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Strategies for Analytical Characterization of Multi-specific Proteins

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Abstract

The development of multi-specific protein therapeutics represents a transformative advancement in biopharmaceutical innovation, yet these complex molecules present unprecedented analytical challenges throughout the CMC lifecycle. Multi-specific formats, including bispecific antibodies, antibody-drug conjugates, and fusion proteins, require comprehensive characterization strategies that extend beyond conventional monoclonal antibody analytical paradigms.

This presentation will explore integrated analytical approaches for characterizing multi-specific proteins from early-stage development through commercial manufacturing. Key considerations include establishing robust methods for confirming correct chain pairing, assessing product heterogeneity, and monitoring critical quality attributes that directly impact safety and efficacy. Mass spectrometry-based techniques, including intact mass analysis, peptide mapping, and native MS, provide orthogonal confirmation of molecular architecture and enable detection of product-related variants such as unpaired chains, aggregates, and post-translational modifications.

Special emphasis will be placed on analytical strategies that support CMC regulatory submissions, including method development considerations for stability-indicating assays, comparability assessments following process changes, and establishing appropriate specifications that balance product complexity with manufacturing capability. How advanced characterization techniques inform process development decisions and risk mitigation strategies during scale-up will be discussed.

The presentation will address practical challenges encountered when transferring analytical methods across development phases and manufacturing sites, highlighting the importance of method robustness and fit-for-purpose validation strategies. Additionally, emerging technologies including high-resolution native mass spectrometry, multi-attribute methods, and advanced chromatographic separations will be discussed in the context of next-generation multi-specific formats.

Given the expanding pipeline of multi-specific therapeutics and increasing regulatory expectations for comprehensive characterization, developing streamlined yet thorough analytical strategies is essential for successful product development. This presentation aims to provide actionable insights for analytical scientists, CMC leaders, and quality professionals navigating the analytical complexities inherent to multi-specific protein therapeutics. By establishing robust characterization platforms early in development, organizations can accelerate timelines, reduce technical risk, and ensure consistent product quality that meets both regulatory standards and patient needs in this rapidly evolving therapeutic landscape.

CMC Strategy for Multi-Specific Proteins: Fit-for-Purpose Analytics that De-Risk Development: From discovery through PPQ and commercial lifecycle management

Why this matters (1–2 bullets):

- Multi-specific modalities introduce new failure modes beyond mAbs (misassembly, chain mispairing, variant complexity), increasing the risk of late-stage surprises and comparability delays.
- A stage-appropriate, orthogonal analytics strategy is essential to connect structural characterization to CMC decisions and regulatory confidence.

Participants will leave with a practical framework to:

- Define fit-for-purpose characterization plans across the development lifecycle
- Select an orthogonal analytical toolbox (MS + chromatography + functional/biophysical) that scales with risk
- Confirm correct assembly and chain pairing and detect misassemblies early
- Build stability/comparability strategies aligned to control strategy and submission readiness

