass spectrometry revealed that the lysine-modified ACCs exhibited a broad distribution of species compared to the more uniform product observed with the cysteine-modified ACCs (cys-diabodies). Differences in the charge and structure of the attached Df and NODAGA chelators also impacted the chemical properties of the resulting ACCs.

Conclusions: The colorimetric assays described herein can provide rapid and accurate estimates of the average chelation ratio for conjugated proteins and serve as a useful tool to support both early-stage conjugation process development and cGMP manufacturing.

Sample Overview – Cys Diabody (Cys-Db) Chelator Conjugates



A. Unconjugated Cys-Db **B. Cysteine Conjugated Cys-Db** **C. Lysine Conjugated Cys-Db**

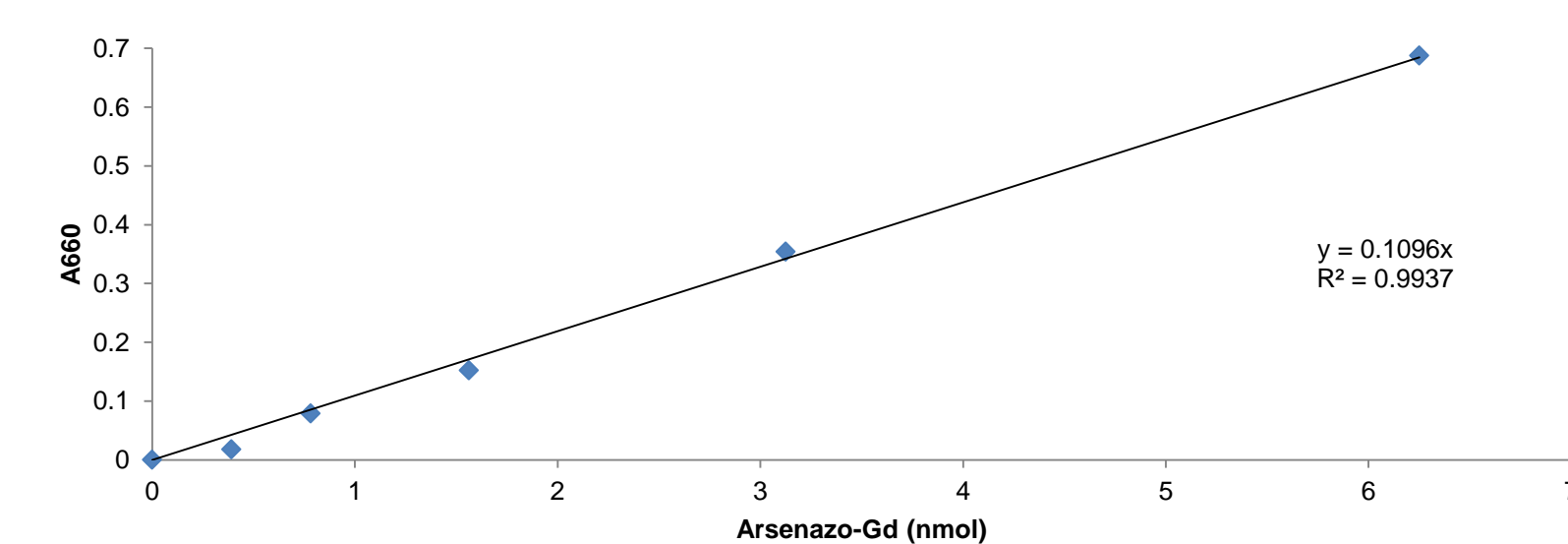
- The cys-diabody (Cys-Db) is an engineered antibody fragment dimer consisting of two cross-paired scFvs linked by a disulfide bond at the C-terminus
- Cys-Db fragments can be functionalized by thiol-reactive molecules at the two C-terminal cysteines (B) or by amine-reactive molecules at surface lysines (C)
- Cys-Dbs were conjugated on cysteine and lysine residues with the chelators NODAGA and Df

Colorimetric Quantification of Chelator Attachment

Arsenazo III/Gadolinium Assay for NODAGA Quantification

- Arsenazo III:Gd Standard Curve
- Dilution in 0.25 M ammonium acetate, pH 7.
 - Microplate format with 215 μ L total volume per well.

