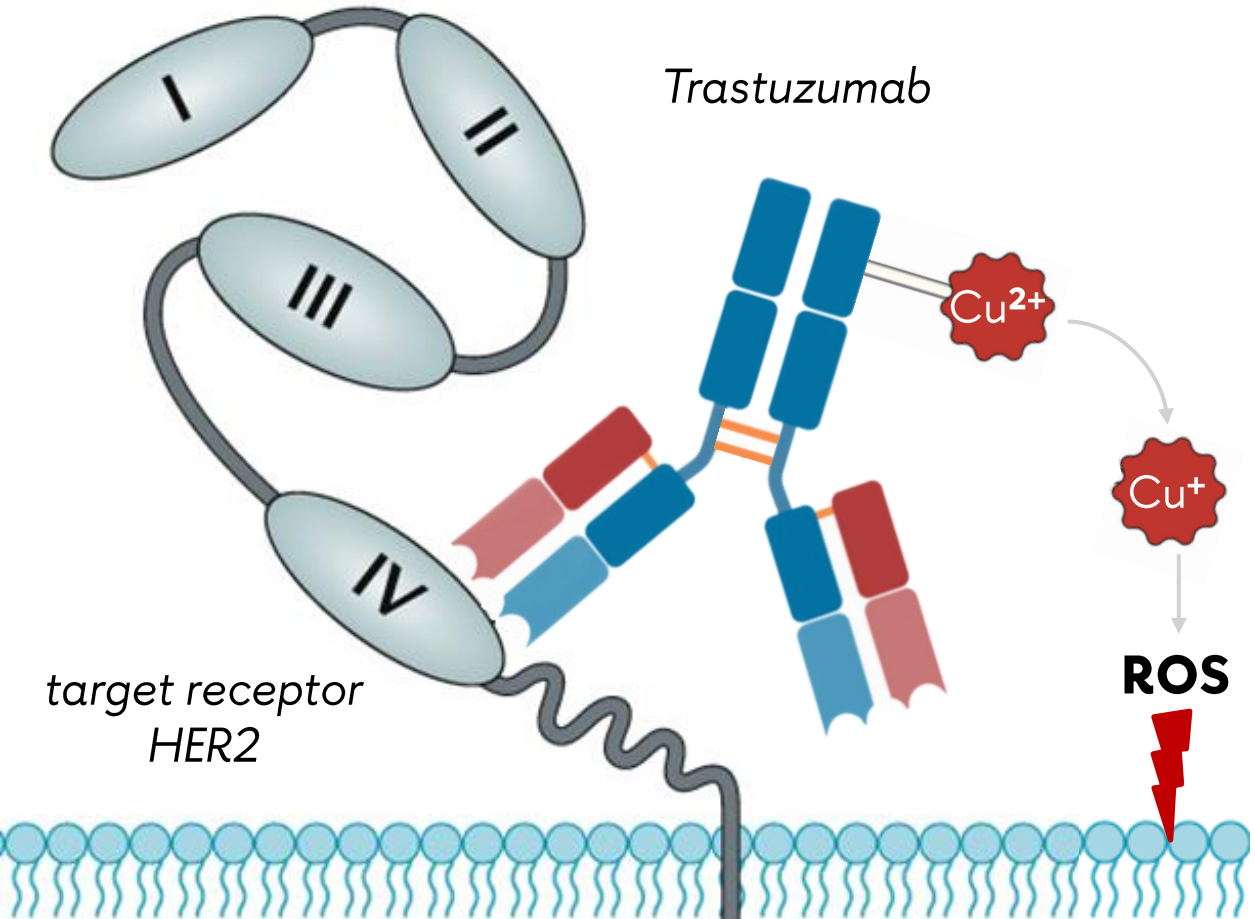
Computational design of trastuzumab conjugates with high chelator-to-antibody ratios

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XXXII Symposium on Bioinformatics
and Computer-Aided Drug Discovery

A new strategy to overcome multidrug resistance in **HER2-positive breast cancer:** immunoconjugate with copper(II)-chelators.



Aim of study:
design trastuzumab conjugate with the highest number of copper ions per antibody without worsening its physicochemical properties.

Therapeutic mechanism:

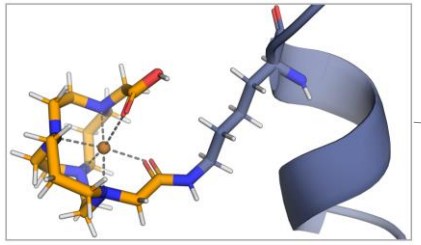
1. Cu^{2+} -> Cu^{+}, reducing agents:
   - endogenous (glutathione);
   - exogenous (N-acetylcysteine).
2. Fenton reaction:

   $Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + HO^{\cdot} + HO^{-}$

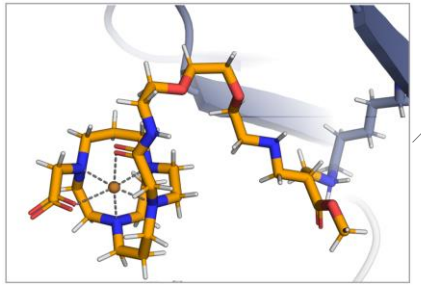
CAR (Chelator-to-Antibody Ratio)
average number of conjugated chelators per antibody.

Hypothesis:
combining site-specific conjugation methods achieves the **maximum and fixed CAR** while preserving key antibody properties.