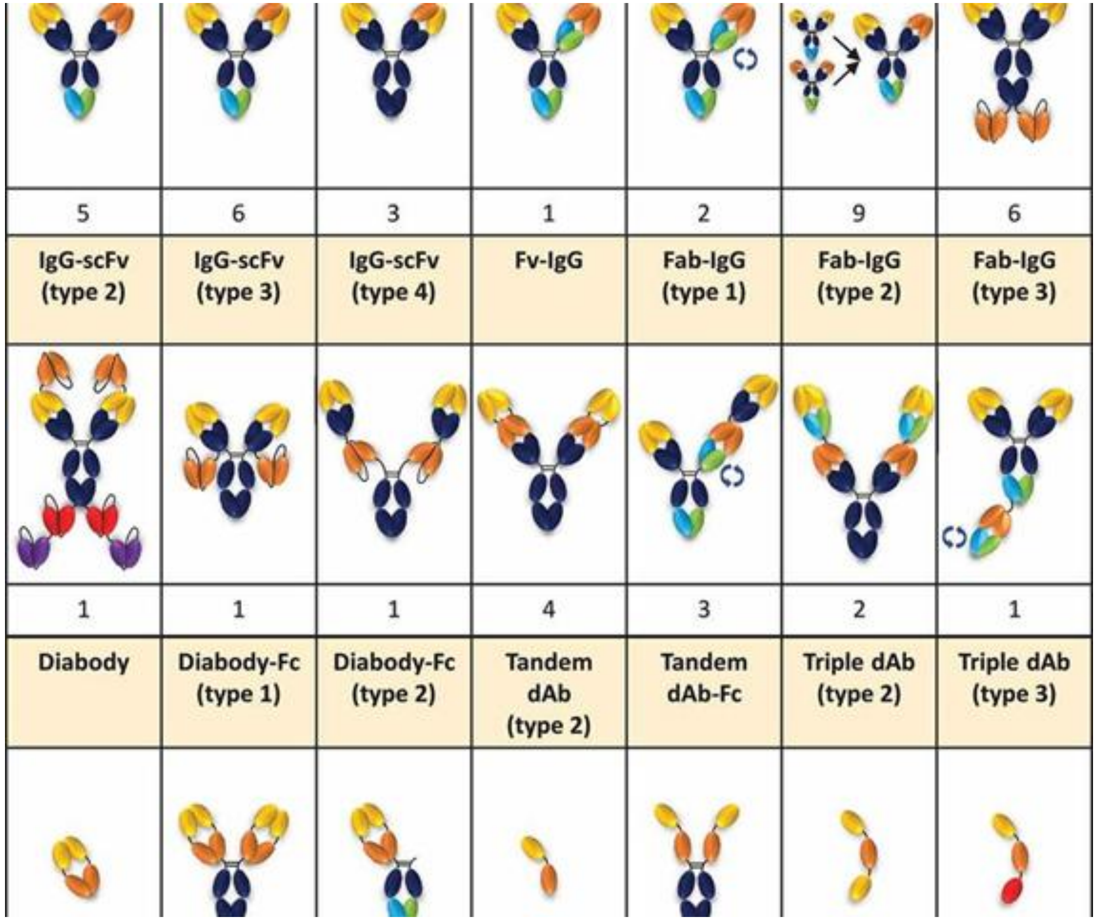
Where it fits in the CASSS program:

- Aligns with “Making Creative Protein Therapies a Reality for Patients” by translating innovation into a regulatory-ready CMC strategy
- Supports discussion on CMC risk management, comparability, and lifecycle control for next-generation biologics

Problem Statement: Multi-specifics Break the "mAb Playbook"

- **Multispecific biologics** are advanced protein-based drugs **engineered** to **bind to two or more distinct targets simultaneously**, offering enhanced precision, efficacy, and reduced side effects by coordinating multiple therapeutic actions, such as bringing immune cells to cancer cells or blocking multiple disease pathways at once

- Multi-specifics (complexity): **architecture + heterogeneity + multiple mechanisms**



Problem Statement: Multi-specifics Break the “mAb Playbook”

Areas where unanticipated product- and process-related risks commonly emerge include chain pairing fidelity, misassembled species, truncations and fragmentation products, aggregate formation, post-translational modification heterogeneity, and payload-related variability (where applicable).

CMC impacts include: **comparability, stability, specification setting, release readiness**

A CMC-first framework: “Fit-for-Purpose” characterization

Decision required	**Early** (Discovery / IND-enabling)	**Late** (Phase 2/3)	**Commercial** (PPQ* / Lifecycle)
Definition / selection	Lead selection: • identity + architecture • major variants / liabilities	Process selection: • comparability triggers • attribute risk ranking	Specification direction: • CQA set + controls • release strategy inputs
Change / comparability	Early change impact: • orthogonal confirmation • baseline trending	Comparability package: • similarity assessment • stability relevance	Lifecycle change: • PACMP inputs • continued verification
Claims / control	Early stability signal: • degradation pathways • method suitability	Shelf-life claim: • stability-indicating set • acceptance criteria	PPQ readiness: • validated methods • control strategy evidence

Development stage

Key question: *What decision does this dataset support? (lead selection, process change comparability, shelf-life claim, PPQ readiness)*

