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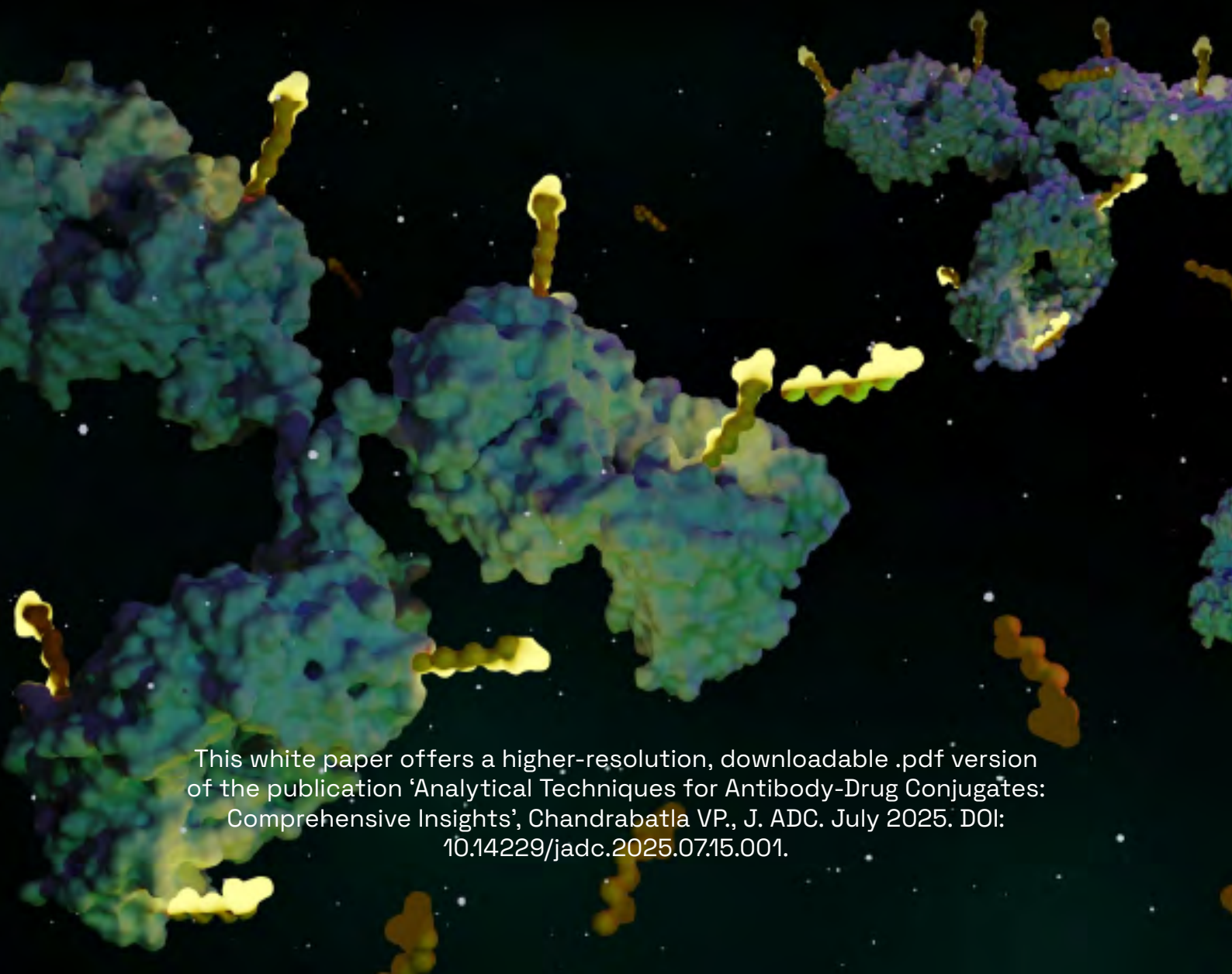
White Paper:

Analytical Techniques for Antibody-Drug Conjugates (ADCs): Comprehensive Insights



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Abstract

Antibody-drug conjugates (ADCs) are a cutting-edge class of biopharmaceuticals that combine the selectivity of monoclonal antibodies with the cytotoxic potency of small molecule drugs. This targeted delivery system has redefined oncology treatment by reducing systemic toxicity and improving efficacy.

ADCs are composed of a tumor-targeting antibody, a cytotoxic agent, and a chemical linker, which together create a complex structure that presents various analytical challenges. These challenges include determining the drug-to-antibody ratio (DAR), ensuring specificity at the conjugation site, and assessing the stability of the linker, all of which significantly influence pharmacokinetics, pharmacodynamics, and potential toxicity. This review highlights critical analytical techniques necessary for the characterization of ADCs, emphasizing their physicochemical properties, biological activity, stability assessments, and regulatory implications. Employing robust analytical methods is essential throughout the ADC development process to guarantee both therapeutic effectiveness and safety, ultimately positioning ADCs as a vital element in contemporary cancer treatment strategies.

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- Over a decade of experience with multiple ADC linker-payload systems
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- Expertise in chromatography for purifying complex, hydrophilic linkers
- Extensive technical knowledge combined with specialized equipment and GMP facilities
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- Proven track record in handling highly potent compounds with safety measures to protect operators and the environment

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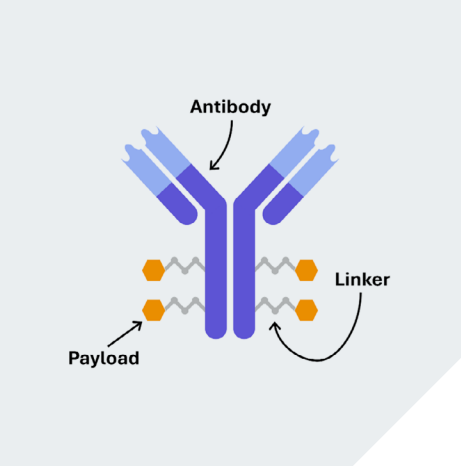
Contents

1.0 Introduction

Antibody-drug conjugates (ADCs) have emerged as a transformative class of biopharmaceuticals that harness the specificity of monoclonal antibodies (in this context, simply referred to as antibodies) to deliver potent cytotoxic agents directly to tumor cells. This targeted mechanism allows for enhanced therapeutic efficacy in cancer treatment, minimizing collateral damage to healthy tissues. [1] Antibody-drug conjugates unite three critical components (Figure 1): a tumor-specific antibody, a cytotoxic payload, and a chemical linker that secures the drug to the antibody while facilitating controlled release in the target environment. [2,3]

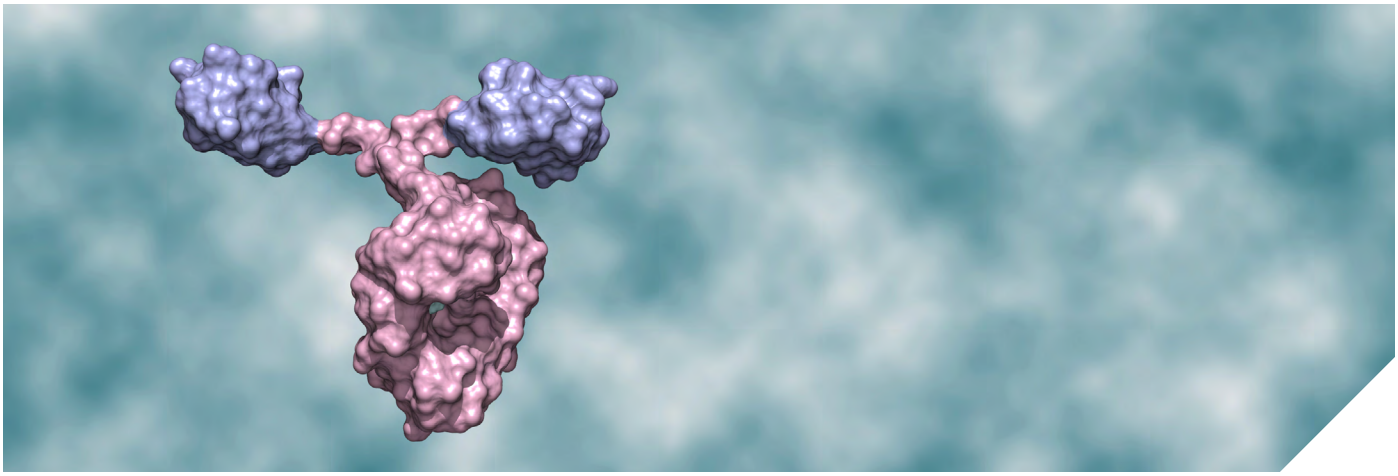


Antibody-drug conjugates (ADCs) consist of three main components:

- **Antibody** - engineered to target specific antigen(s), mainly as mAb but can be bi-specific.
- **Linker** - conjugated between a cytotoxic drug and an antibody at Cys or Lys residue, mainly as cleavable but can be non-cleavable.
- **Drug** - synthetic small molecule functioning as cytotoxin (warhead or payload).