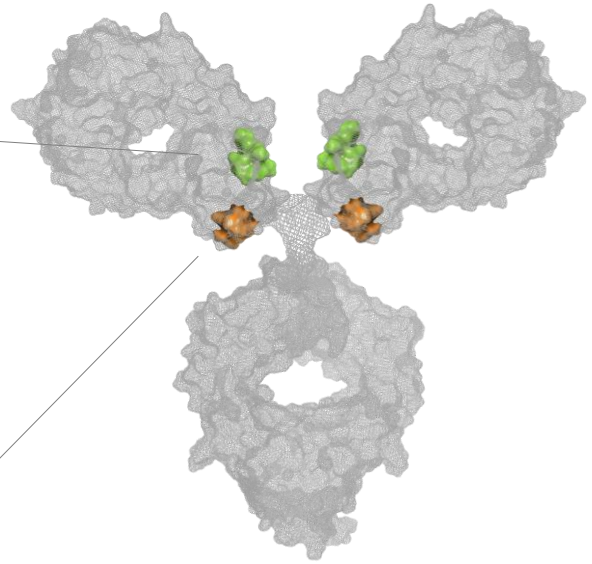
Lys188
Linker: -



Lys207
Linker: diamine-PEG2

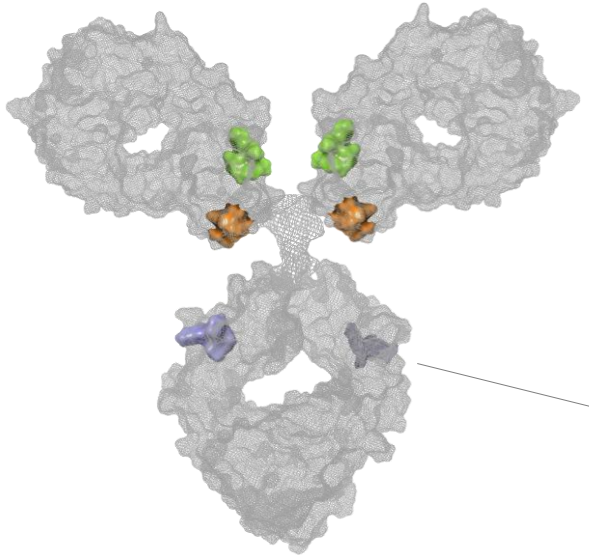


CAR **4**



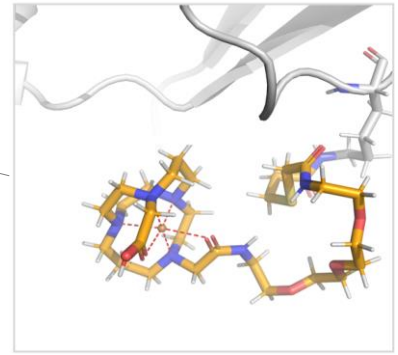
Lys188
Lys207

CAR **6**



Lys188
Lys207
Lys288

Lys288
Linker: acetamide-diamino-PEG3



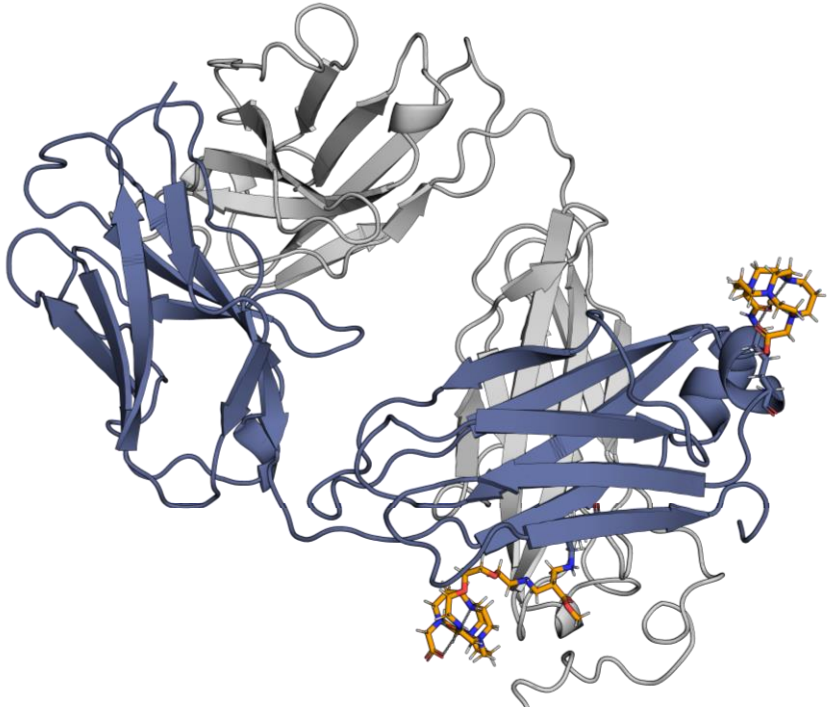
Covalent Docking
Conjugation of chelator-linker to trastuzumab

Conjugate MD
200 ns, pH 6.5 (tumor) and pH 7.4 (healthy)

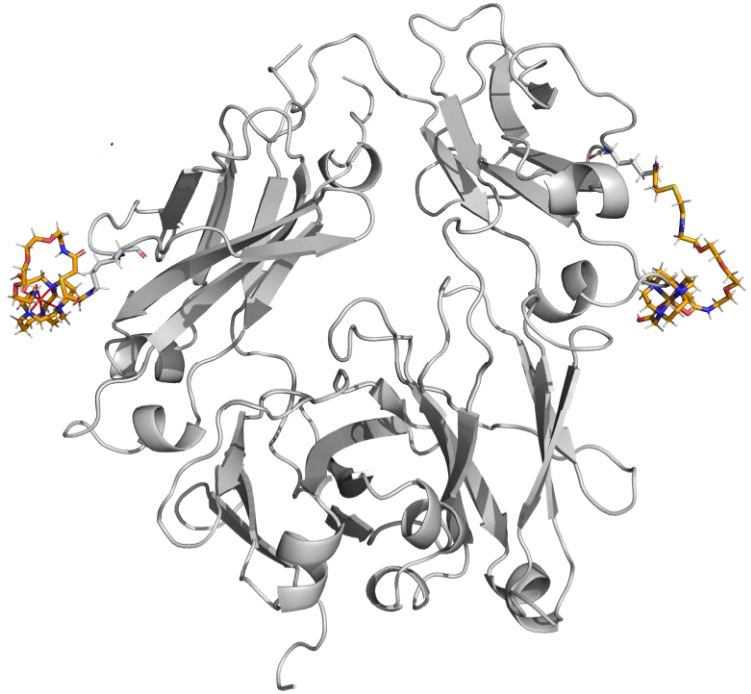
Structural Analysis
RMSD, RMSF, SSE metastable structure search via clustering

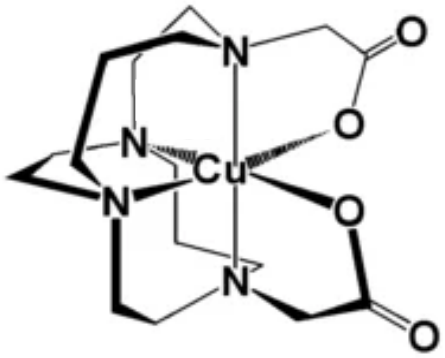
Aggregation Analysis
AggScore

Fab fragment of Trastuzumab (CAR 4 and CAR 6)



Fc fragment of Trastuzumab (CAR 6)





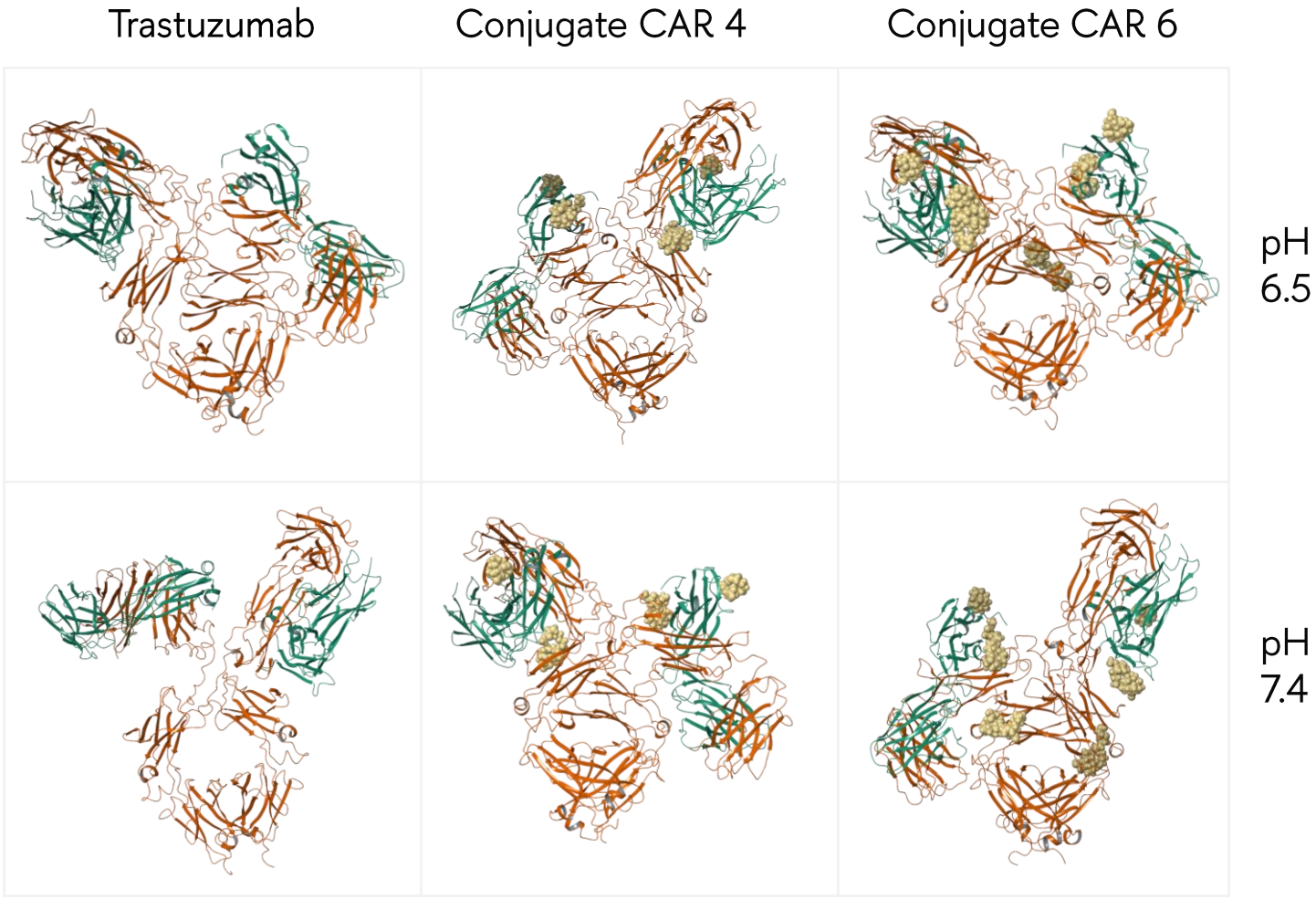
Chelator
CB-TE2A-copper(II)

