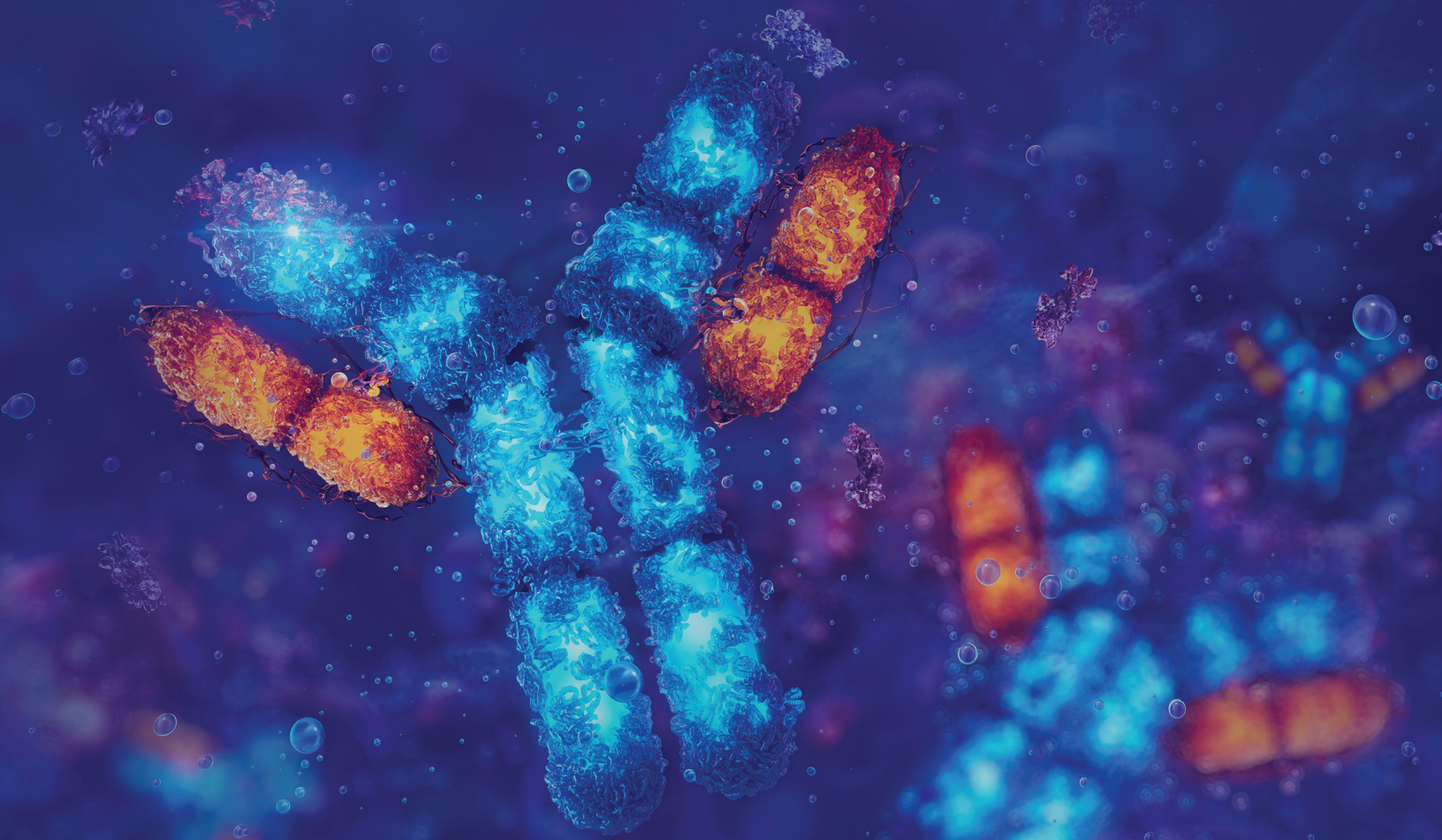
aragen

Whitepaper

PROTAC-BASED DEGRADER ANTIBODY CONJUGATES: COMBINING PROTACS WITH MONOCLONAL ANTIBODIES FOR PRECISION THERAPY



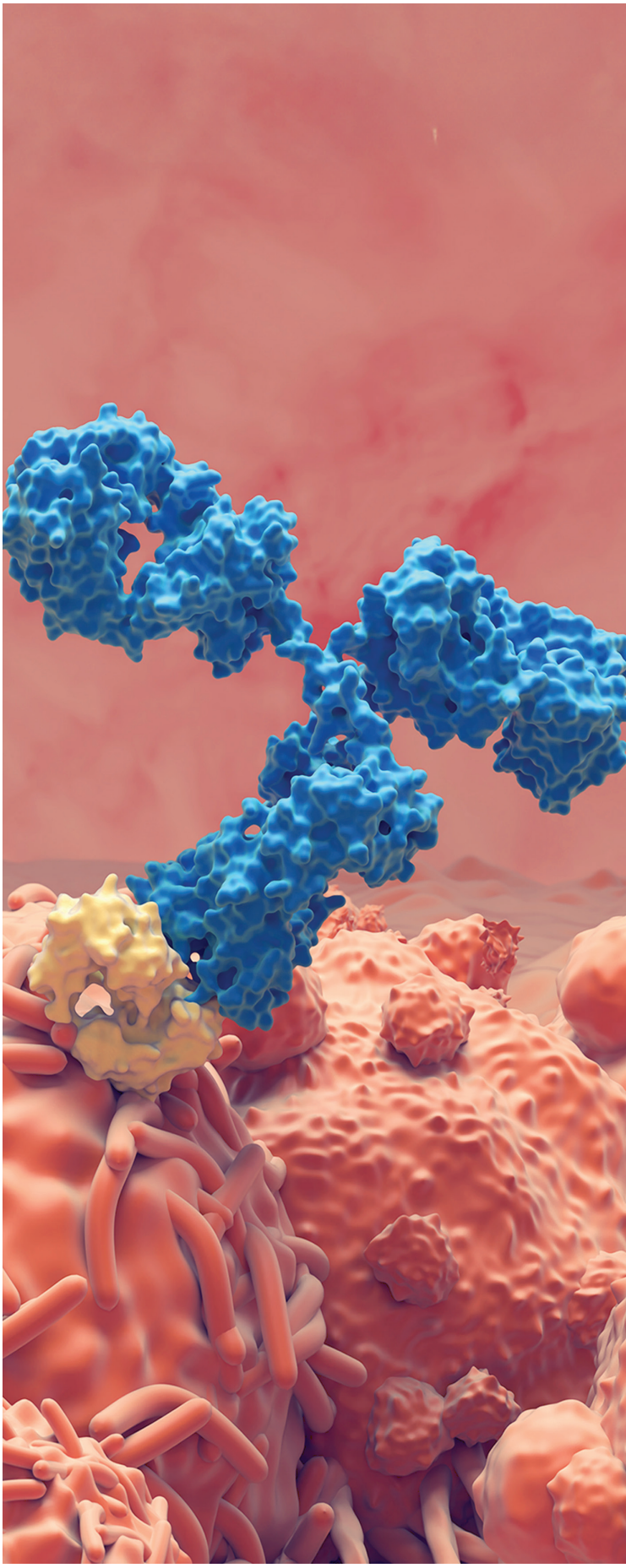
In every molecule is the possibility for better health.

Executive Summary

Degrader-Antibody Conjugates (DACs) are novel class of therapeutics designed for selective protein degradation inside target cells. Among these, PROTAC-based DACs stand out for their potent and selective protein degradation. The PROTAC-based DACs combine a target-specific monoclonal antibody (mAb) with a PROteolysis-TArgeting Chimeras (PROTACs) or molecular glues via a linker. This approach offers advantages over traditional PROTACs, including improved physiochemical properties, solubility, permeability, bioavailability, and target tissue specificity.

The development of PROTAC-based DACs requires careful selection of tissue target- and target-specific antigens, antibody generation, design of target protein-specific PROTAC molecules, appropriate linker selection, precise conjugation technologies and in-house purification processes. Of these steps, conjugation of the PROTAC to the antibody—typically using cysteine-based methods—followed by thorough purification, is essential for developing high-quality PROTAC-based DACs.

This whitepaper presents a case study showcasing Aragen's expertise in PROTAC-antibody conjugation using a cysteine-based strategy, demonstrating our capability to deliver robust, high-quality PROTAC-based DACs.