Figure 1: Schematic diagram of an antibody-drug conjugate (ADC)

Over the past decade, several ADCs have advanced through clinical trials and gained regulatory approval for specific cancer indications. These novel therapeutics, however, are notably more complex than traditional monoclonal antibodies or small-molecule drugs. Their structural intricacies give rise to several analytical challenges. Parameters such as drug-to-antibody ratio (commonly expressed as DAR), conjugation site specificity, and linker stability directly influence the pharmacokinetics, pharmacodynamics, and toxicity profiles of antibody-drug conjugates. Consequently, robust analytical methods are required at every stage of development, from initial discovery to large-scale manufacturing and final quality control. [4]



2.0 The Triple Threat to Tumors: Antibody, Linker, Payload – A Game Changer in Oncology

ADCs aim to maximize anti-tumor potency while minimizing systemic toxicity. Conventional chemotherapies often expose both healthy and malignant cells to cytotoxic compounds, resulting in adverse side effects that reduce patient quality of life. [5] By contrast, ADCs use antibodies that bind to tumor-associated antigens with high specificity, directing the cytotoxic payload to cancer cells selectively (Figure 2).

2.1 Enhanced Therapeutic Index

The antibody component of an antibody-drug conjugate recognizes antigens that are predominantly expressed on cancer cells, thereby achieving selective drug accumulation at the tumor site. This strategy increases the therapeutic index of the cytotoxic agent, allowing for higher potency with fewer systemic effects. [6]

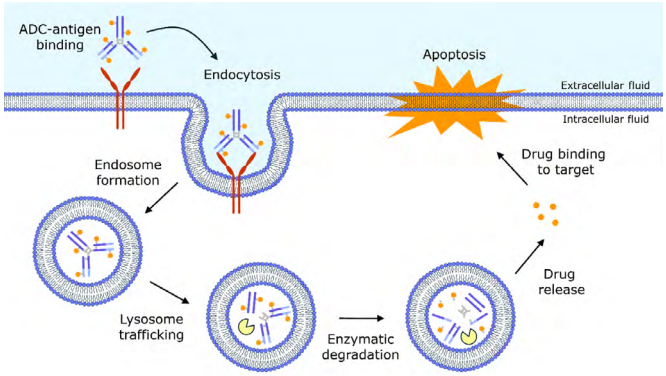


Figure 2: ADC mechanism of action

2.2 Controlled Drug Release

The chemical linker between the cytotoxic agent and the antibody is designed to remain stable during circulation but to release the drug in the tumor microenvironment. This release may be driven by environmental cues, such as pH shifts or enzymatic activity in the tumor region. [7]

2.3 Potential for Combination Therapy

ADCs can be used in conjunction with other anticancer strategies (for example, immunotherapy or radiation therapy), potentially leading to synergistic effects and improved patient outcomes. [8]

Despite the promise of antibody-drug conjugates, their unique structure adds considerable complexity to manufacturing, characterization, and regulatory assessment. Developers must carefully consider the interplay between the antibody, linker, and payload to optimize therapeutic efficacy and safety.

3.0 Conjugation Chemistries of ADCs

ADCs are composed of a monoclonal antibody (mAb) that is covalently linked to a cytotoxic drug via a small molecule linker.

These antibodies are structurally complex molecules that contain multiple drug attachment sites (multiple lysine residues, surface-exposed lysine residues, and cysteine residues) per antibody. Moreover, the conjugation of the linker-derivatized cytotoxic drug is stochastic. [9] Hence, the production of ADCs leads to a heterogeneous mixture with varying drug-to-antibody ratios. This variability in composition can impact the efficacy and safety profiles of the resulting ADCs, making it essential to carefully consider these differences during development and application. ADCs with drugs conjugated to lysine residues are more structurally diverse than ADCs with drugs attached at cysteine residues due to the increased number of attachment sites (Figure 3).

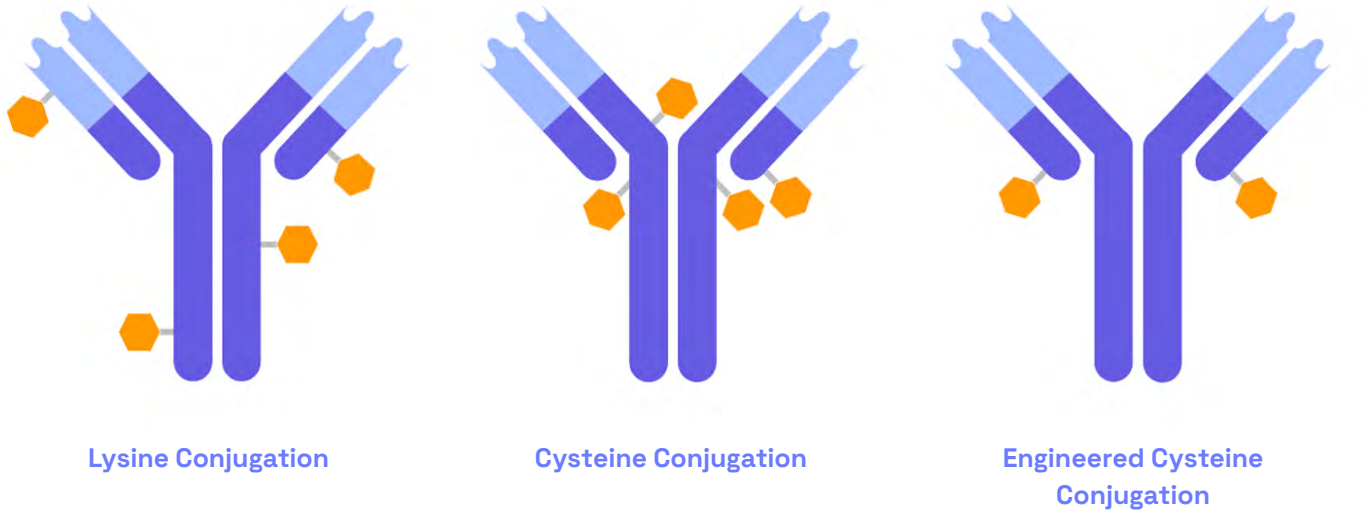


Figure 3: Various ADC conjugations

Because the distribution of attachment sites can vary for reasons like conjugation chemistry and mAb structural complexity, DAR and linker design need to be well characterized to meet regulatory requirements for approval. [9]

The drug-to-antibody ratio (DAR) can impact the efficacy of the ADC as well as the therapeutic index. An ADC with low DAR may be less potent and require higher concentrations to be effective. The inherent heterogeneity of ADCs requires a thorough characterization of the total drug load, distribution of conjugation sites, and the amount of free antibody, linker, and drug to determine the overall DAR. [10]

Various conjugation chemistries, their considerations, and approved ADC examples are shown in Table 1. Furthermore, the altered surface properties of ADCs can prompt aggregation, and the conformational states of the ADC need to be evaluated. A comprehensive analysis of the molecular and functional landscape is essential for understanding the impact of conjugation chemistry on the safety and efficacy of ADCs.