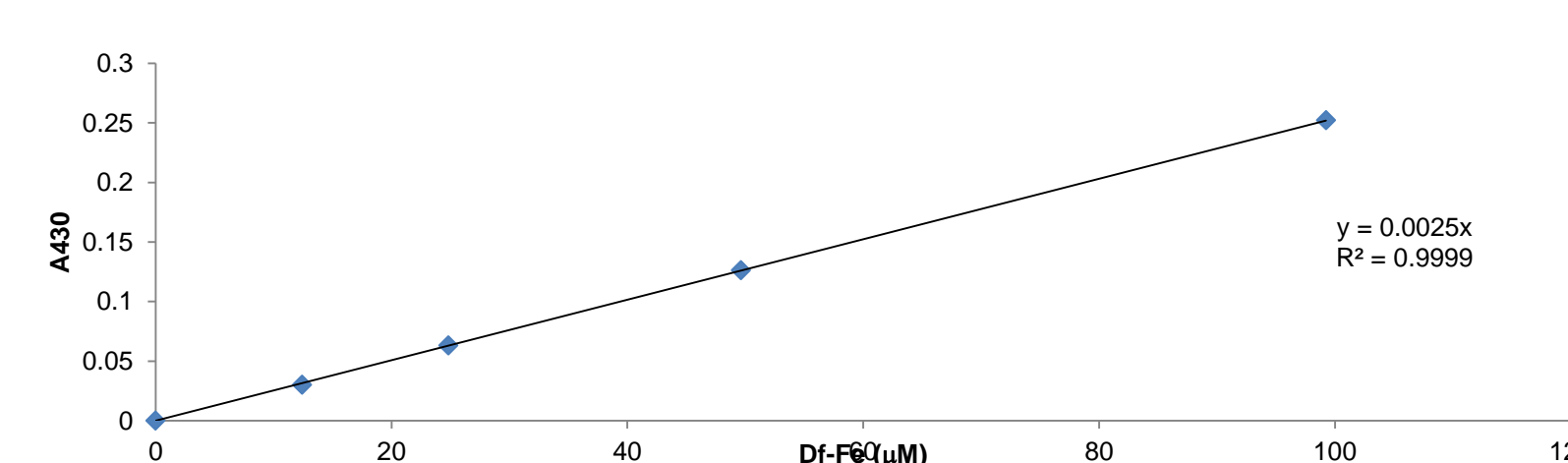
Arsenazo III binds Gd and absorbs at 660 nm. Gd is added to the analytical sample and the A660 is compared to a Gd positive and negative control.



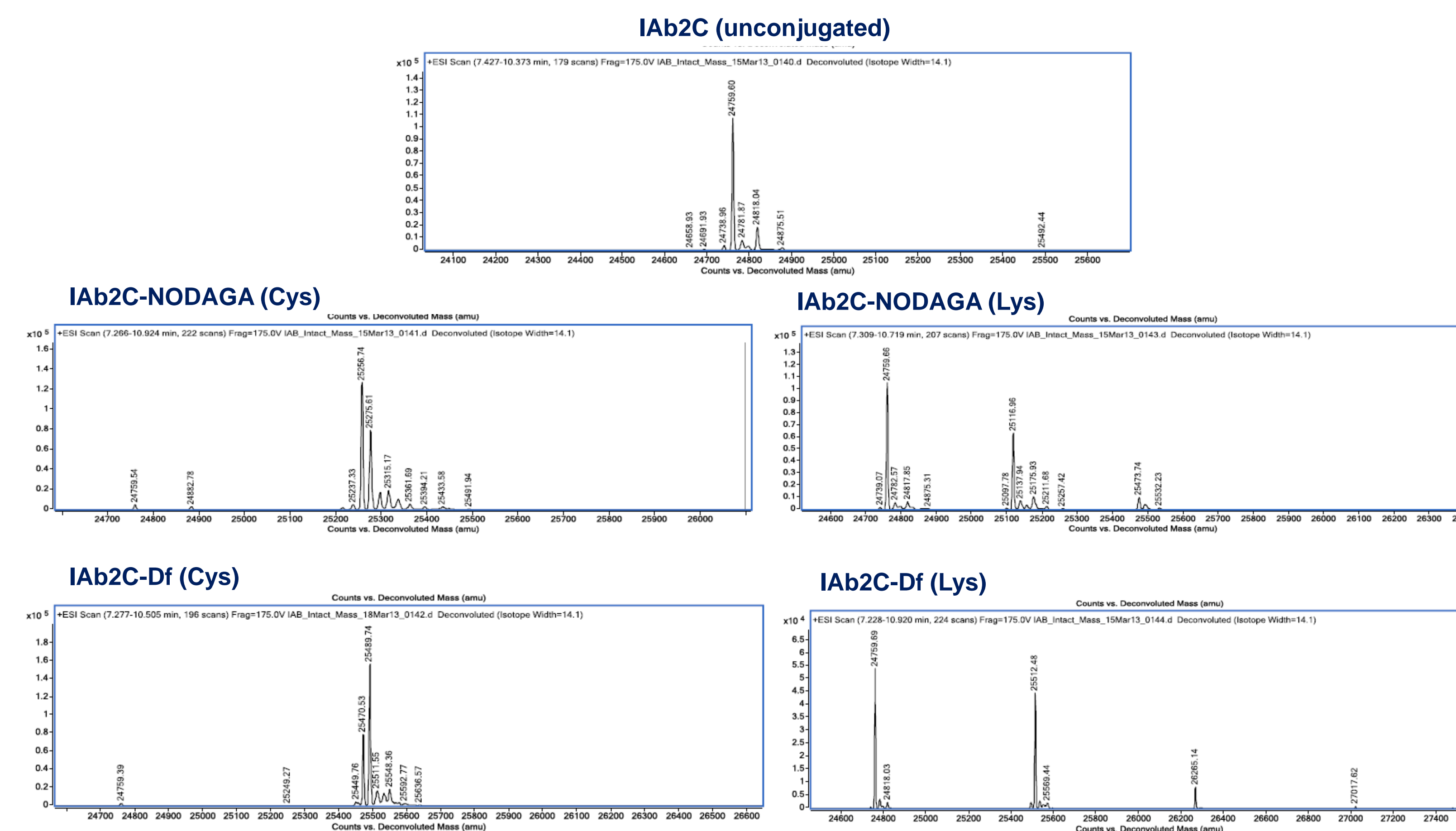
Ferrioxamine Assay for Df Quantification