Overview: The Need for Degrader Antibody Conjugates

Targeted protein degradation (TPD) strategies including PROTACs, LYTACs, molecular glues etc.—are redefining drug discovery by enabling the elimination, rather than merely inhibition of disease-driving proteins. PROTACs as innovative bifunctional molecules, are at the forefront, allowing therapeutic targeting of previously "undruggable" proteins by hijacking the cell's ubiquitin-proteasome system. Since their landmark introduction in 2001 by Craig Crews and Raymond Deshaies, PROTACs have made significant advances, reaching clinical trials (Phase 1-3) against a range of challenging targets. However, most PROTACs fall outside the *"beyond Rule of 5"* space, limiting their solubility, permeability, and oral bioavailability.

The emergence of Antibody Drug Conjugates (ADCs) has also revolutionized drug development. Since the first USFDA approval of an ADC (Mylotarg in 2001, for AML), over 15 ADCs have received approvals for various indications. While ADCs have achieved selective and tissue-specific delivery of cytotoxic payloads, challenges such as drug resistance, off-target toxicity (due to premature payload release), and other side effects persist.

Building on the success of both the modalities, PROTAC-based DACs represent a promising evolution in TPD and targeted therapy. They integrate an antibody's antigen-binding specificity and prolonged half-life with PROTAC-mediated protein degradation activity. This synergy enhances therapeutic index, tissue specificity, and pharmacokinetic/pharmacodynamic (PK/PD) profiles, offering a promising platform for precision medicine across oncology, autoimmune, and neurodegenerative diseases. Successful PROTAC-based DAC development requires a careful selection of target antigens and antibodies, design of selective PROTACs, optimized linker chemistry, and robust conjugation and appropriate purification workflows.

PROTAC-based DAC Design and Mechanism of Action

The design of PROTAC-based DAC hinges on the combination of antibody-mediated targeting of specific tissues and cell types along with the intracellular protein degradation facilitated by the degrader PROTAC payload. Each component must be meticulously engineered to maintain conjugate stability, specificity, and potency. A typical PROTAC-based DAC consists of three essential components (Figure 1):

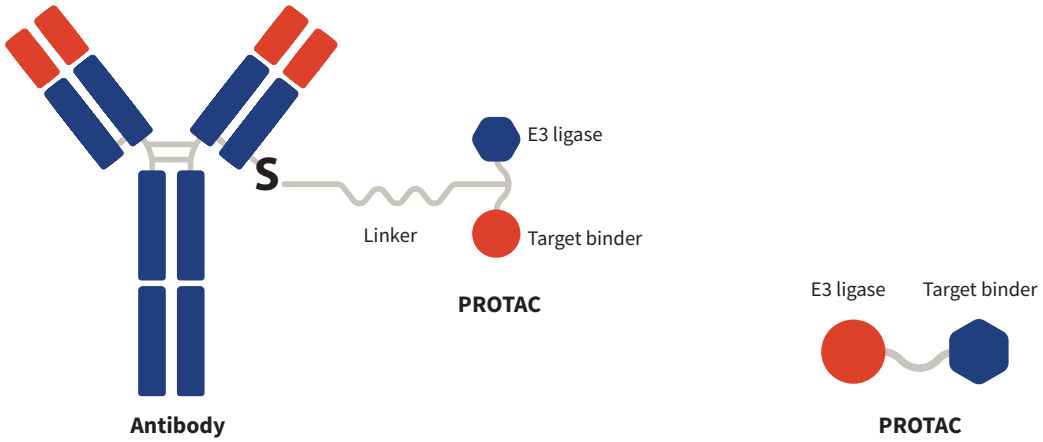


Figure 1: Typical design of a PROTAC-based Degrader Antibody Conjugate (DAC) and PROTAC components (E3 ligase, target binder and linker).

- **Monoclonal or Bispecific Antibody:** Targets and binds specific cell surface antigens (e.g., HER2, CD33), ensuring precise delivery. Antibody selection is guided by disease-specific target, ability of the target antigen (protein) to undergo internalization, and antibody's binding affinity to the antigen.
- **PROTAC Payload:** It is a bifunctional molecule linking a ligand for the target protein and E3-ubiquitin ligase complex (e.g., VHL, CRBN, MDM2, IAP etc.) via a linker/spacer. PROTAC recruits target protein to an E3-ubiquitin ligase complex, triggering poly-ubiquitination of the protein, followed by its proteasomal degradation. Selection of PROTAC for DAC development depends on its potency and selectivity.
- **Chemical linker:** Connects the antibody and the PROTAC payload and can be cleavable (enzyme or pH-triggered) or non-cleavable. Cleavable linkers facilitate efficient PROTAC payload release but are at risk of premature cleavage in blood circulation and consequent toxicity. Non--cleavable linker releases the PROTAC payload upon antibody degradation and remains attached to the payload. It augments DAC's in vivo stability but may slow payload release kinetics due to its dependency on complete antibody degradation. Chemical linker design depends on the conjugation strategy like cysteine, lysine and/or click chemistry. This strategy determines the chemical groups at each end of the linker, which affects how efficiently the conjugation occurs.