Covalent Docking
Conjugation of chelator-linker to trastuzumab

Conjugate MD
200 ns, pH 6.5 (tumor) and pH 7.4 (healthy)

Structural Analysis
RMSD*, RMSF, SSE metastable structure search via clustering

Aggregation Analysis
AggScore



Compared to unmodified trastuzumab, **both conjugates** exhibit:

- identical structural stability (SSE);
- increased structural rigidity (RMSF);
- reduced overall aggregation profile (AggScore).

Conjugate CAR 6 shows locally increased aggregation potential in Fab region:

- Light chain CDRs;
- Heavy chain CH1 domain.

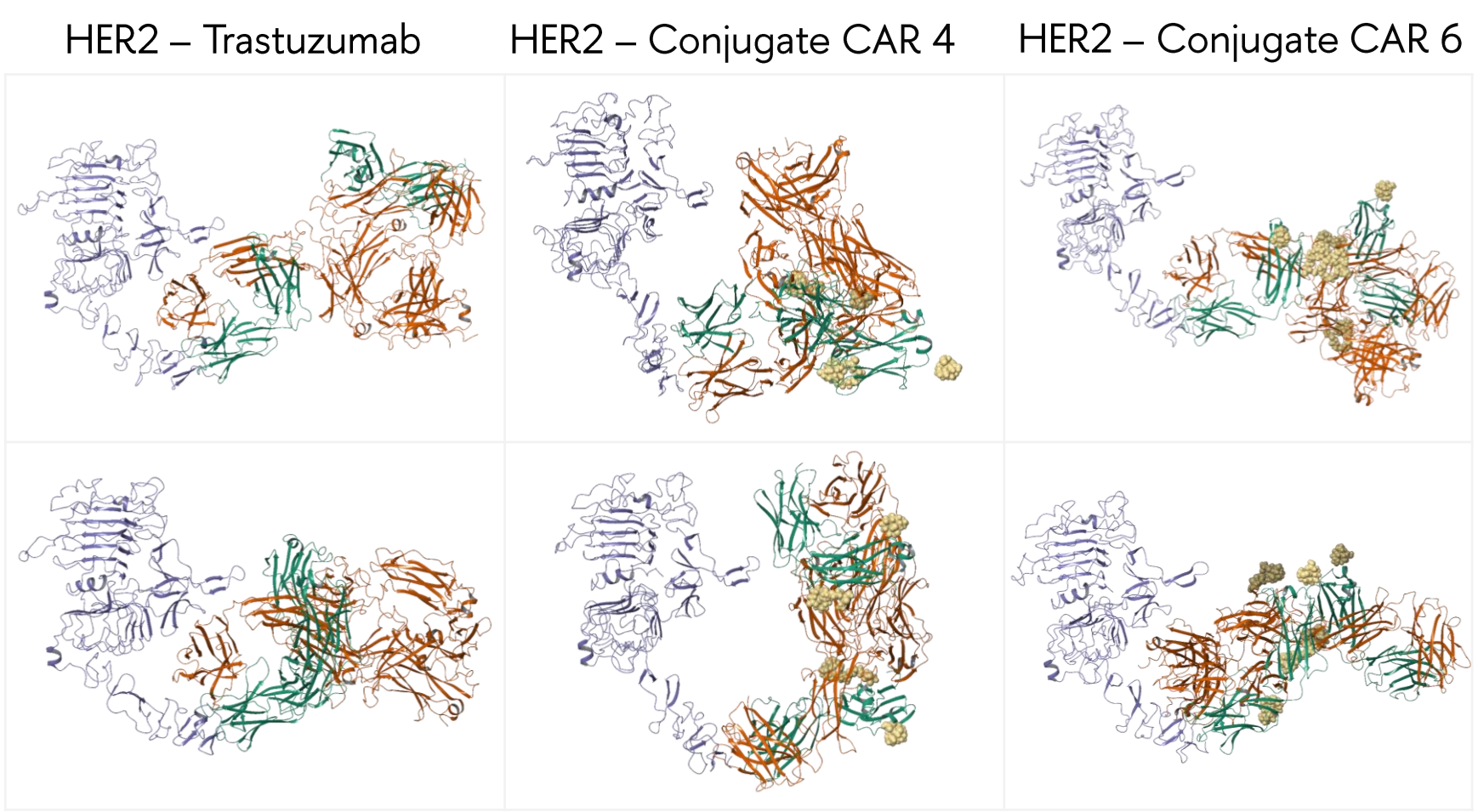
*Structural analysis was performed on equilibrated systems (RMSD plateaued).

Protein-protein docking
Conjugate–HER2,
algorithm PIPER*

Conjugate–HER2 MD
200 ns, pH 6.5 (tumor)
and pH 7.4 (healthy)

Structural Analysis
RMSD**
metastable structure
search via clustering

Binding energy
MM-GBSA



*Best poses were selected by alignment to the reference (PDB: 1N8Z; all RMSD < 2.5 Å).
**Structural analysis was performed on equilibrated systems (RMSD plateaued).

Protein-protein docking
Conjugate–HER2,
algorithm PIPER

Conjugate–HER2 MD
200 ns, pH 6.5 (tumor)
and pH 7.4 (healthy)

Structural Analysis
RMSD
metastable structure
search via clustering

Binding energy
MM-GBSA

Molecule	pH	MM-GBSA ΔG_{bind}, kcal/mol
Trastuzumab	6.5	**-108.25**
	7.4	-55.15
Conjugate CAR 4	6.5	**-169.06**
	7.4	-57.82
Conjugate CAR 6	6.5	**-150.33**
	7.4	-63.91

- Conjugation does not impair **pH-dependent receptor binding;**

pH selectivity allows the antibody to accumulate mainly in acidic tumor tissue (pH 6.5).

- **Both conjugates show more favorable binding energy to the tumor-associated HER2** than trastuzumab;
- The CAR 4 conjugate exhibits the lowest binding free energy;

Thus, increasing CAR from 4 to 6 did not improve predicted HER2 binding.

- **Both conjugates show higher pH selectivity**, suggesting wider therapeutic window.