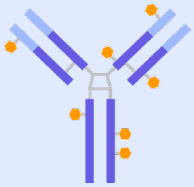
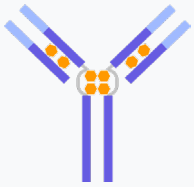
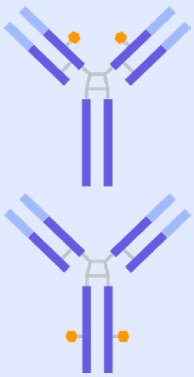
Conjugation type	Conjugation Chemistry	Considerations	Examples/ Comments
Lysine Conjugation  40 to 60 lysine residues are surface-accessible and available for conjugation.	Targets the amino group of lysine residues present on the antibody's surface. The ε-amino group of lysine residues reacts with activated esters or electrophilic groups by an **amide bond.** Commonly, N-hydroxy succinimide (NHS) esters or maleimide-activated linkers are used to form stable covalent bonds with lysine residues on the antibody. The reaction occurs under mildly basic conditions (pH ~8-9), where the lysine residues are nucleophilic.	**Advantages:** Simpler to implement and doesn't require additional modifications to the antibody. High drug-loading capacity due to the abundance of lysine residues in antibodies. Versatile, as lysine residues are naturally present in most antibodies. **Disadvantages:** Heterogeneous population: ADCs exhibit varied DARs due to random conjugation. Risk of affecting the antibody's functionality or antigen-binding regions. Reduced batch consistency in terms of pharmacokinetics and efficacy.	**Kadcyla** (ado-trastuzumab emtansine) Target: HER2 receptor Cytotoxic Payload: DM1 (a maytansine derivative) Conjugation Chemistry: Random lysine conjugation forms amide bonds. Details: Kadcyla uses a non-cleavable linker and demonstrates great success in targeting HER2-positive breast cancer, despite its heterogeneous drug-antibody ratio (DAR).
Cysteine Conjugation  When disulfide bonds are reduced, up to **8 cysteine residues** per antibody become available for conjugation. Typically, target ranges from 2 to 4 in cysteine-conjugated ADCs.	Disulfide bonds in antibodies are reduced to expose thiol groups for conjugation. These thiol groups then react with electrophilic groups such as maleimides or iodoacetamides present in the linker by **thioether bond.** After conjugation, some protocols incorporate mild oxidation to reform disulfide bonds between unreacted cysteines, ensuring structural stability.	**Advantages:** Easier control over DAR compared to lysine conjugation (usually around 4 drugs per antibody). Can produce ADCs with more predictable pharmacokinetic properties. Disulfide bond reduction is reversible, enabling recovery of the native structure if needed. Thioether bonds are highly stable and resistant to hydrolysis under physiological conditions. **Disadvantages:** Requires careful reduction of disulfide bonds to avoid structural destabilization. Risk of aggregation or premature drug release, compromising stability.	**Adcetris (brentuximab vedotin)** Target: CD30 receptor Cytotoxic Payload: MMAE (monomethyl auristatin E) Conjugation Chemistry: Reduced inter-chain disulfide bonds expose cysteine residues, which are linked via thioether bonds. Details: Adcetris showcases controlled DAR (~4), enabling better pharmacokinetics and efficacy for conditions like Hodgkin lymphoma and systemic anaplastic large-cell lymphoma.
Site-Specific Conjugation 	**Engineered Thiol Groups:** This modern approach involves engineering specific residues (unnatural cysteines) or motifs into the antibody for targeted conjugation by thioether bond. **Enzyme-based approaches:** Uses enzymes like transglutaminase/sortase to attach payloads. Transglutaminase catalyzes the reaction between the γ-carboxamide group of glutamine residues and amino-linkers in payloads by amide bond. **Non-canonical amino acids:** Genetically engineered antibodies with unnatural amino acids e.g., p-acetyl phenylalanine, for unique conjugation by covalent bonds such as oxime or hydrazone bonds.	**Advantages:** Produces homogeneous ADCs with defined DAR, improving pharmacokinetics and batch-to-batch consistency. Maintains antibody functionality and binding affinity. Enhanced safety and efficacy profile due to precise payload placement. **Disadvantages:** Complex manufacturing process requiring additional engineering or chemical modification. Higher costs compared to lysine or cysteine conjugation. May face regulatory challenges due to novel techniques.	**Example 1: Enhertu (trastuzumab deruxtecan)** Target: HER2 receptor Cytotoxic Payload: Topoisomerase I inhibitor (deruxtecan) Conjugation Chemistry: Site-specific conjugation through engineered cysteine residues or enzymatic modification. Details: Enhertu's homogenous DAR and tumor-specific release mechanisms contribute to its improved safety and effectiveness in treating HER2-positive metastatic breast cancer. **Example 2: Polivy (polatuzumab vedotin-piiq)** Target: CD79b receptor Cytotoxic Payload: MMAE Conjugation Chemistry: Utilizes site-specific engineered thiol residues for conjugation. Details: Approved for diffuse large B-cell lymphoma, Polivy combines homogeneity with precise targeting.

Table 1: Conjugation chemistry considerations and approved ADC examples [11]

4.0 The Evolution of Antibody-Drug Conjugates from Inception to Innovation

Over three generations, ADC development has shown significant changes driven by innovative technologies. Site-specific conjugation methods have enabled more precise control over the DAR, while maintaining the binding affinity and specificity of the antibody. Novel linkers and their associated triggers, designed to improve the specificity and safety of ADCs. This system enables a regulated release of cytotoxic drugs specifically within the tumor microenvironment minimizing the risk of off-target toxicity.

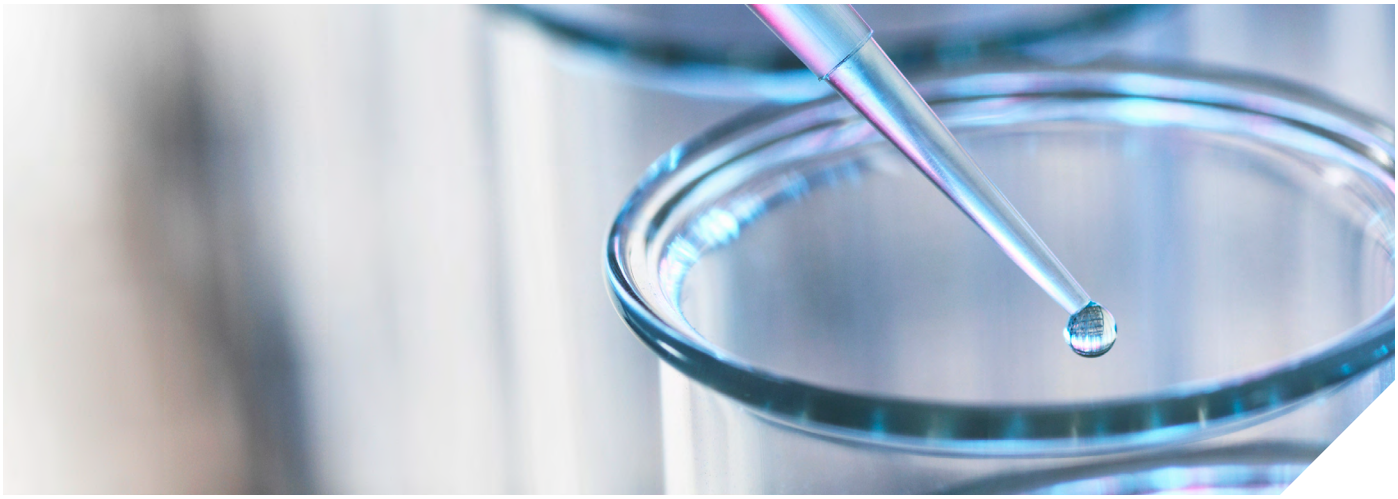
Newer strategies include [12,13]

- Fe(II)-responsive linkers - these respond to the higher levels of ferrous in iron tumors
- Enzyme-cleavable linkers - glycosidases and phosphatases
- Photo-responsive linkers - sensitive to light exposure
- Bio-orthogonal linkers - require biorthogonal cleavage pairs such as Cu(I)-BTAA)
- Dual-enzyme cleavable linkers

Advances in antibody engineering and selection have also enhanced the binding affinity and pharmacokinetics, increasing the potency and efficacy of antibody-drug conjugates. [14] A generational comparison of ADC attributes, as well as approved ADC examples is given in Table 2.

Overall, these technological advancements have addressed many challenges in ADC development, highlighted by the increase in the number of clinical trials and growing investments in the field.

What follows is a comprehensive examination of the current analytical framework for antibody-drug conjugates (ADCs), emphasizing key aspects such as physicochemical and biological characterization, functional assessment, stability testing, and the latest innovations, along with the challenges faced in maintaining product quality. By integrating these critical areas, both researchers and manufacturers can enhance their processes, ensure therapeutic reliability, and clarify regulatory requirements, ultimately advancing the clinical efficacy of ADCs.



	1st Generation ADCs	2nd Generation ADCs	3rd Generation ADCs
Antibodies	Mouse-original or chimeric humanized antibodies	Humanized antibodies	Fully humanized antibodies or Fabs
Linkers	• Unstable • Monovalent • Non-cleavable • Acid-labile	• Improved stability (Cleavable/ Non-cleavable) • Monovalent	• Stable in circulation
Payloads	Low potency (duocarmycin, doxorubicin)	Improved potency (auristatins, maytansinoids)	Low potency (camptothecins & novel payloads like immunomodulators)
Conjugation methods	Random lysines	Random lysines and reduced interchain cysteines	Site-specific conjugation
DAR	Heterogeneous (generally 0-8)	Heterogeneous (generally 4-8)	Homogenous (generally 2, 4, 8)
Advantages	• Specific targeting • Slightly increased therapeutic window	• Improved specific targeting • More potent payloads • Lower immunogenicity	• Higher efficacy • Improved DAR • Improved stability
Disadvantages	• Heterogeneity • Lack of efficacy • Narrow therapeutic index • Off-target toxicity (premature drug release) • High immunogenicity	• Heterogeneity • Fast clearance for high DAR ADCs • Off-target toxicity (premature drug release) • Drug resistance	• Potential toxicity due to high potency payloads • Catabolism difference across species • Drug resistance
Approved ADCs	Mylotarg®, Besponsa®	Kadcyla®, Adcetris®, Padcev®, Elahere®	Enhertu®, Trodelvy®

Table 2: Comparison across different generations of ADCs [11]

5.0 Principal Challenges in ADC Analytics

Despite the visible progress, several hurdles persist in the analytical characterization and manufacturing of antibody-drug conjugates.

5.1 Structural Heterogeneity

Traditional "random" conjugation approaches may generate a mixture of species with different DAR and conjugation sites. This heterogeneity complicates quality control, as varying species can exhibit different stability, efficacy, and toxicity. Site-specific conjugation techniques offer a more uniform product but introduce higher manufacturing costs and technological demands. [16,17]

5.2 Drug-to-Antibody Ratio Optimization

The DAR significantly impacts efficacy, pharmacokinetics, and toxicity. High ratios can increase potency but may compromise stability and accelerate clearance, whereas low ratios reduce potency. Ensuring a consistent and optimal ratio is a major goal in process development. [16,17]

5.3 Payload Instability

Some cytotoxic drugs are prone to premature release, which leads to systemic toxicity. Analytical methods must confirm that linkers remain intact until they reach the tumor microenvironment, releasing the drug primarily within cancerous tissues. [17]

5.4 Regulatory Complexities

Antibody-drug conjugates sit at the intersection of biologics and small-molecule drug regulations, requiring complete data packages that address both domains. Regulators expect detailed evidence of manufacturing consistency, long-term stability, and patient safety. The complexity of ADCs, particularly those with novel linkers or payloads, can lengthen approval timelines. [16]

These challenges necessitate ongoing collaboration among pharmaceutical companies, academic researchers, and regulatory bodies to refine both the science and the regulations governing antibody-drug conjugates.

