Current, State of the Art and Emerging Technologies for the Analysis of Monoclonal Antibodies

Outline and Abstract

The proposed book will be the first to attempt to take a modern approach to the characterization of monoclonal antibodies using the Methods in Enzymology series approach. It will detail what aspects of monoclonal antibodies need to be characterized in order to demonstrate it is a well characterized biological. It will then demonstrate the current and state of the art methods as applied to commercially available monoclonal antibodies. The methods and data will be provided along with the book so that researchers can purchase the standards and the book. Emerging technologies will also be discussed and demonstrated on the standards.

The book can be segmented into 3 books; Background and Overview, Current and State of the Art Biochemical and Biophysical Characterization Methods and Emerging Technologies to Answer Unmet Needs.

Book 1: Monoclonal Antibody Therapeutic Structure, Function, and Regulatory Space.

Preface

Ch1. On the Need for Reference Material for Biopharma

Anthony Mire-Sluis, Amgen
Darryl Davis, Janssen
John Schiel, NIST

1. Describe biopharmaceutical production process
    1.1. Heterogeneity due to biological origin
2. Brief description of mAb heterogeneity
    2.1. How different than small molecule
3. mAb historical background
4. Uses of reference standards and different types (USP, WHO, etc.).
5. Defining the process and keeping the process in control requires characterization
6. Characterization and comparability requires in-house product-specific standard
    6.1. Current state of-the-art in-house standards
        6.1.1. Interim, primary, working
    6.2. Indicates process is in control, and that method is reproducible.
7. Reference material supplement current strategies (how this material fits into bigger picture)
    7.1. Define RM and SRM
    7.2. Can show method Conformance to expectation
    7.3. Secondary analyte to determine
        7.3.1. Method suitability
        7.3.2. LOD
        7.3.3. Etc.
    7.4. Describe difference between this and small molecule
        7.4.1. Challenges
        7.4.2. Qualitative
        7.4.3. Quantitative
8. Summary

Ch2. On the Importance of Monoclonal Antibodies: Mechanism of Action

Roy Jefferis (Univ. of Birmingham).

CHAPTER COMPLETE

1. Introduction
2. Human antibody isotypes
3. The polypeptide structure of IgG
4. Human IgG gene polymorphism
5. Allotype expression and amino acid correlates
6. Allotypy of licensed chimeric mAb therapeutics
7. The quaternary structre of human IgG
8. Quaternary structure of IgG-Fc
9. The IgG Fc oligosaccharide moiety
10. IgG-Fc protein oligosaccharide interactions
11. IgG-Fc ligand binding, activation, and modulation
    - 11.1. Cellular IgG-Fc receptors
        - 11.1.1. IgG-Fc receptors (FcyR) mediating antigen clearance
        - 11.1.2. FcyR binding sites on IgG
    - 11.2. FcRn: catabolism and transcytosis
    - 11.3. Compliment Activation
    - 11.4. Role of IgG glycoforms in recognition by cellular FyRs
12. Quaternary Structure of Fab
13. Human antibody isotpyes ofther than IgG
14. Concluding remarks

Ch3. Heterogeneity of IgGs: Structure Function as related to process parameters.