Conclusion

- Both designs of the trastuzumab-CB-TE2A-copper(II) possess a fixed, high CAR while retaining key antibody properties:
  structural stability, rigidity, low aggregation propensity, and pH-dependent binding;
- Both **CAR 4 and CAR 6 conjugate designs are promising candidates**
  for further ***in vitro*** studies;
- **CAR 4 conjugate** is selected as the **main candidate for synthesis**.
  It uses two site-specific methods at light-chain lysines (Lys207 and Lys188) and shows the highest receptor affinity.

Acknowledgments

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- National Research University of Information Technologies, Mechanics and Optics.

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1) Valle I. et al. *Antibody-drug conjugates in breast cancer: mechanisms of. 2 resistance and future therapeutic perspectives //* NPJ Breast Cancer. – 2025. – T. 11, № 1. – C. 10;

The addition of N-acetylcysteine (NAC) enhances copper cytotoxicity:

2) **Tsymbal S. A.** et al. *Copper-Containing Nanoparticles and Organic Complexes: Metal Reduction Triggers Rapid Cell Death via Oxidative Burst //* International Journal of Molecular Sciences. – 2021. – T. 22, № 20. – C. 11065;

Conjugation at different sites affects the structural properties of the molecule:

3) Croitoru A. et al. *Harnessing computational technologies to facilitate antibody-drug conjugate development //* Nat Chem Biol. – 2025. – T. 21, № 8. – C. 1138-1147;

Site-specific lysine conjugation methods used in our study:

4) Matos M. J. et al. Chemo- and Regioselective Lysine Modification on Native Proteins // J Am Chem Soc. – 2018. – T. 140, № 11. – C. 4004-4017;
5) Pham G. H. et al. Tuning a Protein-Labeling Reaction to Achieve Highly Site Selective Lysine Conjugation // Chembiochem. – 2018. – T. 19, № 8. – C. 799-804;
6) Fujii T. et al. Yamada K. AJICAP Second Generation: Improved Chemical Site-Specific Conjugation Technology for Antibody-Drug Conjugate Production // Bioconjug Chem. – 2023. – T. 34, № 4. – C. 728-38;

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Trastuzumab structure (*in silico* model):

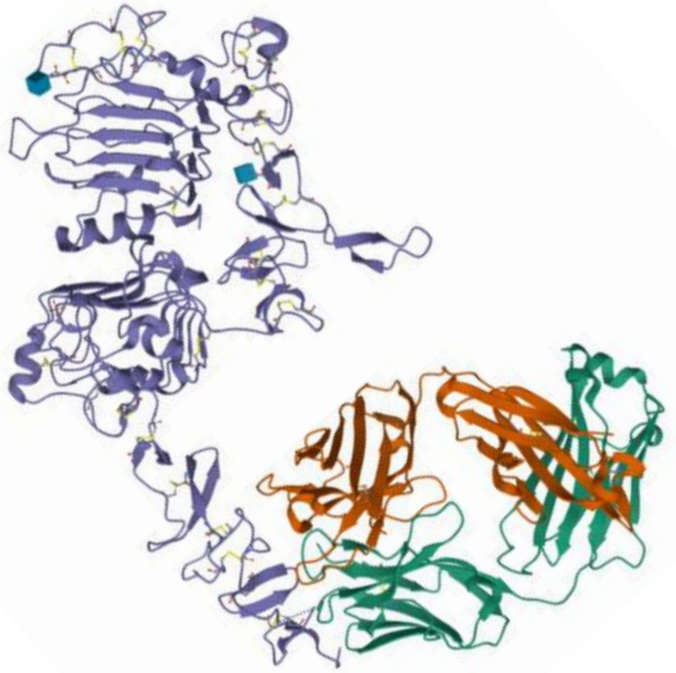
7) Brandt J. P. et al. *Construction, MD simulation, and hydrodynamic validation of an all-atom model of a monoclonal IgG antibody //* Biophys J. – 2010. – T. 99, № 3. – C. 905-13;

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8) PDB Entry 1N8Z // RCSB PDB. — URL: https://www.rcsb.org/structure/1N8Z;
9) Wong E. H. et al. *Synthesis and Characterization of Cross-Bridged Cyclams and Pendant-Armed Derivatives and Structural Studies of Their Copper(II) Complexes //* Journal of the American Chemical Society. – 2000. – T. 122, № 43. – C. 10561-10572;

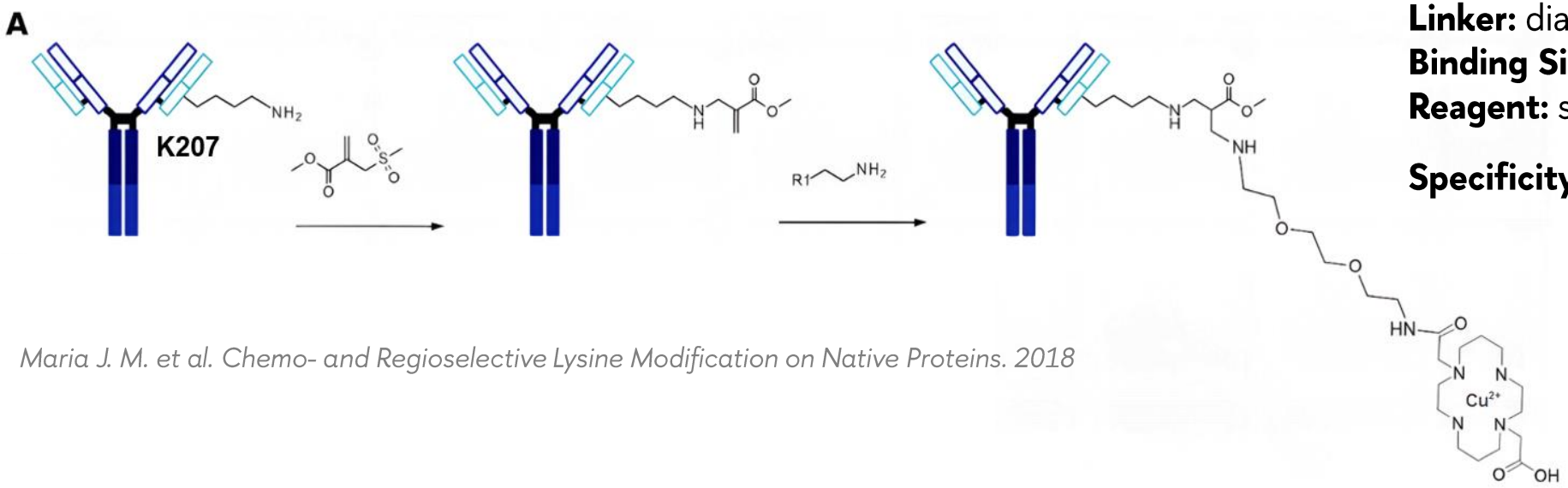
Aggregation profile prediction tool:

10) Sankar K. et al. AggScore: Prediction of aggregation-prone regions in proteins based on the distribution of surface patches // Proteins. – 2018. – T. 86, № 11. – C. 1147-1156.