6.0 Keystones of ADC Development: Robust Analytical Testing and Quality by Design

Comprehensive analytics serve as the foundation for the development of antibody-drug conjugates. Thorough testing and characterization guarantee that every batch adheres to stringent quality standards, ensuring that any changes in production or formulation do not affect efficacy or introduce new safety issues.

Ensuring the paramount quality, safety, and efficacy of ADCs necessitates the establishment of meticulous analytical methodologies. These methods are imperative to corroborate the ADC's identity, purity, potency, conjugation chemistry, and retention of stability during storage. The synergy of monoclonal antibodies' precision targeting with potent therapeutic agents in ADCs enhances the therapeutic assault on malignant cells, while simultaneously minimizing collateral damage to healthy tissues. Comprehensive analytical scrutiny of ADCs demands a multifaceted approach, ensuring the integrity and performance of both the linker and the payload components. [18,19]

A Quality by Design (QbD) approach demands a complete understanding of the critical quality attributes (CQAs) of ADCs, such as the appropriate drug-to-antibody ratio, uniformity of attachment sites, and stability of the linker. Robust analytical methods facilitate accurate monitoring and management of these attributes. [20] Many of these quality attributes are expected to be crucial for ADCs; nonetheless, experimental validation is necessary to ascertain their criticality for a specific ADC.

As per ICH guidelines Q8 (R2), a risk assessment for each quality attribute must be evaluated for potential impact on the patient (Table 3). This process requires a scoring system that considers both the impact and uncertainty associated with each attribute. Additionally, variations in critical quality attributes (CQAs) are expected due to the three components involved in antibody-drug conjugates (ADCs).

For antibody-drug conjugates, identifying CQAs requires an understanding of their unique molecular structure, which includes a mAb, cytotoxic drug (payload), and the linker connecting them. Each of these contributes to the safety, efficacy, and stability of the final ADC product.

Category	Impact (on Safety and Efficacy)	Uncertainty	Risk Ranking	CQA (ADC)
Primary Structure	**High** Primary structure determines identity and integrity. Differences can impact safety and efficacy directly.	**Low** It has relatively lower analytical uncertainty due to advanced MS techniques like LCMS/MS.	**Moderate**	**Yes**
Higher Order Structure	**High** Structural misfolding affects functionality, with some uncertainty due to indirect correlation methods (e.g., CD or DSC).	**Medium** Variability in analytical method sensitivity increases uncertainty.	**High**	**Yes**
Post-Translational Modifications	**High** Post-translational modifications (PTMs) affect efficacy and stability.	**Medium** Variability in analytical method sensitivity increases uncertainty.	**Moderate**	**Yes**
Drug to Antibody Ratio (DAR)	**High** High level of DAR affects the safety profile of the ADC. Low level of DAR leads to a decrease in the efficacy of ADC.	**Medium** It can be measured by advanced methods like HIC and LCMS, reducing uncertainty and mitigating heterogeneity.	**High**	**Yes**
mAb Size Variants (Aggregates)	**High** Size variants (aggregates, particles, fragments) can directly affect the efficacy and safety of ADCs.	**Medium** Aggregation risks can vary depending on storage conditions and manufacturing processes.	**Moderate**	**Yes**
Payload Distribution	**High** Random distribution can cause un-controllable efficacy and safety.	**High** Distribution depends on the conjugation process, linker design, and stability, all of which can introduce variability.	**High**	**Yes**
Impurities: **Unconjugated / Naked Antibodies**	**High** Unconjugated antibody competes with ADC for binding antigen, reducing efficacy. Unconjugated antibodies are susceptible to aggregation upon exposure to high temperatures, increasing the immunogenicity issue.	**Medium** While quantifiable through analytical methods like size-exclusion chromatography (SEC) and binding assays, sources of un-conjugation (e.g., process inefficiencies) may introduce variability.	**Moderate**	**Yes**
Impurities: **Unconjugated Drug (D0)**	**High** ADC without drug (no payload or D0), premature release of drug outside of targeted cancer region, can cause safety concern (off-target cytotoxicity). In addition, D0 also competes with ADC for binding antigen, reducing efficacy.	**High** Variability arises from unstable linkers or inefficient purification processes. Environmental factors during manufacturing and storage can exacerbate risks.	**High**	**Yes**
Charge Variants	**High** Charge variants (acidic, main, and basic) can potentially influence the stability and biological activity.	**Medium** It depends on multiple factors, including the stability of the linker and conjugation process.	**Moderate**	**Yes**
Biological Activity	**High** The binding capacity and cytotoxic nature of the drug directly impact safety, efficacy, and immunogenicity	**High** Biological assays face high variability and directly impact efficacy, making this a critical area of focus.	**High**	**Yes**

Table 3: Hypothetical framework for conducting a risk assessment for identifying CQAs of ADCs, aligned with ICH Q8(R2)

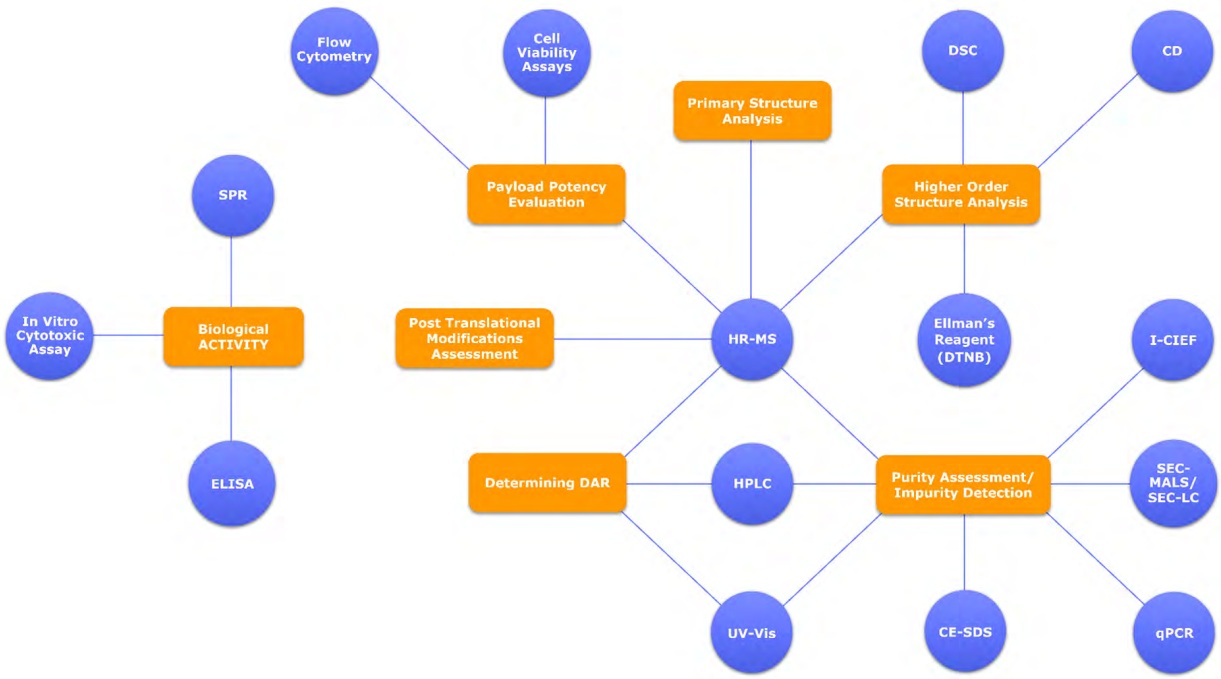
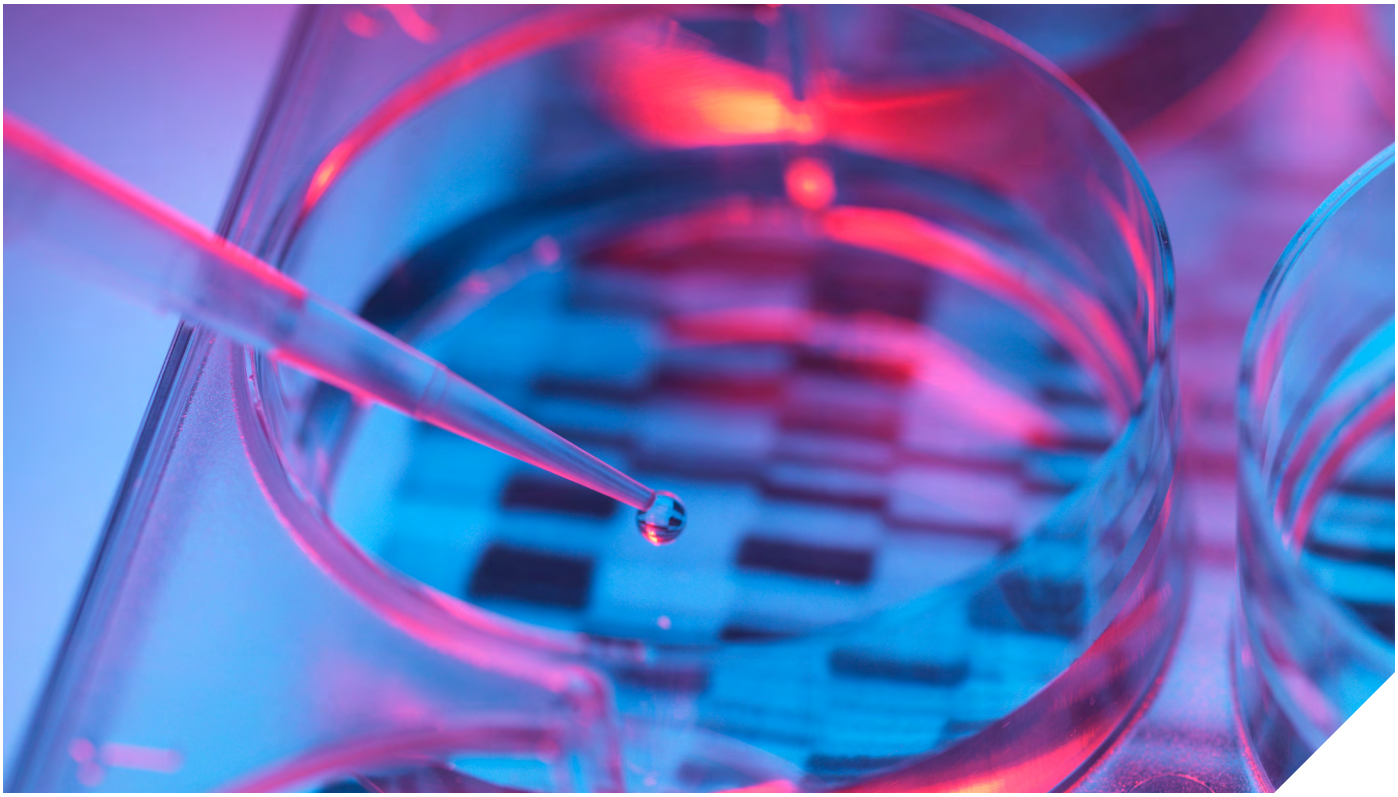