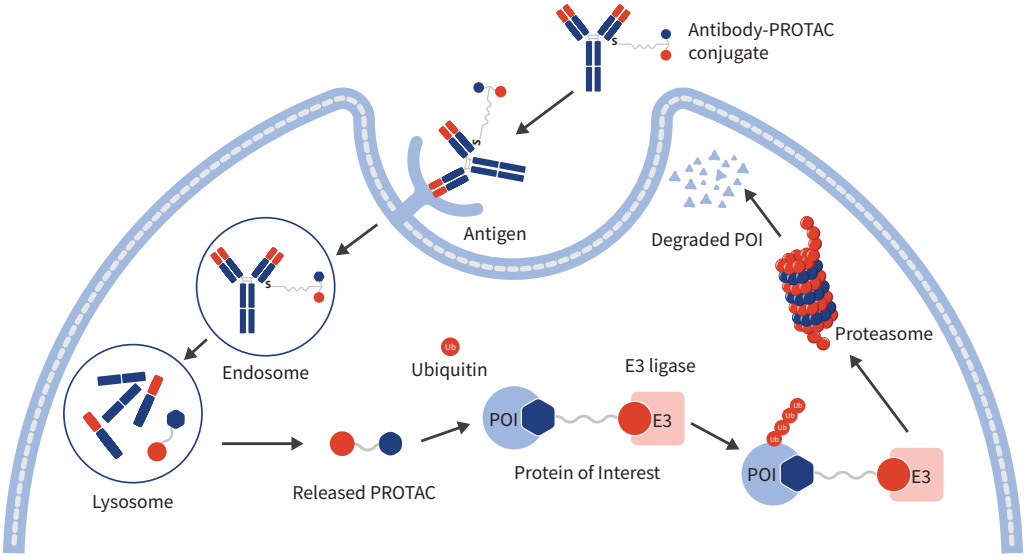
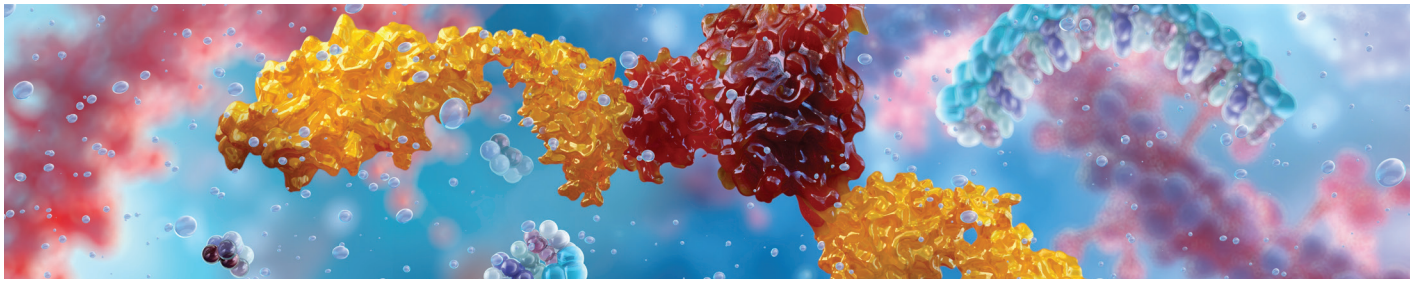


Figure 2: Mechanism of action of PROTAC-based Degrader Antibody Conjugate (DAC).

Mechanistically, the antibody component of PROTAC-based DAC docks on the overexpressing antigen of the target cells. Thereafter, PROTAC-based DAC-antigen complex gets internalized into the cell via receptor-mediated endocytosis to form an endosome. The endosome inside the cell is trafficked to lysosome where antibody and linker is cleaved leading to release of PROTAC payload in cytosol. Upon release, PROTAC forms ternary complex with target protein and E3-ubiquitin ligase, facilitating polyubiquitination and proteasomal degradation of the protein. This mechanism achieves potent intracellular protein clearance while minimizing systemic exposure and off-target toxicity (Figure 2).