X-ray structure of the complex of the trastuzumab Fab fragment with the HER2 receptor **(PDB: 1N8Z)**

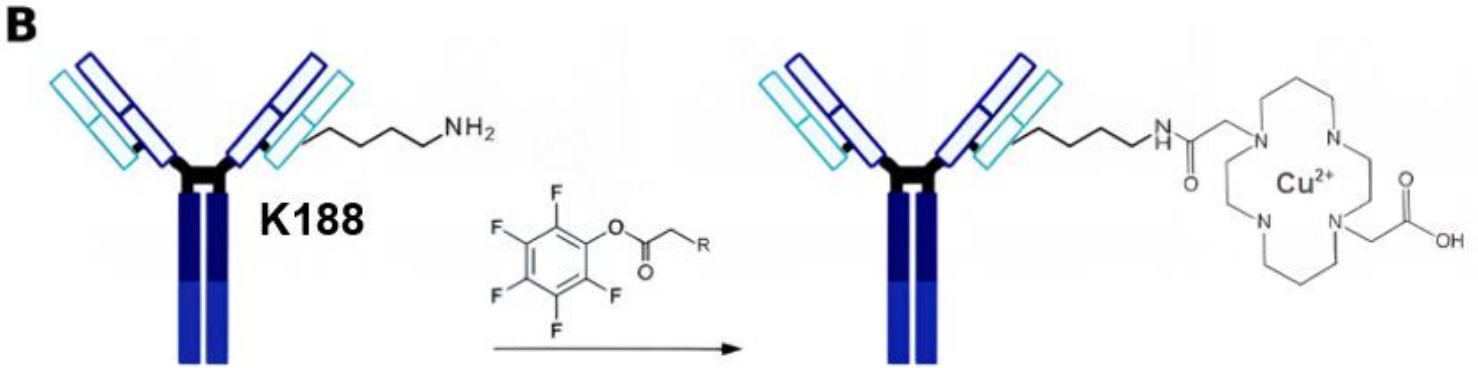
Site-specific lysine conjugation methods



Maria J. M. et al. Chemo- and Regioselective Lysine Modification on Native Proteins. 2018

Linker: diamine-PEG2
Binding Site: light chain Lys207
Reagent: sulfonyl acrylate

Specificity mechanism: lowest pKa at pH = 8

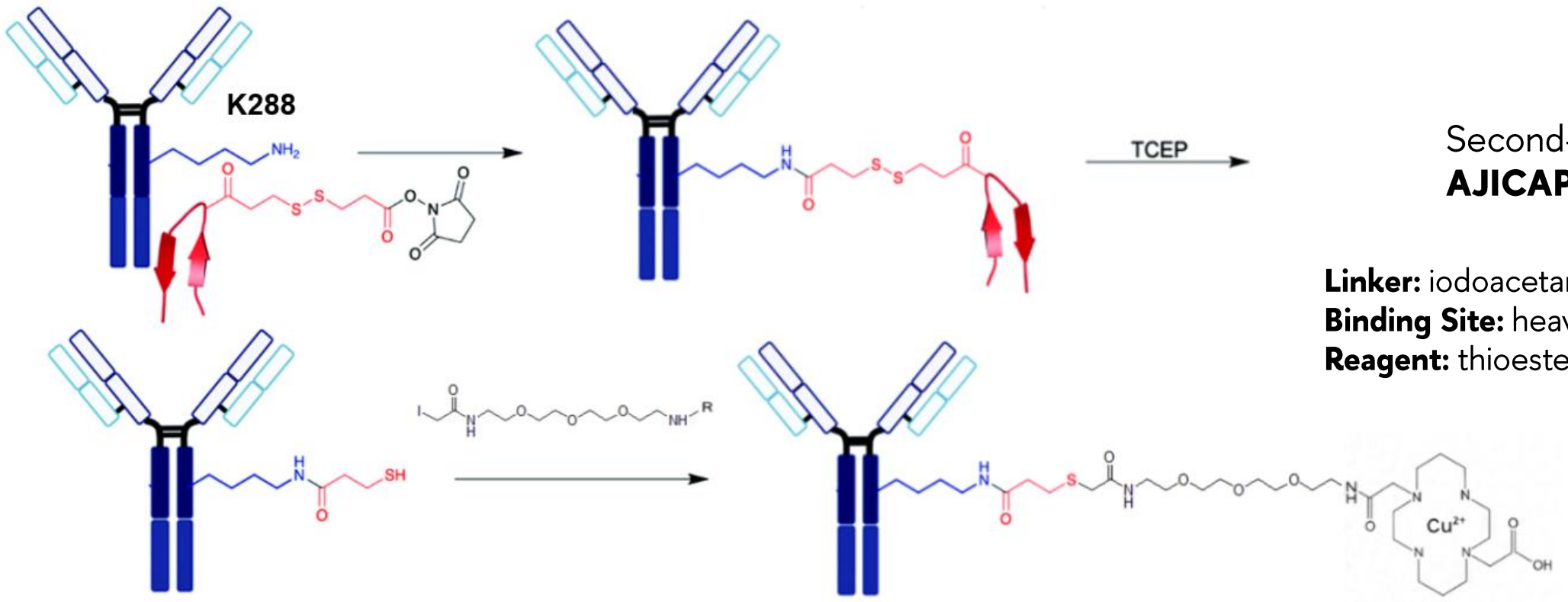


Grace H. P. et al. Tuning a Protein Labeling Reaction to Achieve Highly Site Selective Lysine Conjugation. 2018

Linker: -
Binding Site: light chain Lys188
Reagent: pentafluorophenyl ester (PFP)

Specificity mechanism: microenvironment of Lys188 (defined by Asp151 and His189 residues)