Figure 4: Common analytical techniques (circles) for ADC characterization (rectangles)

Due to their intricate structure, comprehensive analytical characterization is essential to ensure their safety, efficacy, and consistency. Various analytical techniques are employed to assess critical quality attributes such as primary structure, DAR, conjugation sites, stability, and potency (Figure 4). [22]

Furthermore, the altered surface properties of ADCs can prompt aggregation, and the conformational states of the ADC need to be evaluated. [23]



7.0 Testing and Analytical Evaluation of ADCs

7.1 Drug-linker Intermediate and General Tests

Chemistry, manufacturing, and control (CMC) release tests for payloads and linkers are designed to verify their quality, purity, and stability. This includes the characterization of their structure, impurities, and reactivity, which are essential for the efficacy and safety of ADCs (Figure 5). The following tests are recommended as quality attributes for comparability studies involving linker and drug intermediates. [24]

- Appearance and purity/impurity profile
- Drug-linker related purity and impurities
- Elemental analysis
- Residual solvent
- Heavy metals
- Water content
- Chiral purity (if applicable)

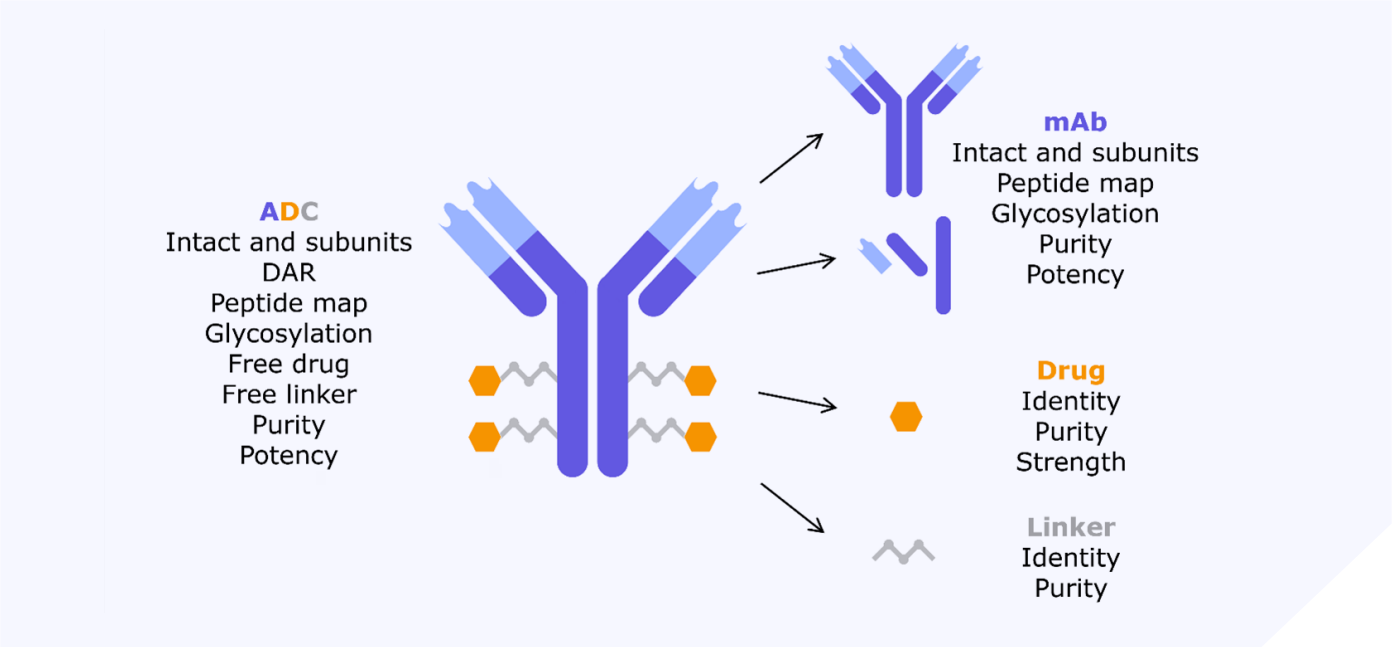


Figure 5: Parameters that need to be evaluated for ADCs and their components

7.2 ADC Characterization and Release Tests

Characterization and release testing are essential to the development and quality control of ADCs. Table 4 outlines essential ADC parameters that need to be evaluated including the analytical techniques used, their purpose, and the expected characterization outcome.

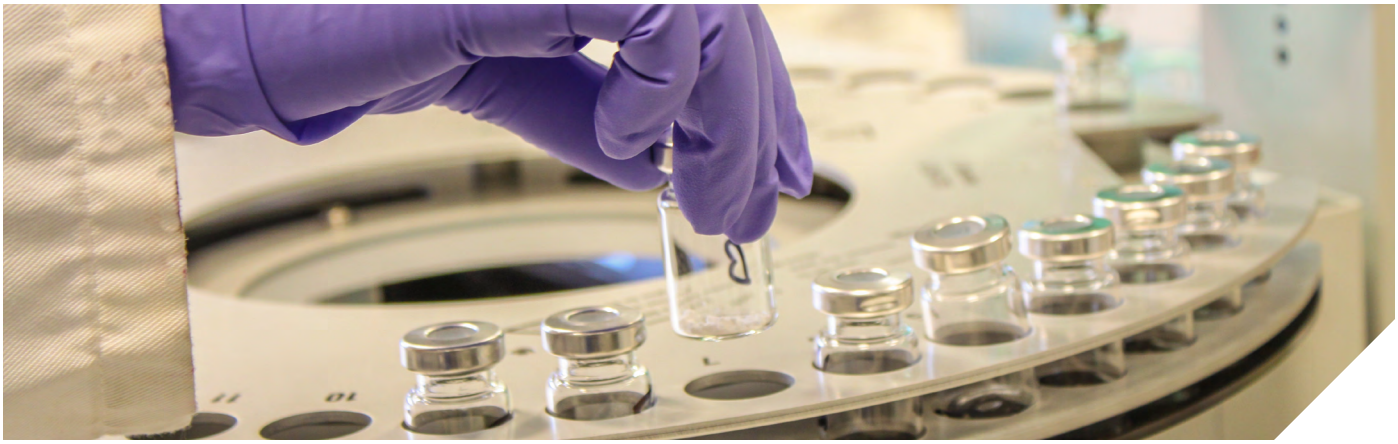
7.3 Connecting Mechanisms of Action and Potency Assays

- Antigen binding assay: Demonstrates a critical step in the ADC mechanism of action (MOA) (Figure 2).
- Cell-based cytotoxicity assay: Demonstrates the ADC MOA, including target binding, internalization, drug release, and cell killing.
- Bystander effect: If bystander effect is a proposed MOA for an ADC, bystander effect activity should be characterized.