*PPQ-Process Performance Qualification

Bispecific Ab Production & Purification

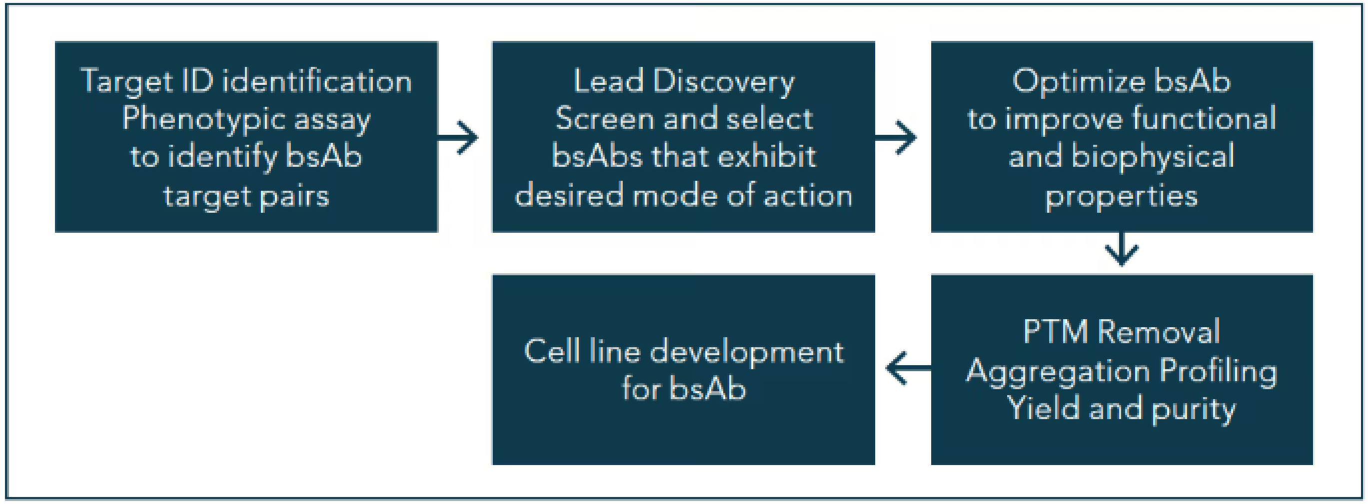


Figure 2: Outline of the bsAb production and screening.