PROTAC-based DAC Development Considerations

- **Conjugation Chemistry:**
  Conjugation can be random (often targeting cysteine or lysine) and site-specific (using engineered antibodies, e.g., THIOMAB or enzyme-based methods). Conjugation chemistry directly determines Drug-to-Antibody Ratio (DAR), affecting PK, efficacy, and safety. Once the right conjugation chemistry is identified, optimizing conjugation reaction conditions (buffer composition, molar ratios, temperature, duration) is crucial for achieving the desired DAR while maintaining antibody integrity.
- **Drug-to-Antibody Ratio:**
  DAR represents the average number of payload molecules (PROTACs) attached per antibody. It directly influences efficacy, PK, and safety of the DAC. The DAR depends on the conjugation chemistry and the reaction conditions. Since most payloads are hydrophobic, a high DAR can induce antibody aggregation and impair DAC functionality. Conversely, a low DAR may reduce the amount of therapeutic payload delivered, lowering potency. Therefore, achieving an optimal and homogeneous DAR is crucial for successful DAC development. Post-conjugation, the DAC is purified to eliminate any unconjugated payload, and then DAR is measured using techniques by RP-HPLC, LC-MS, or UV-Vis spectrophotometry.
- **Post-conjugation Purification:**
  Post-conjugation, purification removes free payloads, aggregates, unconjugated antibodies, and reaction byproducts, reducing off-target toxicity by free payloads and ensuring DAC quality. Common purification methods include affinity chromatography, Gel Filtration Chromatography (GFC), Hydrophobic Interaction Chromatography (HIC) or RP-HPLC, buffer exchange and tangential flow filtration.
- **PROTAC-based DAC Characterization:**
  Following purification, characterization of PROTAC-based DACs is essential to determine key properties like DAR, purity, percentage aggregation level and structural integrity. These assessments are crucial for meeting regulatory requirements and accelerating clinical translation. The most common DACs characterization approaches are:
  - **Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE):** Evaluates molecular integrity, conjugate formation, and purity.
  - **Size Exclusion Chromatography-HPLC (SEC-HPLC):** Assesses purity and aggregation levels.
  - **Liquid Chromatography-Mass Spectrometry (LC-MS):** Determines DAR, identifies conjugation sites and quantifies free payload.
  - **Reverse Phase-HPLC (RP-HPLC):** Analyzes DAR, assesses DAR heterogeneity, and quantifies free payload.
  - **SPR Binding Assay:** Determines antibody–antigen binding and payload–protein of interest interactions.
  - ***In-vitro/vivo* Studies:** Characterizes functional activity and biological performance.



Case Study: Developing PROTAC-based DACs With Optimal DAR–Challenges and Solutions

Challenge

The development of PROTAC-based DACs is a complex technical challenge that lies at the intersection of chemistry, process engineering, and analytical precision. Achieving a defined and reproducible DAR in PROTAC-based DACs remains a critical challenge in bioconjugation chemistry. It requires meticulous optimization of conjugation parameters, significant resource investments, and robust purification workflows. Furthermore, implementation of suitable purification strategies is essential to eliminate free payloads, aggregates, and reaction by-products to significantly improve the product quality and therapeutic efficacy.

Aragen's Approach

At Aragen, we utilized our expertise in protein bioconjugation to develop and characterize PROTAC-based DACs. The process includes screening of various linkers, fine-tuning of conjugation reaction conditions, and developing post-conjugation purification strategies. Our standard experimental workflow is outlined below:

- **Antibody Functionalization:** The antibody is prepared for conjugation, which involves buffer exchange and/or treatment with a reducing agent.
- **Bioconjugation:** The antibody is conjugated with a linker and PROTAC molecule.
- **Purification:** Upon completion of the conjugation reaction, the mixture undergoes purification using GFC or affinity chromatography (MabSelect SuRe), followed by buffer exchange using desalting columns or ultrafiltration/diafiltration (UF/DF) methods.
- **QC and DAR Determination:** Analytical techniques such as SDS-PAGE, SEC-HPLC and mass analysis using LC-MS are employed. DAR values are calculated from LC-MS data.