Attribute	Analytical Techniques	Purpose	Expected Characterization Outcome
Primary Structure	Intact Mass Determination by mass spectrometry HR LCMS (Ex: Orbitrap)	To verify the molecular intact/reduced/ deglycosylated mass of ADC.	Measured intact mass shall be consistent with the theoretical mass along with possible PTMs such as N & C terminal amino acid modifications, glycosylation, and linker-payload conjugation on both heavy chains.
	Reduced mass determination by mass spectrometry		Measured reduced mass shall be consistent with the theoretical mass of light and heavy chain along with possible PTMs such as N & C terminal amino acid modifications, glycosylation, and 1 linker-payload conjugation.
	Deglycosylated mass determination by mass spectrometry		Measured deglycosylated reduced mass of light and heavy chain shall be consistent with the theoretical mass along with possible PTMs such as N & C terminal amino acid modifications, glycosylation, and 1 linker-payload conjugation.
	Peptide mapping by mass spectrometry (LCMS/MS)	To confirm the amino acid sequence of the antibody and linker-drug components.	The amino acid sequences of measured peptide fragments of ADC shall be consistent with theoretical sequences of enzyme digested peptide fragments.
	Conjugation site confirmation by LCMS/MS	To verify the conjugation site(s).	The same possible/expected conjugation sites shall be present.
Higher Order Structure	Disulfide bond linkage confirmation by LCMS/MS	To confirm the intactness of disulfide bonds.	The observed experimental data shall support the predicted disulfide assignments and the intactness of the secondary structure.
	Free sulfhydryl estimation by biochemical assay (kit based)	To determine free -SH content, there by determining secondary structure indirectly.	The free-SH content shall be correlated with LCMS/MS results and also the content shall not show significant difference between test and reference samples.
	CD analysis (circular dichroism)	To confirm 2° and 3° structure of ADC.	The profiles and reporting values of alpha helices and beta sheets shall be consistent with the reference standard or expected values.
	DSC (differential scanning calorimetry)	To determine thermal and conformational stability.	No significant difference shall be observed in T onset, Tm1, Tm2, and Tm3 between the test sample and reference standard.
Post Translational Modifications (PTMs)	N-glycan analysis by HILIC-HPLC or normal phase chromatography with 2-AB labeling	To determine the glycan profile/ pattern and relative distribution on N-glycans.	The proportions of N-Glycoforms (such as G0F, G1F, G2F, fucosylated forms) shall be reported, and shall be comparable with the reference standard.
	Peptide mass fingerprinting (PMF) by mass spectrometry	To determine and quantify PTMs.	The PTMs, such as those given below, must be determined for their presence. 1. N-Glycosylation 2. Glycosylation site identification 3. Oxidation 4. Deamidation 5. Succinimide formation 6. N-terminal glutamine to glutamate modification 7. Loss of c-terminal lysine
Size Variants	SEC-MALS	To determine molecular weight/ size of monomer, fragments, and aggregates.	The results must be comparable with reference standard or report the values.

Attribute	Analytical Techniques	Purpose	Expected Characterization Outcome
Purity & Impurities	SEC-HPLC/UPLC	To determine relative purity based on differences in molecular size of different variants present in the product.	The % of monomer, fragments and aggregates shall be consistent between test and reference standard.
	Reduced capillary electrophoresis-SDS (r-CE SDS)	To quantify (by reduction of the ADC) the heavy and light chains, any fragments, free drug, and free linker based on differences in molecular charge/size of different variants present in the product.	The results must be comparable with reference standard or report the values.
	Non-reduced capillary electrophoresis-SDS (n-CE SDS)	To determine relative purity by separating/quantifying variants based on differences in molecular charge/ size of monomer, LMWs and HMWs.	The % of monomer, LMWs, and HMWs shall be consistent between test and reference standard.
	icIEF	To determine charge variants such as acidic, basic, and neutral (main peak) variants.	The results must be comparable with reference standard or report the values.
DAR	Ultraviolet-visible (UV-Vis) spectrophotometry	To determine DAR: this technique measures absorbance at specific wavelengths to calculate DAR based on the extinction coefficients of the antibody and drug.	The results must be comparable with reference standard or report the values.
	Hydrophobic interaction chromatography (HIC)	To determine DAR: this technique separates ADC species based on hydrophobicity, allowing for DAR determination.	
	Liquid chromatography-mass spectrometry (LC-MS):	To determine DAR and Distribution: LC-MS provides detailed information on DAR and drug load distribution by analyzing intact ADCs or their reduced forms.	
Biological Activity	Binding Assays: • ELISA • 2.SPR	To evaluate the binding affinity of the ADC to its target antigen.	The results must be comparable with reference standard or report the values.
	In Vitro Cytotoxicity Assays: • MTT or MTS Assays - Assess cell viability based on metabolic activity. • LDH Release Assays - Measure cell membrane integrity as an indicator of cytotoxicity.	To measure the ability of ADCs to kill target cancer cells.	

Table 4: Essential parameters that need to be evaluated for ADCs [15]



7.4 Potential Impurities/Contaminants of ADC Products

The potential process- and product-related impurities and contaminants that can be present in an ADC drug substance (DS), their possible origin, and possible detection analytical methods are presented in Table 5.

Source	Impurity/Contaminant	Analytical Techniques
Product-Related Impurities		
ADC product (DS)	Fragments: Unconjugated antibodies, free payload (cytotoxic drug), and charge variants	SEC-HPLC, CE-SDS, icIEF
	Aggregates	SEC-HPLC
Process-Related Impurities		
Cell substrate	Host cell DNA	Quantitative polymerase chain reaction (qPCR)
	Host cell protein	HCP Immunoassay/ELISA
Purification	Protein A residues	Protein-A ELISA
Payload	Free drug	HPLC
Catalyst	Chemical elements such as Phosphorous etc.	ICP-MS
Process-Related Contaminants		
Input materials: Cell line	Endogenous viruses	TEM
	Adventitious viruses	qPCR, in-vitro culture
Input materials: Microbiological contaminants	Mycoplasma	USP/Ph. Eur general chapter methods
	BET	
	Molds and bacteria	

Table 5: Impurities in ADCs can stem from raw materials, manufacturing, or storage. Common types include residual solvents, unreacted materials, and degradation products. Techniques like HPLC, mass spectrometry, and ELISA are used to detect and quantify them, ensuring product safety and quality.

8.0 Stability Assessments and Quality Control

ADCs are sensitive to a range of environmental and processing conditions. Inability to maintain adequate conditions may lead to aggregation, cytotoxic payload loss, or other degradation pathways.

Stability and quality control studies seek to characterize how ADCs withstand these challenges and maintain product integrity through manufacturing, storage, and administration.

Stability-indicating tests are essential for evaluating the quality, safety, and efficacy of ADC substances over their shelf life. FDA and EMA guidelines emphasize the need for developing validated analytical methods to assess CQAs and detect potential degradation pathways. [25, 26]

Table 6 includes stability-indicating tests for ADC drug substances in line with regulatory guidelines.

Test Category/ Quality Attribute	Impact	Key Monitoring/Assessment	Tests that can be employed
Physicochemical Parameters			
Drug-to-Antibody Ratio (DAR)	Potency and pharmacokinetics	Stability testing monitors any changes in DAR over time, as these may indicate degradation or payload loss.	Hydrophobic interaction chromatography (HIC) or mass spectrometry
Charge variants	Immunogenicity, antigen binding (efficacy), and payload stability*	Changes in the ADC's charge profile due to deamidation, oxidation, or glycosylation are tested.	Ion-exchange chromatography (IEX) and capillary electrophoresis
Purity	Potency and pharmacokinetics	Purity testing complements stability studies by establishing the product's quality at the start. However, stability-indicating tests are needed to identify and monitor specific degradation pathways (e.g., linker cleavage, protein oxidation).	SEC-HPLC, CE-SDS
Free payload	Potency, toxicity, and pharmacokinetics	High free payload levels can signal linker instability or degradation.	LC-MS or HPLC can be used to ensure the linker does not prematurely release the payload.
Aggregates	safety (e.g., immunogenicity risks) and efficacy	**Structural Instability:** The presence of aggregates signals instability in the ADC's protein structure, potentially indicating degradation pathways like oxidation, deamidation, or fragmentation. **Conjugation Integrity:** Aggregation may be linked to issues in linker or payload attachment, affecting the overall stability of the ADC.	Size-exclusion chromatography (SEC) or dynamic light scattering (DLS)
Unconjugated antibody	Potency, pharmacokinetics, and process consistency	**Linker Instability:** Over time, the linker-payload may degrade or detach from the antibody, leading to an increase in unconjugated antibodies during storage. This signals possible instability of the ADC under specific storage conditions.	Size-exclusion chromatography (SEC), hydrophobic interaction chromatography (HIC)