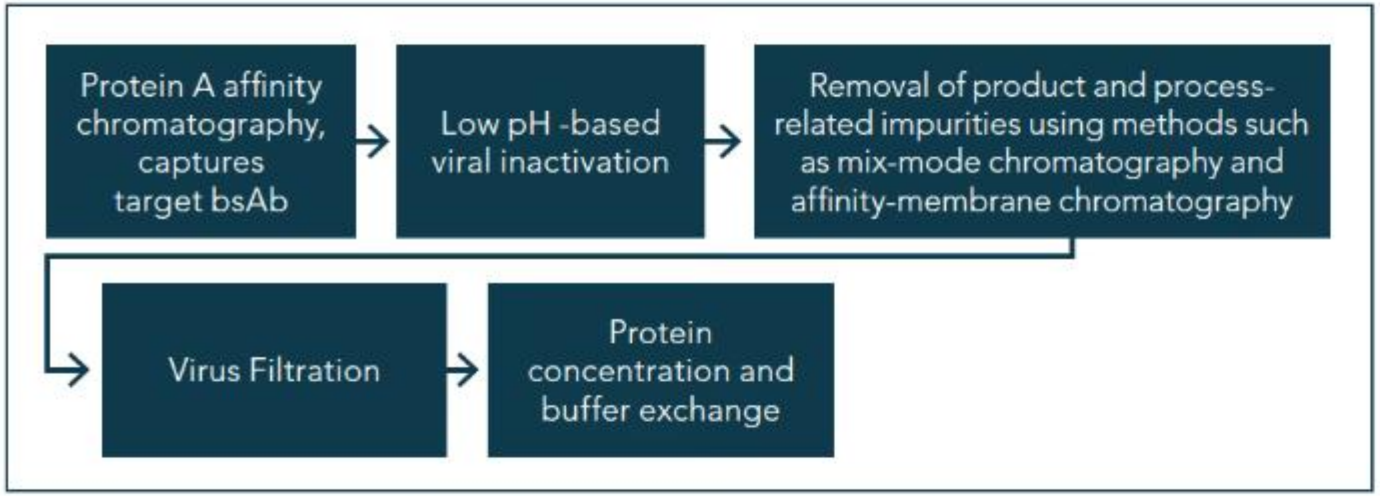


Figure 1: Outline for downstream purification of bsAsb

Modality risk map: what's most likely to fail

- Correct chain pairing & assembly integrity
- Product-related variants (PTMs, clipping, glycoforms)
- Higher-order structure sensitivity
- Aggregation pathways
- Charge variants and isoforms
- Residuals/impurities and fragmentation products

Pick assays that best discriminate **failure modes**.

Core toolbox: orthogonal methods, mapped to CQA decisions

Pick the appropriate assay and/or tool; Know the question you are trying to answer:

- **Intact MS**: mass confirmation, gross variants, mispairing flags
- **Peptide mapping**: PTM localization, sequence variants, clipping
- **Native MS** (emerging): assembly / stoichiometry / noncovalent complexes
- **SEC/MALS, CE-SDS, icIEF**: aggregates, fragments, charge heterogeneity
- **HIC/RP/2D-LC**: hydrophobic variants, co-eluting forms
  Message: “One method never suffices — multi-specifics demand orthogonality.”

Bispecific Characterization

bsAb Sample	Liquid Chromatography method	Physical Property
Denatured	Reversed Phase	Hydrophobicity
Native	Ion Exchange	Charge
	Size Exclusion	Hydrodynamic Volume
	Hydrophobic Interaction	Hydrophobicity
	Mixed-mode size exclusion	Hydrophobicity and hydrodynamic volume

Table II: A list of liquid chromatography methods used in tandem with MS to characterize and/or purify IgG-like bispecific antibodies.

How can we prove correct chain pairing / correct assembly?

Primary evidence (direct confirmation)

- Intact mass / native MS
- Reduced & non-reduced LC–MS
- Subunit analysis (e.g., IdeS / Fab–Fc mapping)

Supportive evidence (orthogonal)

Orthogonal chromatography:

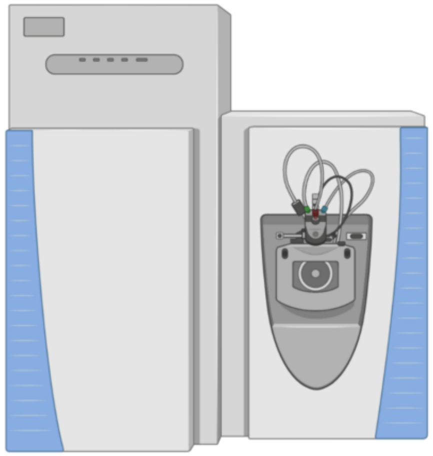
- SEC (aggregates / misassemblies)
- IEX (charge variants)
- HIC (hydrophobic variants)

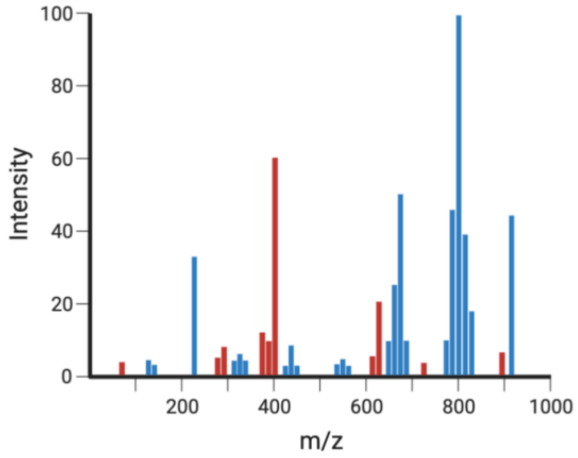
Use to corroborate architecture and detect off-target species.