- Ferrioxamine Standard Curve
- Dilution in 0.2 M sodium citrate, pH 7.
 - Cuvette (1 cm) format.

Df binds Fe and absorbs at 430 nm. Based on the A430, the concentration of Df can be calculated.



HPLC/Mass Spectrometric Analysis of Chelator Attachments



- Deconvoluted mass spectra of intact and chelated Cys-Dbs showing intact masses following sample reduction and HPLC/MS analysis
- The degree of modification was more uniform when conjugation occurred on engineered cys

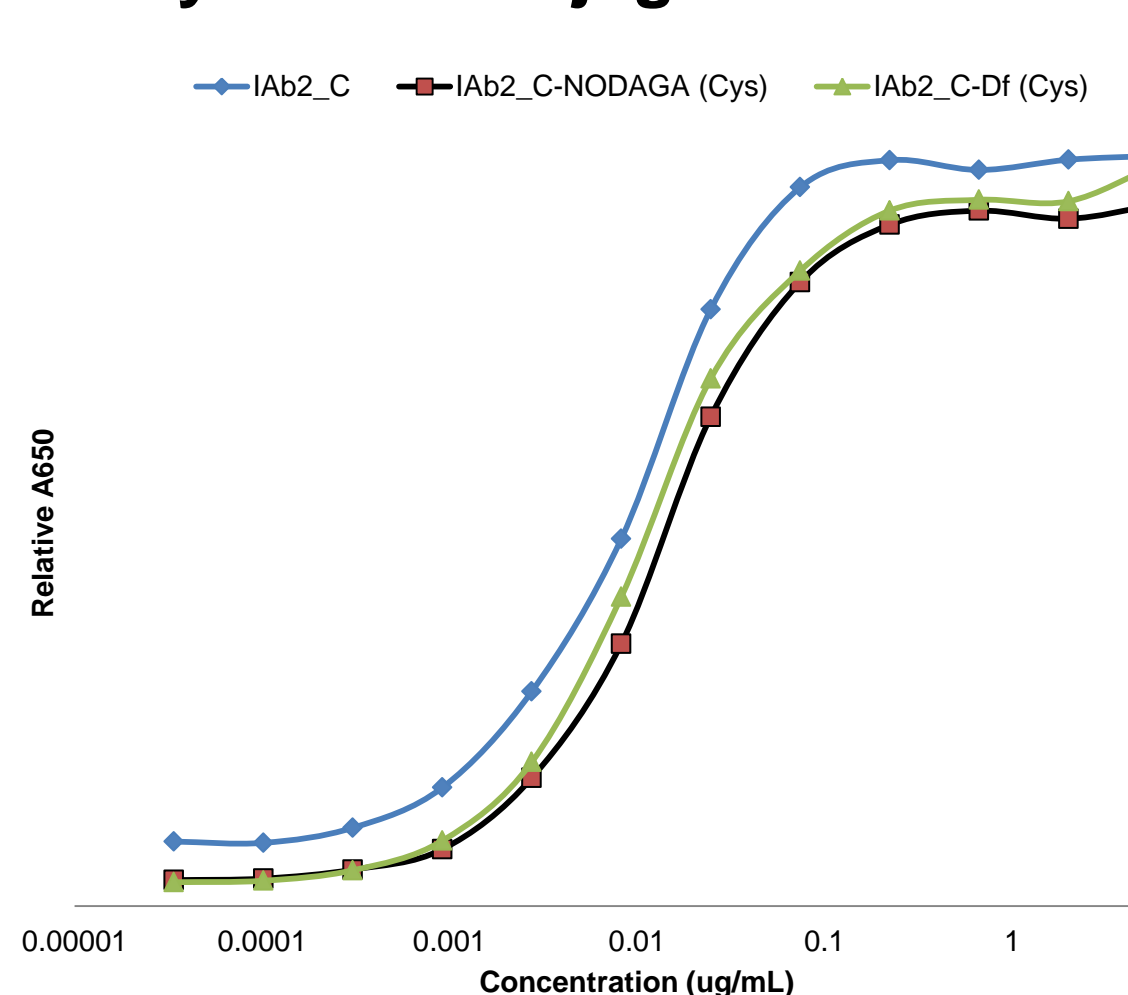
Colorimetric vs. HPLC/MS Analysis of Chelator Attachments