Site-specific lysine conjugation methods



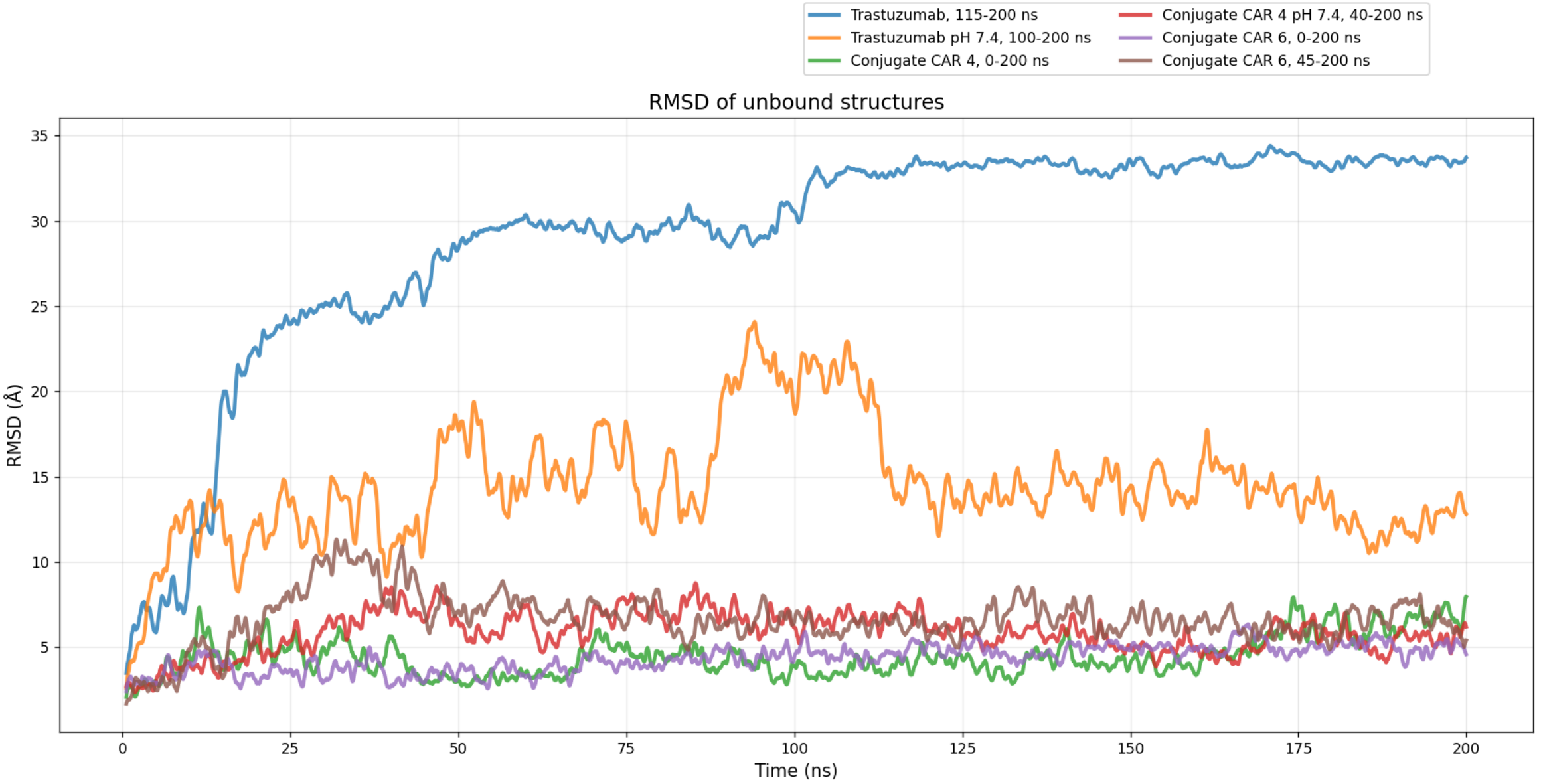
Second-Generation
AJICAP Technology

Linker: iodoacetamide-diamino-PEG3
Binding Site: heavy chain Lys288
Reagent: thioester

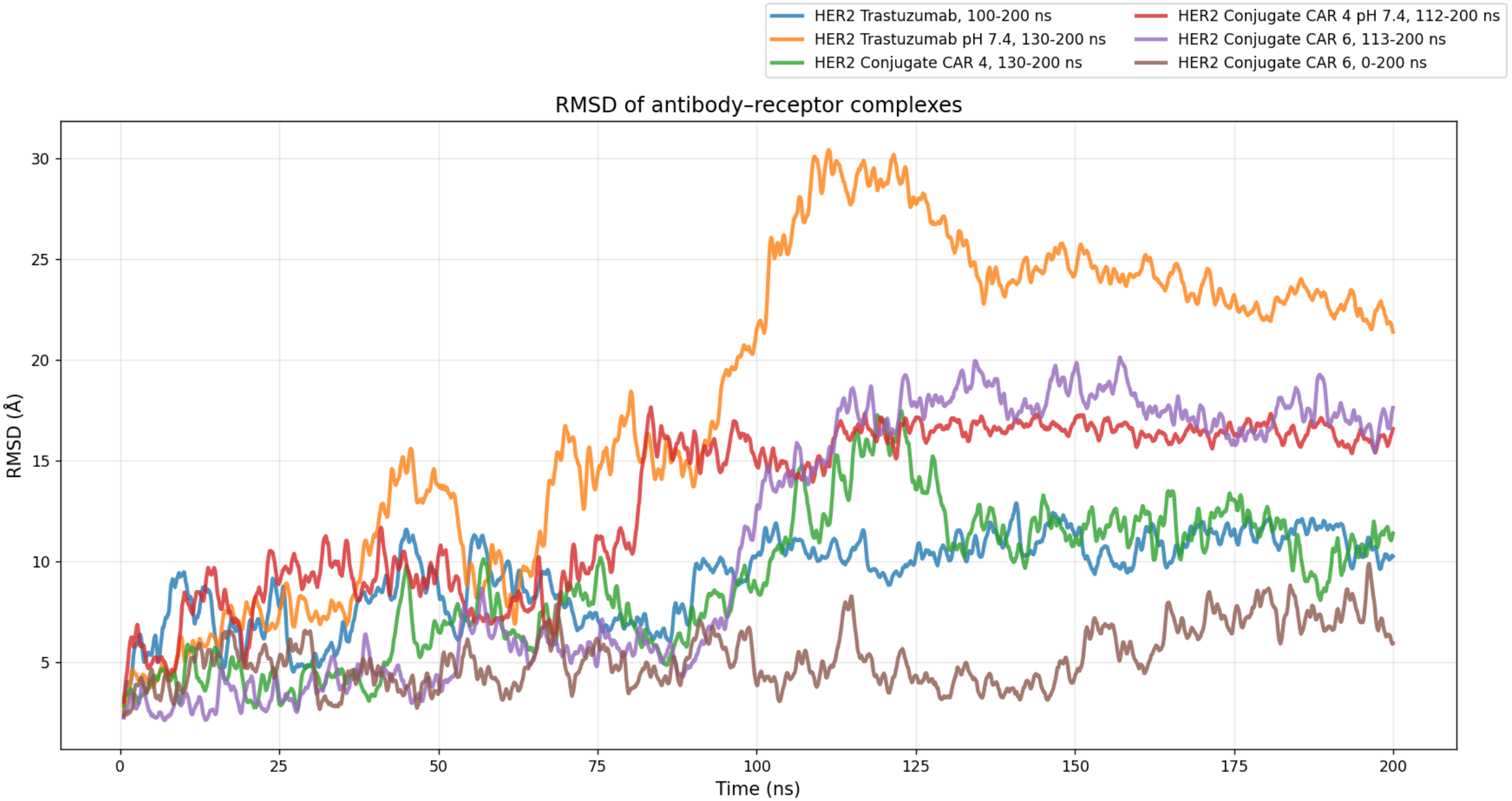
Specificity mechanism: Fc-affinity peptide directs covalent binding to Lys288 via a thioester group; after peptide cleavage, the linker-chelator attaches to the remaining thiol group at Lys288.

Fujii T. et al. AJICAP Second Generation: Improved Chemical Site-Specific Conjugation Technology for Antibody–Drug Conjugate Production 2022

Root-mean-square deviation (RMSD)



Root-mean-square deviation (RMSD)