Fit-for-purpose: combine direct MS-based confirmation with orthogonal chromatographic evidence for a defensible control strategy.

Mass spec in CMC: the practical value (not just “cool data”)

- **What MS changes operationally**: speed, sensitivity, specificity
- **Risk reduction**: earlier detection of misassemblies & subtle variants
- **Avoid the trap**: collecting data without a decision pathway (what do you want to know or prove)

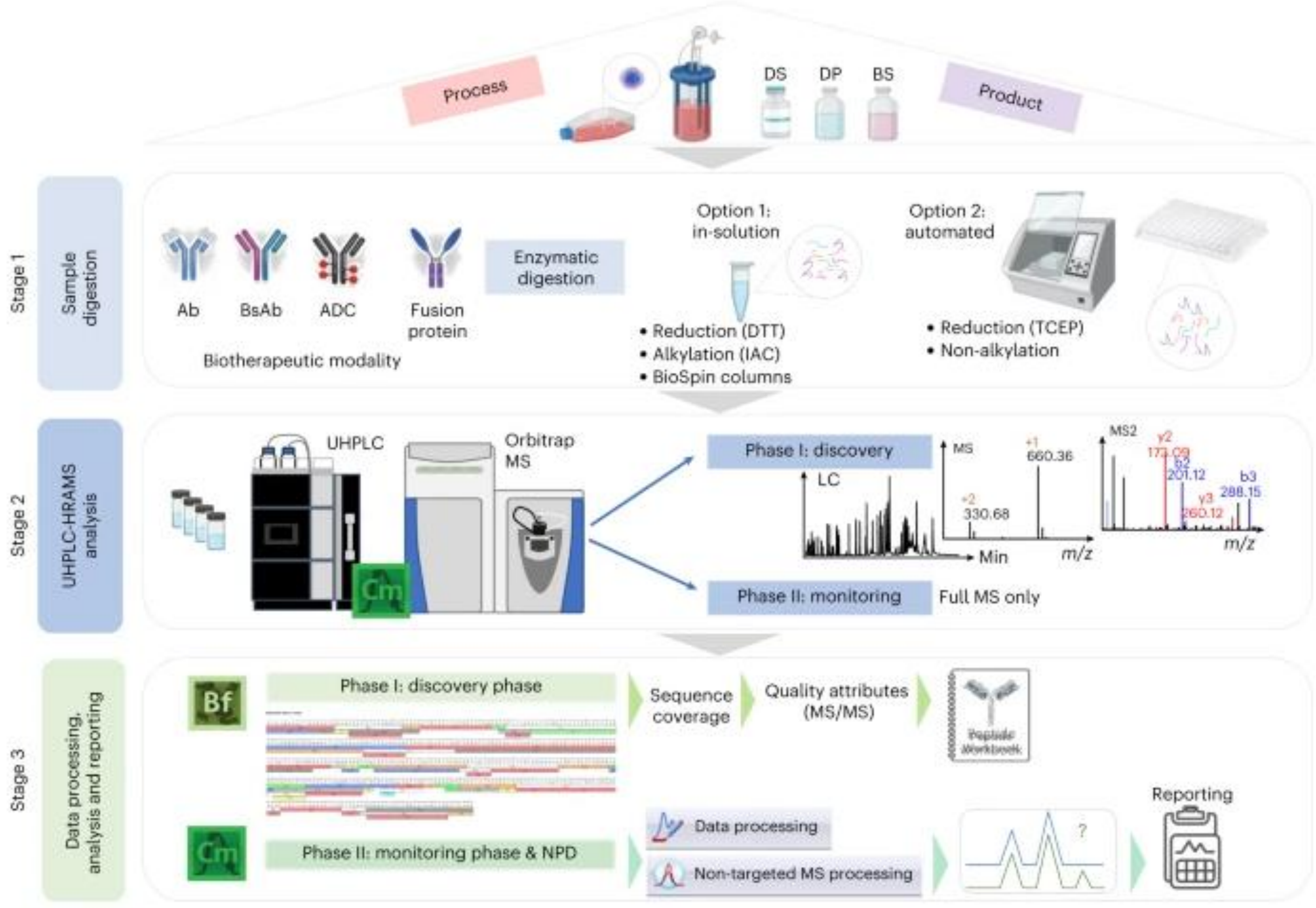




Multi-Attribute Method (MAM): where it fits and where it doesn't

- Best use cases: **routine PTM monitoring**, trending, comparability support
- Watch-outs: reference standards, peptide coverage, inter-lab transfer, data QC
- Clear message: "MAM works when it's designed like a QC assay, not a research experiment."