Cys Diabody	Chelator	Reaction Site	Colorimetric Assay Results ^a	LC/MS CAR Results (Relative Abundance as % of total) ^b			
				IAb2C + 0 Modifications	IAb2C + 1 Modifications	IAb2C + 2 Modifications	IAb2C + 3 Modifications
IAb2C-NODAGA (cys)	NODAGA-Maleimide	Cysteine	1.6	1.3	98.7	-	-
IAb2C-Df (cys)	Df-maleimide		2.2	5.4	94.5	-	-
IAb2C-NODAGA (lys)	NODAGA-NHS ester	Lysine	2.4	58.9	35.6	5.5	0
IAb2C-Df (lys)	Df-p-SCN		2.4	50.2	41.4	7.6	0.9

- ^aFor LC/MS, the Cys-Db was analyzed under reducing conditions which reduced the C-terminus disulfide bond allowing evaluation of the monomeric scFvs
- ^bThe colorimetric method analyzed the Cys-Db as an intact dimer

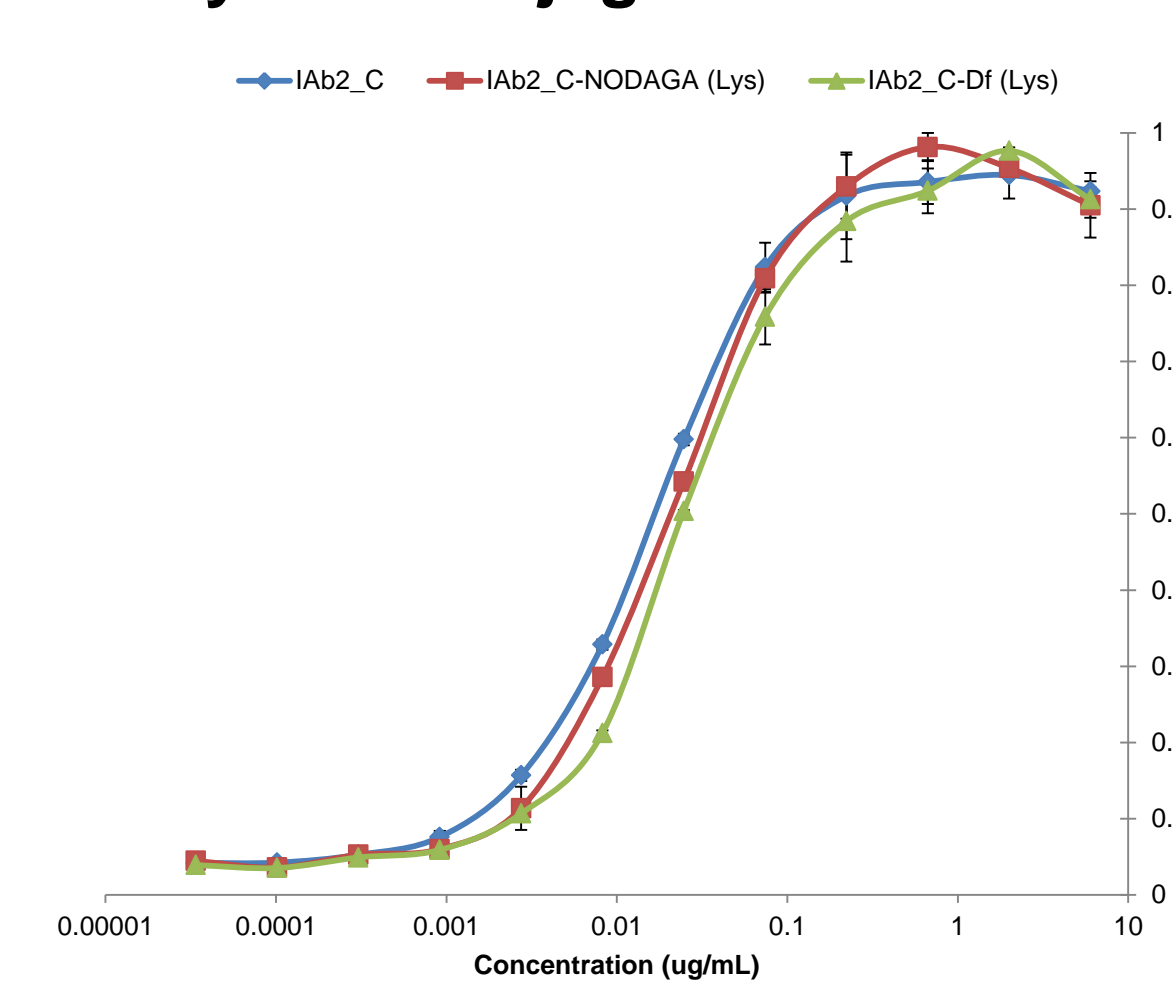
Conjugation Validation – Functional Binding by ELISA