Using this approach, we developed a method, where PROTAC was conjugated to mAb using cysteine-directed conjugation chemistry. The antibody was first buffered into BBS buffer, followed by reduction with 6 molar equivalents of TCEP and incubation at 37°C for 2 hours. After reduction, the temperature was lowered to 20°C, then 8 molar equivalents of a maleimide-based linker (NGM-BCN) were added and incubated at 20°C for 10 min to conjugate the linker to reduced cysteine residues. A subsequent buffer exchange using UF/DF with acetate buffer removed unconjugated linker. Then, 20 molar equivalents of azido-PROTAC (solubilized in DMF) were added and click chemistry-based conjugation was performed by incubating the mixture at 22°C for 4 hours. Figure 3 illustrates the systematic workflow implemented in this case study.

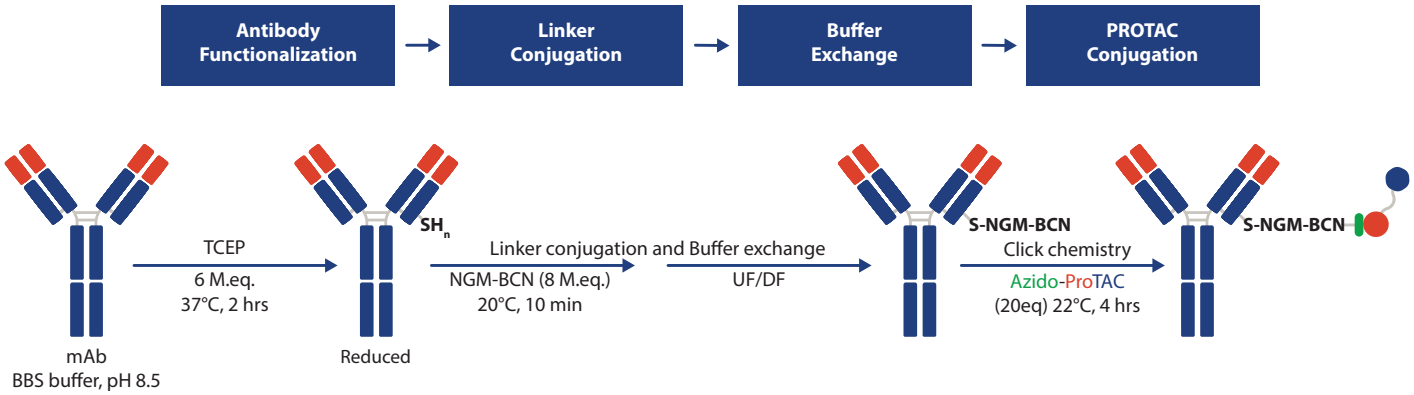


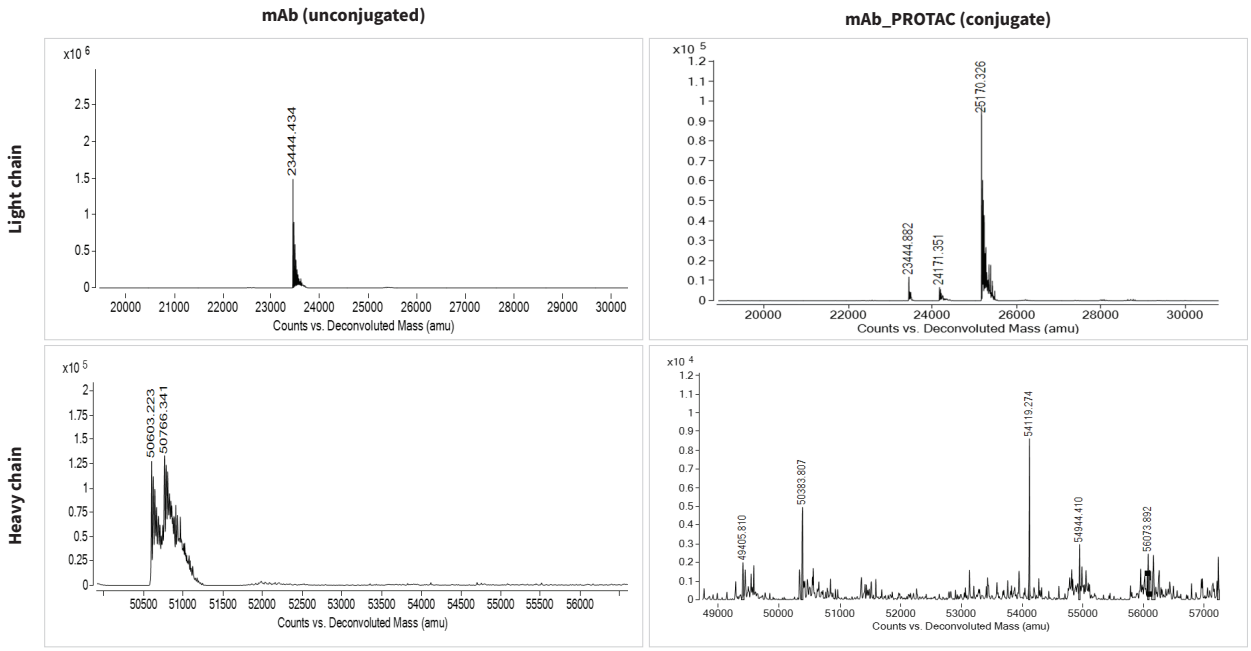
Figure 3: Monoclonal antibody (mAb)-PROTAC conjugation strategy.