Fig. 1: Overview of the MAM workflow for analysis of biotherapeutic proteins from process development to product characterization.



SEC in CMC: the practical value (not just “cool data”)

Figure 1

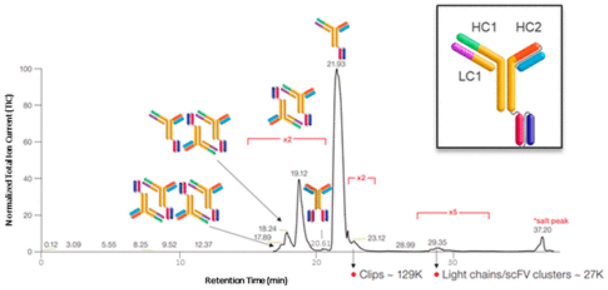


Figure 1. Native size exclusion chromatogram of a trispecific antibody. The base peak shows the expected produc,t with both smaller and larger deleterious products also present at high levels as identified by MS. The inset on the top right describes the naming convention used for this monomeric trispecific antibody.

Figure 2

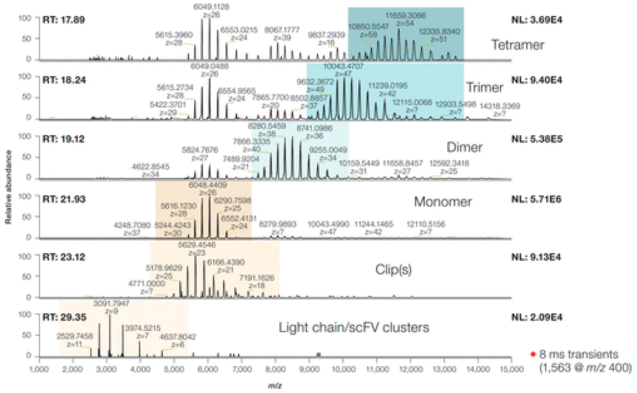


Figure 2. Extracted MS data for the eluted SEC peaks at 17.9, 18.2, 19.1, 21.9, 23.1, and 29.4 min. Highlighted portions of individual spectra correlate with the highest masses observed in each peak, and the corresponding assignment is labeled to the right.

- **Quantitative assessment of size variants:** Measures monomer, HMW, and (when resolved) LMW distributions with high reproducibility
- **CQA linkage**: Aggregation is a common CQA for complex modalities; SEC provides a routine control strategy metric
- **Mechanistic insight via profiles**: Peak shape and HMW distribution inform aggregation mechanism (reversible vs irreversible, oligomer distribution)
- **Method robustness and transferability**: SEC is well-established for validation, transfer, and lifecycle management
- **Orthogonality**: Pairs well with SEC-MALS, AUC, DLS, or native MS to confirm mass and composition of HMW species

CMC deliverables: what regulators expect you to prove

- Identity + structure confirmation
- Control strategy linkage (process → CQA → assay)
- **Stability-indicating** assays tied to degradation pathways
- Comparability: analytical similarity vs clinical relevance narrative
- Fit-for-purpose validation (early vs late)

Method robustness + transfer: the hidden failure point

- Assay lifecycle: dev → qualification → validation → transfer
- Common pitfalls: sample handling, digestion variability, column lifetime, software drift
- What to do: system suitability, reference materials, trending, acceptance criteria

“So what should teams do?”