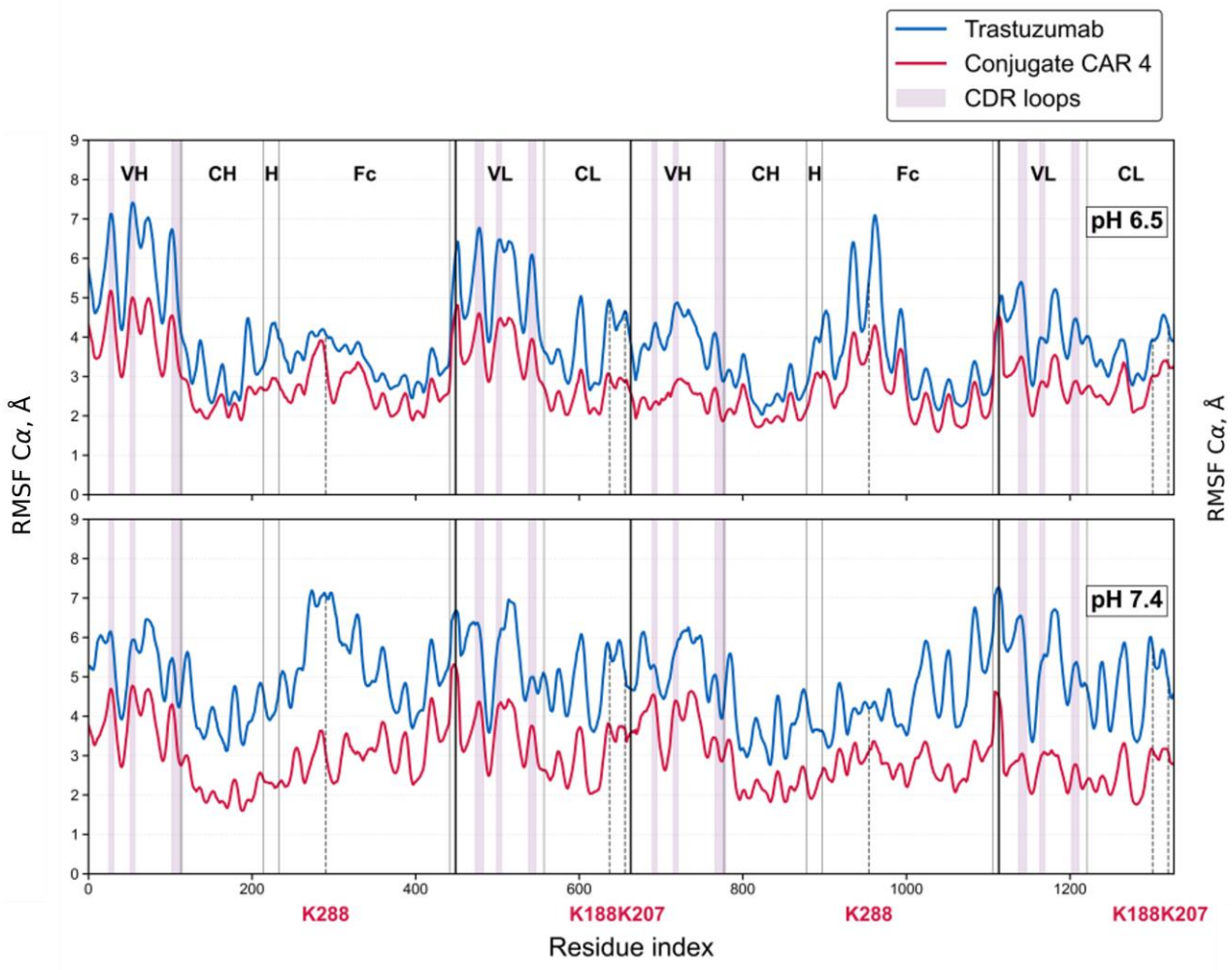


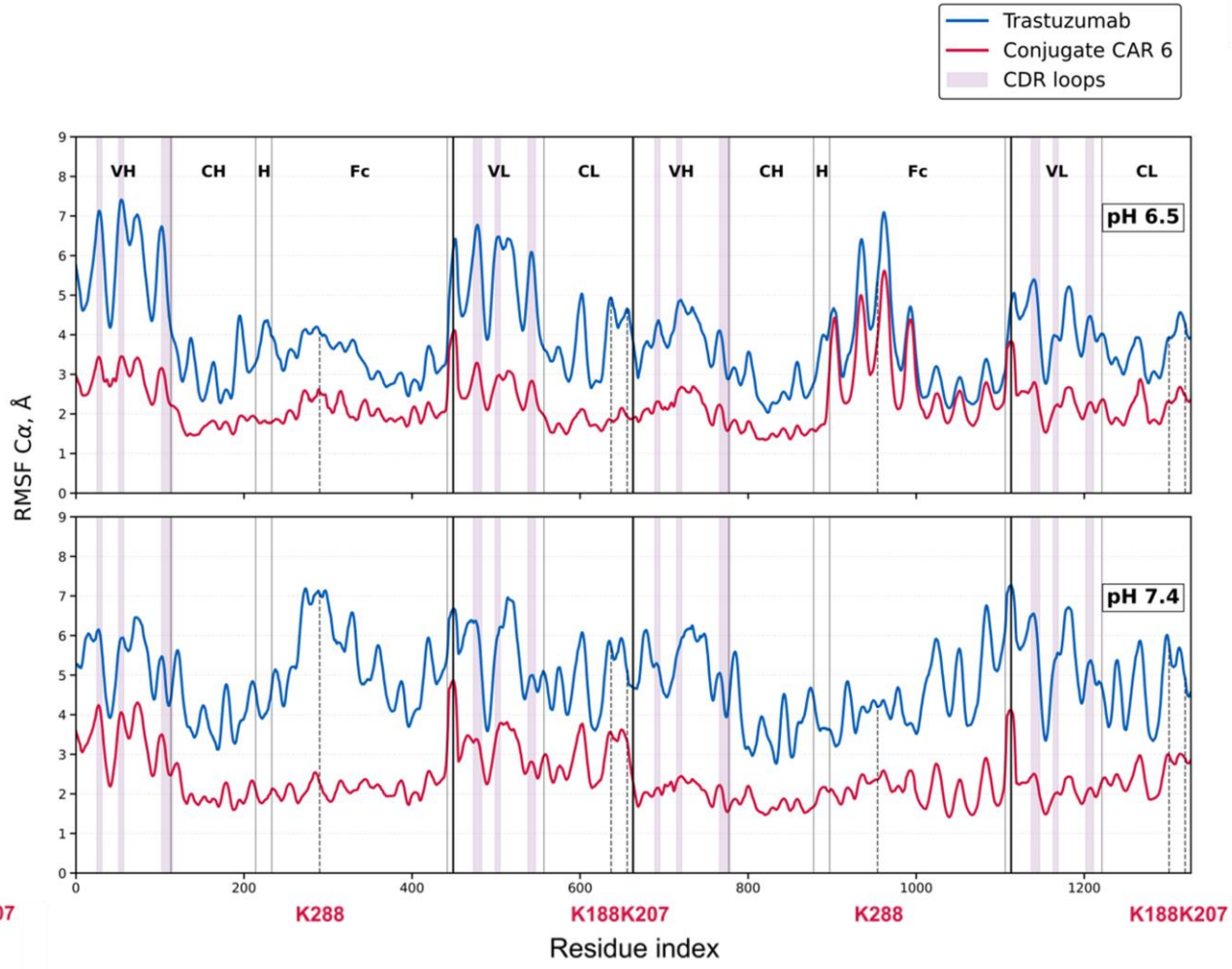
Secondary Structure Content

Proportions of α-helices and β-strands (% of total antibody structure) averaged over the all MD-trajectory.