Test Category/ Quality Attribute	Impact	Key Monitoring/Assessment	Tests that can be employed
Biological Parameters			
Antigen/ target binding activity	Potency and efficacy	Changes in binding activity over time can indicate degradation pathways affecting the antibody, such as glycosylation shifts or conformational changes. The conjugation process itself may alter the antibody's binding regions, making it important to monitor this attribute throughout stability studies.	Tested using ELISA, flow cytometry, or surface plasmon resonance (SPR).
Cytotoxicity	Potency and efficacy	Declining cytotoxicity may suggest instability of the linker or degradation of the cytotoxic payload, impacting its ability to release the drug at the target site. Cytotoxicity testing ensures that the ADC retains its functional capability to induce cell death in target cells, confirming the therapeutic potency. Cytotoxicity tests can reveal premature drug release caused by linker instability, which may lead to off-target effects or reduced efficacy.	Cell-based cytotoxicity bioassay
General tests			
Appearance and color	Safety and efficacy	Changes in color, clarity, or presence of visible particles often serve as the first signs of product instability. Instability in the linker, payload, or antibody may result in visual changes such as precipitation, formation of aggregates or particles, and/or changes in solution color.	Visual Inspection
pH	Safety and efficacy	FDA and EMA emphasize pH testing in stability studies to ensure product quality, consistency, and safety over the ADC's shelf life. pH testing supports determination of optimal storage conditions, establishment of the drug's shelf life, and label claims.	Potentiometry
Particulate matter	Safety and efficacy	**Subvisible particulate:** Dynamic light scattering (DLS) and analytical ultracentrifugation (AUC). **Visible Particulate:** Manual visual inspection under controlled lighting conditions.	DLS
Linker Stability Testing			
Free linker quantity**	Safety, efficacy, and pharmacokinetics	Linkers can degrade under stress conditions like pH changes, elevated temperatures, or exposure to light. Stability testing ensures the linker retains its structural integrity under various conditions. Linker instability can lead to unconjugated antibodies or free payload, affecting the overall composition and consistency of the ADC product.	HPLC, Mass spectrometry

Table 6: Key stability indicating tests. [21]

*Charge modifications can make the ADC more susceptible to enzymatic or chemical degradation, reducing stability.

**Free linker quantity is not usually a direct result of these tests because the focus is on ensuring the linker's functionality and its role in maintaining the overall ADC structure. However, indirect evidence of linker instability is derived from the levels of free payload and unconjugated antibodies.

9.0 CMC Regulatory Considerations for ADCs

As a combination of large and small molecules, ADCs bring certain challenges to CMC supervision due to their complex structural characteristics and production process.

CMC regulatory considerations for the mAb, payload-linker intermediate, drug substance and drug product are given in Table 7. Currently, there are no specific guidelines for the CMC part of ADC products. In the United States, sponsors submit ADC listing applications through Biologics License Application (BLA), but ADCs are regulated by the Center for Drug Evaluation and Research (CDER). The CMC review of ADC products involves two departments: Office of Biotechnology Products (OBP) and Office of New Drug Quality Assessment (ONDQA).[16,27] The OBP is responsible for the reviewing the antibody component of ADCs, while the ONDQA handles the review of the payload and payload-linker intermediate.

The DS and DP if the ADC are jointly reviewed by both offices under the leadership of a reviewer. Therefore, module 3 of the common technical document (CTD) must be comprehensive and reflect the manufacturing processes and overall quality control for each component of the ADC including the antibody, payload-linker intermediate, drug substance, and drug product. [16,27] Currently, there is no globally harmonized CMC declaration format for ADC products. This challenge means sponsors cannot create globally applicable documents.

Monoclonal Antibody (mAb)

- Regulatory requirements for ADC antibody intermediates and their use as final DS are consistent.
- Comparability studies between preclinical materials and clinical materials should be conducted in terms of characterization and quality control.
- Per ICH Q6B, mAb intermediates should be characterized using the same principles as final mAbs.
- The mAb's effector function and binding to Fcγ receptor (FcgR) and neonatal Fc receptor (FcRn) should also be characterized, and the impact of mAb intermediates on target cell signal transduction should be evaluated.
- Antibodies modified to reduce effector function or reduce the formation of IgG4 half-antibodies require further characterization.

Payload-Linker Intermediate

- Regulatory requirements for payload-linker intermediates and final DS are the same.
- The main considerations include optical chirality, polymorphism, impurities (product-related, process-related, free drugs and related substances, residual solvents, heavy metals), biological activity (target specific binding, affinity, effector function) and potency.
- Payload and linker are considered starting materials, and intermediates are combinations of the two.
- The source of starting materials needs to be determined, that is, toxin sources, fermentation sources (such as microbial strains), semi-synthetic compounds resulting from structural modification of natural products, peptides and chemically synthesized compounds.
- The selection of ADC starting materials is usually discussed with the FDA at the EOP2 meeting.
- Impurities generated during the payload-linker coupling process should also be analyzed. Processes for reducing or removing impurities must be specified, and the presence of impurities characterized through method validation. The FDA recommends characterizing the impurities in the payload-linker intermediate and conducting structural identification of individual impurities at levels greater than 0.1% before conducting pivotal clinical trials.

Drug Substance (DS)

- The main considerations for CMC of ADC DS are structural identification and characterization, impurity analysis, and control. Distribution and DAR are important concepts for ADC structural characterization.
- The presence of drug-related substances, free toxins, residual solvents and other process-related impurities (catalysts, metals, etc.) in the DS should be demonstrated through characterization.
- Prior to IND application, drug-related impurities should be identified during the preclinical toxicology study stage, and the comparability of DAR and drug distribution characteristics between batches from preclinical toxicology studies and clinical materials should be demonstrated through the above methods.

Drug Product (DP)

- The characterization of the ADC as a whole is similar to the characterization of the antibody part, usually only the mAb is characterized rather than the payload-linker.
- Although the efficacy of ADC depends on the payload, antigen binding is also important for mechanism of action (MOA). Uncoupled antibodies should also bind to antigens, and it needs to be proven that conjugation will not affect antigen binding.
- If the ADC has multiple MOAs, they should all be characterized. If there are any changes to the mAb, payload, or linker, a comparability study should be conducted on the DS. In addition, comparability studies should be conducted on toxicology batches, clinical batches, and commercial batches.

Table 7: CMC regulatory considerations [16, 27]

10.0 Common Technical Document Module 3: The Quality Module

There are two primary formats for ADC CMC registration declarations (Table 8).

One format (Option B) involves multiple drug substance folders, which include module 3 for the payload-linker intermediate, mAb, and ADC DS. The other format (Option A) consolidates all these elements into a single DS folder. While these formats do not strictly adhere to the electronic CTD guidelines, they can be adapted or combined based on specific circumstances. [28] Additionally, the integration of various ADC components increases the complexity of the ADC process, necessitating thorough characterization of drug intermediates and the final product. It is essential to focus process control on critical quality areas and minimize unnecessary comparability studies or analyses by leveraging a scientific understanding of the manufacturing process alongside a risk-based approach throughout the product development lifecycle.

Option A

3.2.S Drug Substance
S.1 - DS General Information
S.2 - DS Manufacture
DS S.2.1
DS S.2.2
DS S.2.3
DS S.2.4 Contr, Critical Steps and Intermediate
Drug-Linker DI S.2.4
DL DI S.2.4.1.1
DL DI S.2.4.1.2
DL DI S.2.4.1.3
DL DI S.2.4.1.4
DL DI S.2.4.1.5
DL DI S.2.4.1.6
DL DI S.2.4.1.7
mAb DI S.2.4.2
mAb DI S.2.4.2.1
mAb DI S.2.4.2.2
mAb DI S.2.4.2.3
mAb DI S.2.4.2.4
mAb DI S.2.4.2.5
mAb DI S.2.4.2.6
mAb DI S.2.4.2.7
DS S.2.5
DS S.2.6
S.3 - DS
S.4 - DS
S.5 - DS
S.6 - DS
S.7 - DS

Option B

3.2.S - DL DI
S.1 - DL DI
S.2 - DL DI
S.3 - DL DI
S.4 - DL DI
S.5 - DL DI
S.6 - DL DI
S.7 - DL DI

3.2.S - mAb DI
S.1 - mAb DI
S.2 - mAb DI
S.3 - mAb DI
S.4 - mAb DI
S.5 - mAb DI
S.6 - mAb DI
S.7 - mAb DI

3.2.S - DS
S.1 - DS
S.2 - DS
S.3 - DS
S.4 - DS
S.5 - DS
S.6 - DS
S.7 - DS

Table 8: ADC registration declaration Common Technical Documentation Module 3 structure.