Cysteine Conjugated EC50



Cys Diabody	EC50 (nM)
IAb2C	0.18
IAb2C-NODAGA (cys)	0.26
IAb2C-Df (cys)	0.22

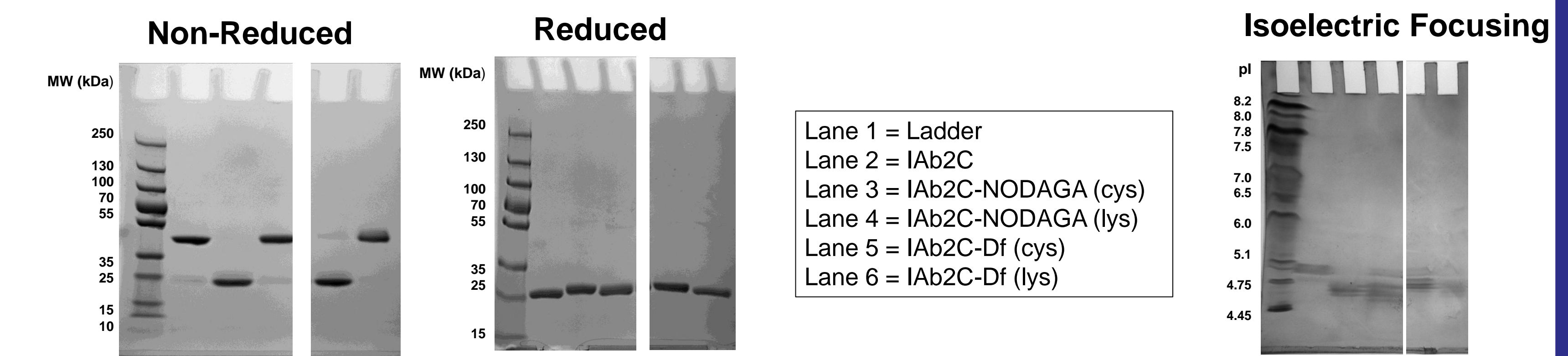
Lysine Conjugated EC50



Cys Diabody	EC50 (nM)
IAb2C	0.32
IAb2C-NODAGA (lys)	0.39
IAb2C-Df (lys)	0.40

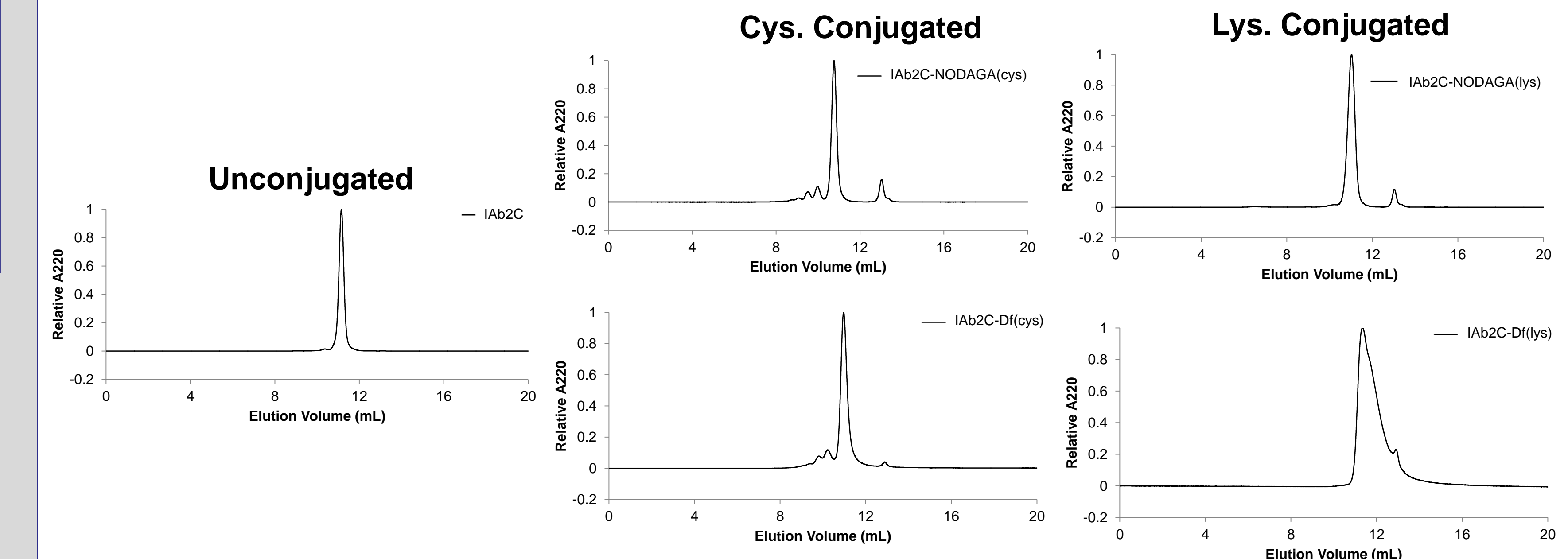
- ELISA binding demonstrated that functional affinity was not reduced after cysteine or lysine modification of the Cys-Db

Conjugation Validation – SDS-PAGE and Isoelectric Focusing



- SDS-PAGE analysis shows that conjugation did not cause significant fragmentation or aggregation
- The unconjugated (Lane 2) and lysine conjugated Cys-Dbs (Lanes 4 and 6) migrated as ~ 50 kDa covalent dimers
- The cysteine conjugated Cys-Dbs (Lanes 3 and 5) migrated as ~ 25 kDa monomers
- IEF analysis shows a slight decrease in pI for all conjugated samples resulting from attachment of negatively charged NODAGA, modification of positively charged lysines and/or hydrolysis of the maleimide ring

Conjugation Validation – SE-HPLC



- Minor increase in the aggregate profile relative to the unconjugated sample was observed after cysteine conjugation
- Samples were analyzed on a TSKgel BioAssist G3SWxl column with mobile phase of 0.1 M phosphate + 0.1 M sulfate + 10% methanol, pH 6.8 for the SE-HPLC analysis
- Slight peak tailing observed for the lysine conjugated IAb2C_Df sample is due to nonspecific hydrophobic interaction with the analytical column

Summary of Results

- Cys-diabodies are single-chain constructs that self-assemble into dimers and provide a versatile platform for controlling both the location and stoichiometry of conjugation
- Determination of the Chelator:Cys-Db ratio following conjugation with NODAGA and Df can be accurately measured using simple colorimetric assays with spectroscopic absorbance readouts
- The results obtained from the colorimetric assays were corroborated using HPLC/MS analysis to determine the intact mass of the conjugated Cys-Dbs
- Additional verification of successful conjugation with minimal impact on protein integrity was demonstrated by SE-HPLC, SDS-PAGE and IEF analyses
- ELISA binding assays showed that conjugation on either cysteine or lysine residues did not impact the functional binding activity of the Cys-Dbs
- These assays provide useful tools to support both early-stage conjugation process development and cGMP manufacturing