The purified PROTAC-based DAC was analyzed by SDS-PAGE, SEC-HPLC and LC-MS. The LC-MS data was used to calculate DAR by determining the number of payloads attached to heavy and light chains based on mass differences before and after conjugation. The percentage area for each species were calculated from mass spectra data (Figure 4). The following formula was used to calculate the average DAR:

$$\text{Average DAR} = \frac{(P1.A1+P2.A2+...+Pn.An)2}{100}$$

Here, "P" represents the number of payloads per chain and "A" denotes the corresponding percentage peak area, and 1, 2, ...n represents different heavy and light chain species post-conjugation.

A



B

mAb Chain	Glycan	Observed Reduced Mass (Da)		Mass difference (AM in Da)	No. of Payload per chain (P)	Mass Peak percentage Areas (A)	DAR
		mAb Pre-conjugation	mAb-NGM-BCN PROTAC Post-conjugation				
Heavy chain	GO	50383	50384	1	0	28	5.07
	GOF	50603	54119	3516	2	30	
	GIF	50765	54994	4229*	2	12	
	G2F	50930	56074	5144	3	11	
	G2F	50930	56162	5232	3	19	
Light chain		23444	23445	1	0	15	
			24171	727*	0	6	
			25170	1726	1	79	

Figure 4: MS analysis of monoclonal antibody (mAb) and Degrader Antibody conjugate (mAb-PROTAC) **(A)** and average DAR calculation **(B)**. LC-MS mass data analysis and calculation of DAR based on pre- and post-conjugation mass differences of heavy and light chain.

*Molecular weight of linker (NGM-BCN): 475.53 Da; PROTAC: 1231.87 Da and linker and PROTAC conjugate (NGM-BCN_PROTAC): 1707.4 Da. *Mass difference suggests presence of NGM-BCN linker without PROTAC.*

Outcome

We successfully optimized the cysteine-based conjugation reaction and purification process, followed by rigorous quality checks and DAR estimation. The average DAR of the resulting PROTAC-based DAC in this study was 5.07. This case study demonstrates our capability in developing high-quality degrader antibody conjugates.

Conclusion

PROTAC-based degrader antibody conjugates combine a PROTAC molecule with a cell surface antigen-specific antibody, representing a powerful next-generation therapeutic approach for selective protein degradation in target tissues such as neoplasms or diseased cells. Producing a high-quality PROTAC-based DAC is a complex process that requires a well-designed systematic workflow, precise conjugation chemistry, optimized purification methods, and rigorous analytical characterization. The presented case study highlights a cysteine-directed conjugation strategy and scalable DAR determination protocol for efficient development of high-quality PROTAC-based DACs.

At Aragen, we continue our DAC development capabilities by expanding our conjugation strategy portfolio. Ongoing advances in conjugation techniques and analytical methodologies will be critical to accelerating DAC production and facilitating clinical translation.

Why Aragen?

With nearly a decade of expertise in antibody engineering and protein bioconjugation, Aragen offers comprehensive end-to-end DACs development solutions:

- **Extensive Expertise:** Skilled in various antibody formats and conjugation chemistries.
- **Reliable Data:** Robust, reproducible conjugation and analytical data supporting confident decision-making.
- **Tailored Solutions:** Flexible conjugation strategies customized to your R&D goals.
- **Integrated Infrastructure:** State-of-the-art facilities supporting discovery, development, and scale-up under one roof.

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At Aragen, we recognize your work is vital, urgent and impacts lives. Our purpose, 'In every molecule is the possibility for better health' motivates us to drive the success of your programs, so that we can together transform hope into health for millions of people around the world.

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