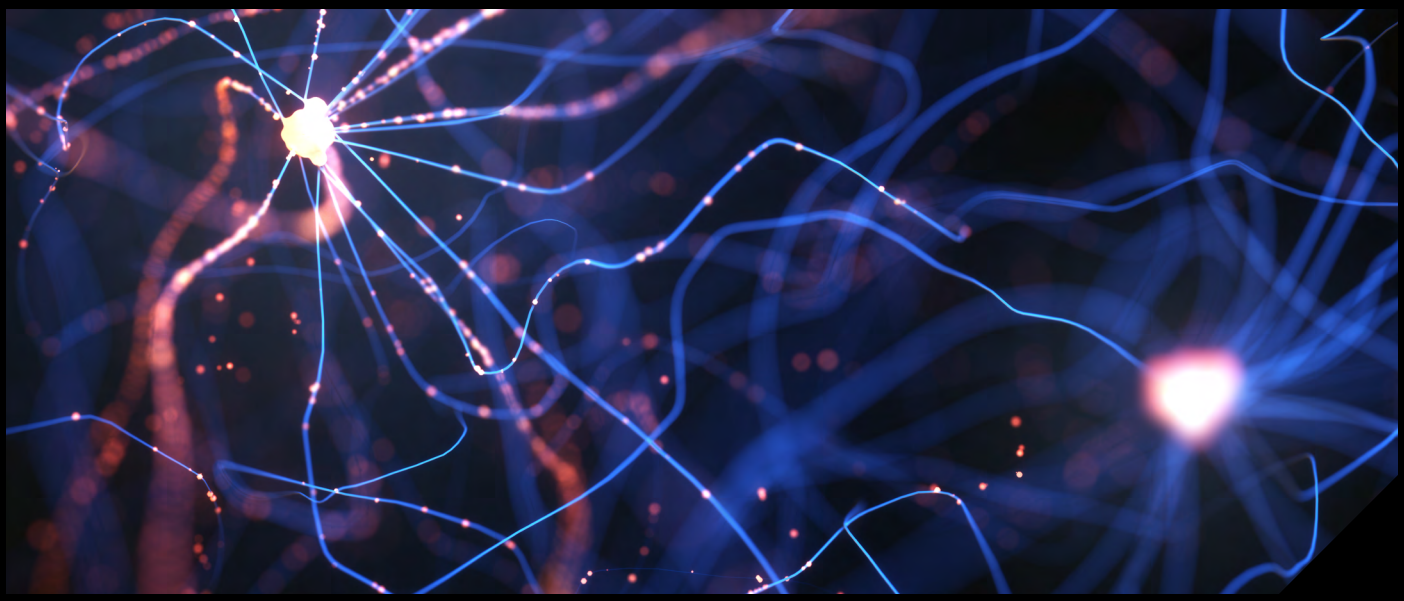
11.0 Conclusion

Antibody-drug conjugates stand as a cornerstone in modern oncology, combining the highly specific targeting capability of antibodies with the potent cytotoxic effect of small-molecule drugs. Their promise lies in delivering effective and focused tumor treatment while mitigating the systemic toxicities often associated with traditional chemotherapy. However, this promise comes hand in hand with the added complexity posed by the conjugation chemistry, linker design, payload stability, and biologic interplay with the patient's immune system.

Robust analytical techniques are indispensable at every stage of ADC development, from initial prototype screening and process optimization to full-scale manufacturing and final quality control. Physicochemical analyses, such as HPLC, mass spectrometry, and various spectroscopic methods, reveal the molecular composition and integrity of the antibody-drug conjugate. Meanwhile, functional assays ensure that the antibody retains its affinity, and the cytotoxic payload remains potent upon delivery to tumor cells. Stability studies underpin these efforts by mapping the susceptibility of the final product to degradation or aggregation.

Regulatory authorities require comprehensive analytical packages prior to the approval of clinical trials or marketing authorization. These packages should encompass data regarding purity, potency, pharmacokinetics, pharmacodynamics, immunogenicity, and overall safety. To satisfy these rigorous standards, well- qualified and validated analytical methodologies are essential.

As antibody-drug conjugates evolve beyond oncology and into other therapeutic areas, the refinement and expansion of analytical capabilities will be crucial. Emerging site-specific conjugation methods demonstrate the field's dedication to overcoming heterogeneity, while personalized medicine frameworks suggest a future in which therapies are tailored to individual patient profiles. In sum, the future of ADCs is both challenging and extraordinarily promising, with analytics at the heart of shaping this next frontier in targeted therapy.



Vara Prasad Chandrabatla

Ph.D. in Analytical chemistry

Associate Director of Analytical at Veranova

Dr. Vara Prasad Chandrabatla is a highly regarded pharmaceutical scientist with over 21 years of expertise in bioanalytical research, CMC analytical development, and quality control. Currently, he holds the position of Associate Director of Analytical R&D at Veranova, where he spearheads innovative initiatives in antibody-drug conjugates (ADCs) analysis. His contributions are vital for advanced product characterization, specification development, and lifecycle management, all in accordance with global regulatory standards.

Dr. Chandrabatla has effectively managed regulatory inspections and compliance efforts with prominent health authorities, including the FDA, ANVISA, UK MHRA, MOH Turkey, and the GCC. His extensive background encompasses first-to-file submissions, regulatory dossier preparation, and ensuring inspection readiness, with a notable impact on resolving complex compliance challenges. His technical proficiency includes high-resolution mass spectrometry, analytical quality by design (AQbD), and computer system validation, having held significant roles at various esteemed organizations such as Penn Life Sciences (USA), Sannova Analytical (USA), Aragen Life Sciences(India), Dr. Reddy's Laboratories(India), and LOTUS Labs(India). Dr. Chandrabatla earned a Ph.D. in Analytical Chemistry and an M.Sc. in Organic Chemistry, and he actively contributes to the scientific community as an editorial board member and author of numerous peer-reviewed articles, along with holding provisional patents in bioanalytical method development.

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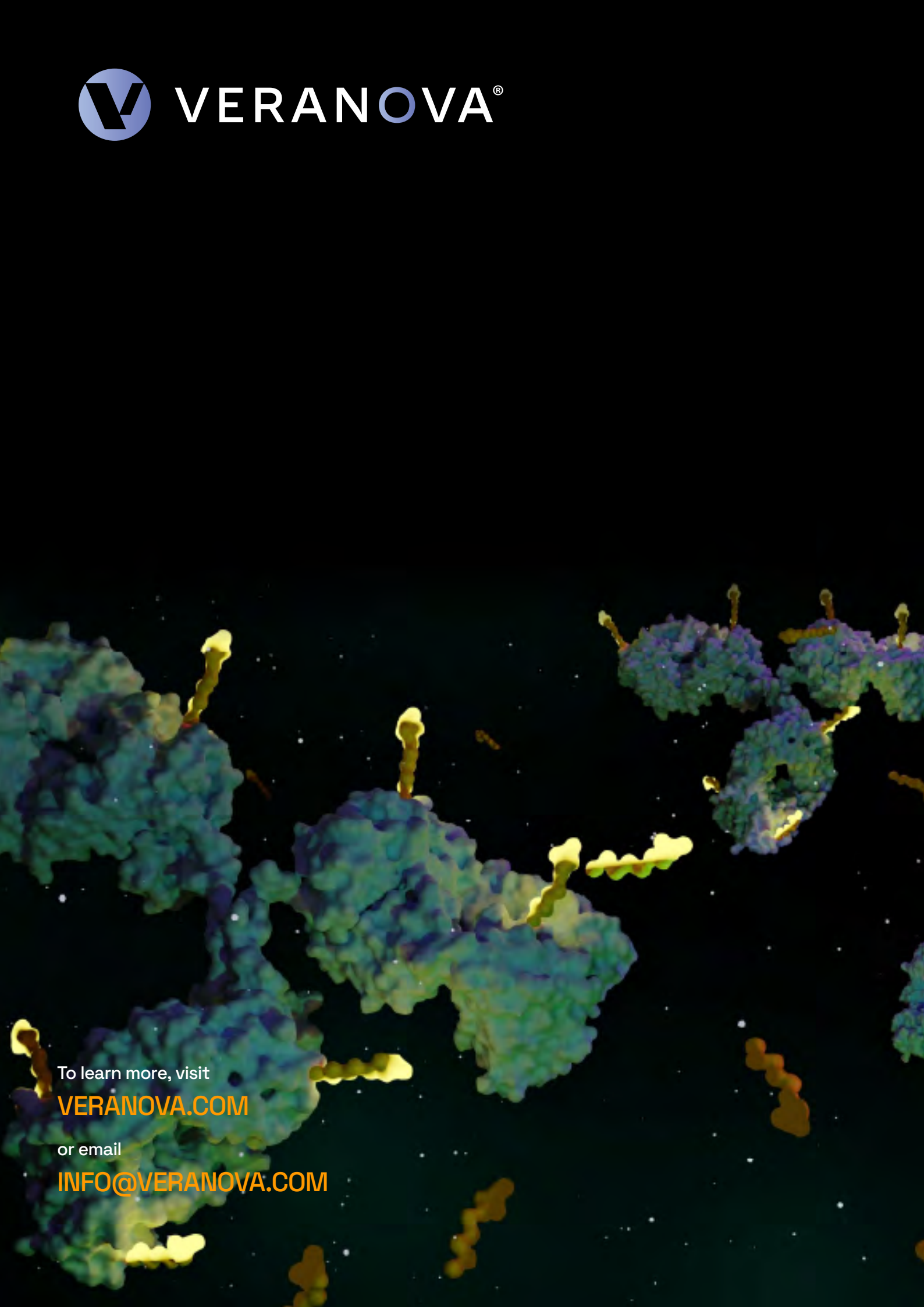

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