Reimagining drug regulation in the age of AI: a framework for the AI-enabled Ecosystem for Therapeutics

Figure 1

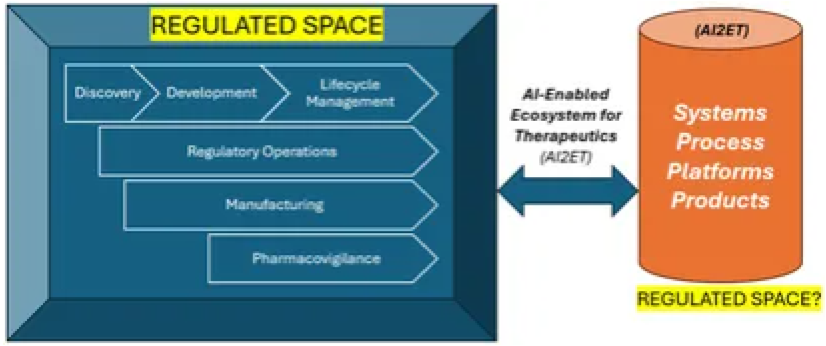


Figure 1. How is *AI-Enabled Ecosystem* for *Therapeutics related* to the various stages of creating human medicines—discovery to lifecycle management (and vice versa).

Figure 2

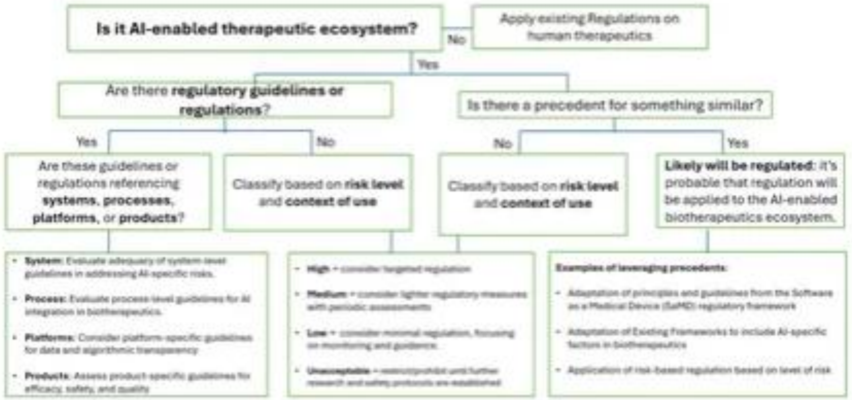


Figure 2. To regulate or not to regulate *AI-Enabled Ecosystem for Therapeutics (AI2ET).*

Center of Bioinnovation @ NU

Advancing Biopharmaceutical Innovation Through AI-Enabled Analytical Characterization and CMC Translation

Mission: Bridge academic excellence and industry impact through cutting-edge analytical science, AI-powered solutions, and global workforce development

- **Strategic Pillars:**
  - **Applied Analytics** | Complex biotherapeutics characterization (multi-specific mAbs, cell/gene therapies, mRNA, ADCs) using advanced mass spectrometry, multi-attribute methods, and AI-integrated workflows
  - **Translational Impact** | Direct industry collaboration addressing real-world CMC challenges across discovery, development, manufacturing, and regulatory submission
  - **Workforce Pipeline** | Seamless continuum from high school (Amgen Biotech Experience) through doctoral training, including BATL's industry-aligned programs and international credential offerings
  - **Global Partnerships** | NIBRT Dublin, Barnett Institute, WHO/ICH/APEC regulatory initiatives, and industry leaders (Takeda, Biogen, Agilent, Pfizer)
  - **Operational Strengths:** BATL (GMP training, 6 industry-sponsored PhDs) | LSTC (CLIA/CAP-certified diagnostics) | Faculty Fellows across Network Science, Engineering, Chemistry
  - **Leadership:** Prof. Jared Auclair (Director) | Prof. Barry Karger (Strategic Advisor) | Advisory Board including Dr. Renee Wegrzyn (former ARPA-H Director)

Conclusion

STREAMLINED BUT THOROUGH CHARACTERIZATION IS ACHIEVABLE WHEN STRATEGY IS DECISION/DATA-DRIVEN.

ASK YOURSELF: WHAT'S YOUR BIGGEST PAIN POINT TODAY: MISPAIRING? COMPARABILITY? METHOD TRANSFER? SPECS?

Questions/Discussion/Comments
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