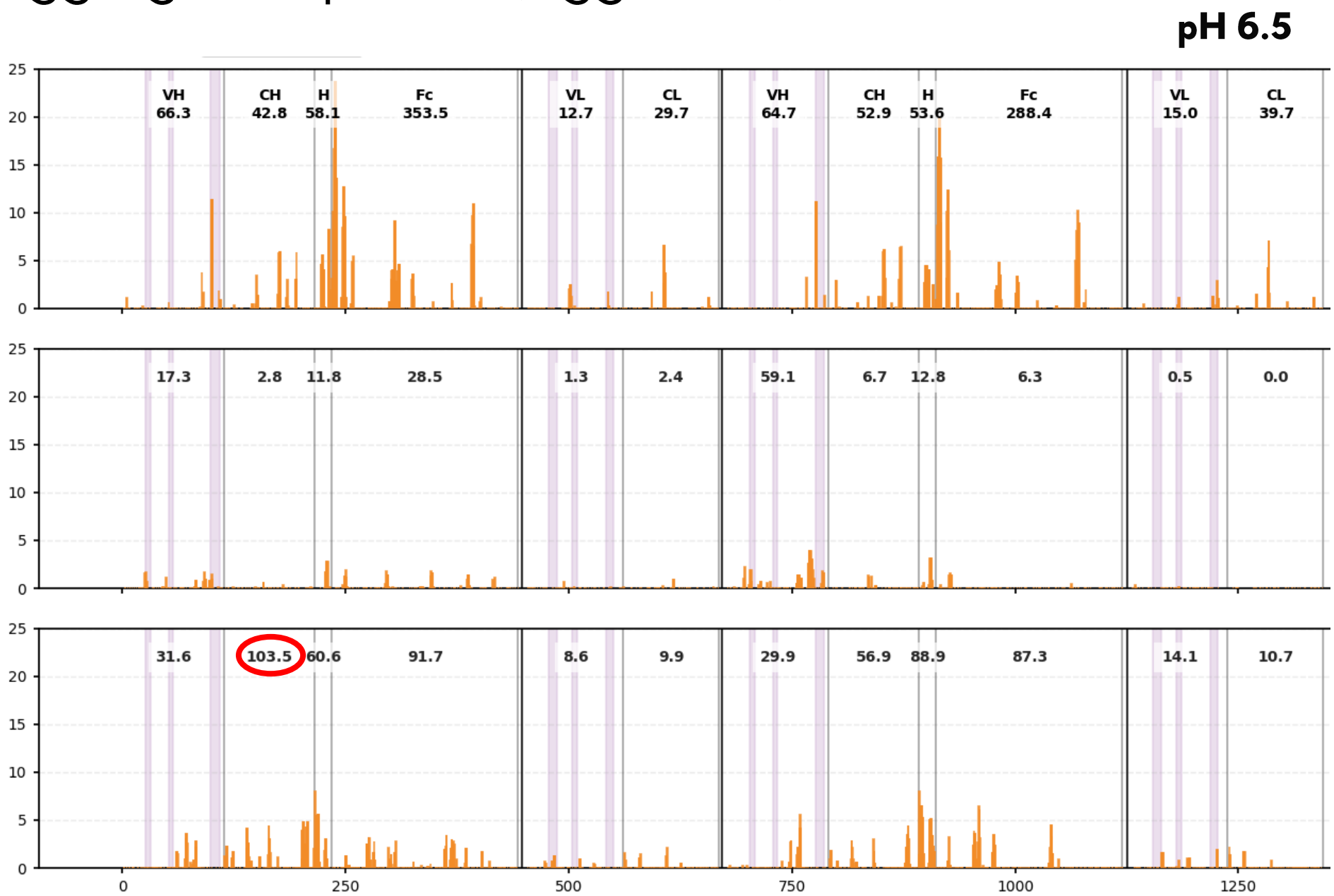
Molecule	pH	α-helices (%)	β-strands (%)	Total (%)
Trastuzumab	6.5	2.69	44.66	**47.35**
	7.4	3.11	44.12	**47.23**
Conjugate CAR 4	6.5	3.08	44.16	**47.24**
	7.4	2.92	44.97	**47.90**
Conjugate CAR 6	6.5	3.14	44.60	**47.74**
	7.4	3.15	44.09	**47.24**

Root-mean-square fluctuation (RMSF)





Aggregation profile (AggScore)

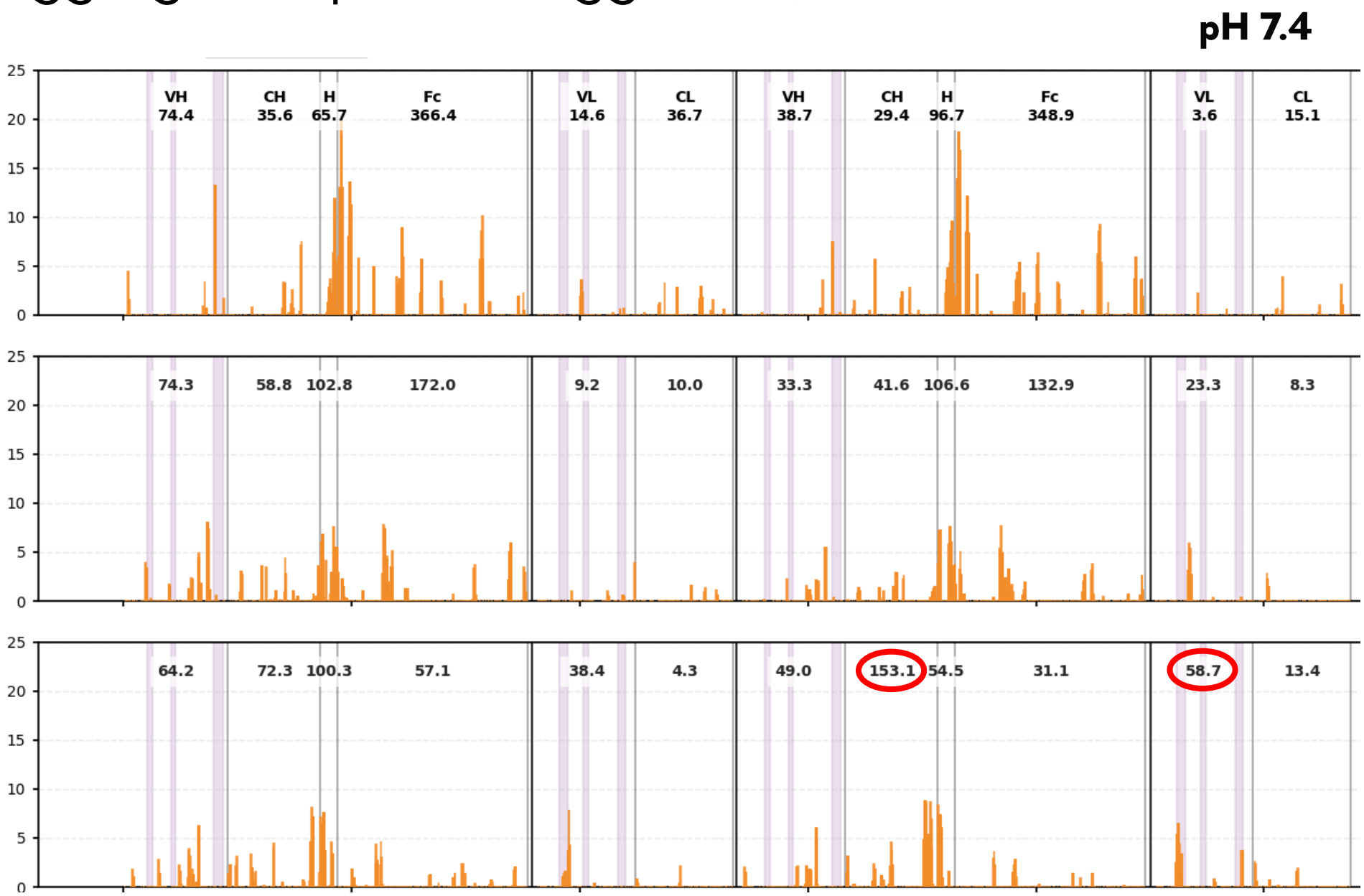


Trastuzumab

Conjugate CAR 4

Conjugate CAR 6

Aggregation profile (AggScore)



Trastuzumab

Conjugate CAR 4

Conjugate CAR 6

Binding free energy components

Molecule	pH	MM-GBSA ΔG_{bind}, kcal/mol	Coulumb, kcal/mol	Covalent, kcal/mol	H_{bond}, kcal/mol	Lipo, kcal/mol	π-π stacking, kcal/mol	SelfCont, kcal/mol	$Solv_{GV}$, kcal/mol	vdW, kcal/mol
Trastuzumab	6.5	**-108.25**	-69.19	+16.75	-4.43	-29.68	-3.41	+0.75	+59.59	-78.63
	7.4	**-55.15**	+1.45	-72.73	+5.01	-12.56	-2.49	-0.05	+103.37	-77.15
Conjugate CAR 4	6.5	**-169.06**	-14.21	+1.09	-12.22	-43.61	-6.29	+1.87	+6.68	-102.35
	7.4	**-57.82**	-21.88	+45.20	-7.46	-33.4	-6.31	-3.65	+48.68	-79.0
Conjugate CAR 6	6.5	**-150.33**	-110.57	-11.07	-9.62	-24.89	-2.66	+0.90	+73.87	-66.29
	7.4	**-63.91**	+169.99	-51.82	+3.35	-10.68	+0.85	-0.14	-45.86	-129.59