Mark Schenerman, MedImmune
Reed Harris, Genentech

1. Overview of Mabs
   1.1. *Discovery*
   1.2. *Use in diagnostics, reagents, medicinals*
   1.3. *Overall market value in diagnostics and healthcare*
1. Intro: why test the material? Need to show a consistent, stable and active product that's demonstrably pure despite its inherent heterogeneity.
2. Test results inform about purity, stability, process consistency, product consistency (= "quality").
3. Common approach for determining Critical Quality Attributes includes assessments of patient impacts due to variation such as bioactivity (or bioactivities), PK (determined by animal PK studies or FcRn models), relative immunogenicity, and safety.
4. Challenges include: multimeric nature of mAbs (where only one of four chains may be modified) and overall molecular size (~1450 amino acid residues).
5. Attribute categories: each one to include a description of the variation, our testing capabilities, how it usually develops (post-translational, cell culture, stability, oxidation etc.), and common or default CQA impacts:
   5.1. size variants: aggregates (dimers, higher aggregates), fragments (includes polypeptide chain cleavage and non-disulfide bonded subunits)
   5.2. Primary structure: truncations (±Lys, ProNH2), extensions (VHS-), pyroGlu
   5.3. Post-Translational Modifications: disulfide variants (thioether, trisulfides), unusual or rare modifications; refer to Jefferis chapter for glycans
   5.4. Degradative modifications: Trp and Met oxidation, Asn deamidation, Asp/isoAsp/succinimide forms,
   5.5. Variation due to cell line/culture: sequence variants due to mutations, misincorporation, glycation
   5.6. Higher-order structure: maybe… is this covered elsewhere?
6. Out of scope: process-related impurities such as host cell proteins, microbiological issues, particles, conjugated forms (PEG, linked drugs), engineered changes

7. Not meant to be a comprehensive review article, can refer to some good reviews that are available such as Beck et al., Vlasak et al. Expect other chapters to go into detail about glycans (Jefferis), aggregates, Higher Order Structure.
8. Something in the summary about current regulatory expectations for early stage, late stage, licensing stage characterization (unless covered by Brorson).

Ch4. What Constitutes a Well Characterized Biological Protein

Kurt Brorson, FDA
Brent Kendrick, Amgen

1. Quality Attribute Overview
2. Analytical techniques
3. New analytical methods.
4. Industry perspective
    1. **Elucidating the structure of the product and product related variants**
        a. **Creating the DNA clone and determining the gene sequence**
        b. **Biochemical Characterization of the drug substance**
            i. **Verifying the primary structure and amino acid sequence (e.g. peptide mapping with MS and MS/MS, whole mass, etc)**
            ii. **Detection of potential primary structure post-translational modifications (e.g N- and C-terminal variants, methionine oxidation, asparagine deamidation, etc)**
            iii. **Verifying Glycosylation site and structures (if applicable)**
                1. **Determination of N-glycosylation Site and Occupancy**
                2. **Non-consensus N-glycosylation**
                3. **Composition of N-linked Glycans**
                4. **Structural Analysis of N-linked Glycans**
                5. **O-linked Glycosylation**
            iv. **Disulfide Structure**
            v. **Free Sulfhydryls**
            vi. **Charge Variants**
        c. **Biophysical Characterization of the drug substance**

i. **Size Variants (high molecular weight and low molecular weight species)**

ii. **Higher Order Structural Characterization (secondary and tertiary structure, thermal unfolding temperature(s)**

d. **Biological Characterization of the drug substance (mechanism of action, biological assays and relevance to mechanism of action)**

2. **Relevant degradation pathways under recommended storage conditions**
3. **Product Quality Attribute Assessment of significant product variants identified from Elucidation of structure (this section will be brief and reference Gregg Nyberg and Greg Flynn's chapter)**

d. **Utilization of platform, literature and other prior knowledge (e.g. IgG1 and IgG2 monoclonal antibody platforms), especially for Fc related variants**

e. **Assessment of biological relevance of product related variants**

c. **Risk assessment and identification of critical quality attributes**

Ch5. Regulatory Guidelines as Applied to Recombinant Proteins
Primary - George N. Saddic, GSK

1. **General overview of Regulatory guidance regarding analytical testing.**
2. **Overview of CTA submission sections regarding analytical testing**
3. **Current trends in Release testing**
4. **Expectations to IND**
5. **Expectations beyond IND**
6. **Current trends in Characterization Assays**
7. **Expectations to IND**
8. **Expectations beyond IND**
9. **If and when comparability needs to be considered and demonstrated**
10. **FIO testing strategy and the need to understand other attributes of a mAb such as particulates, impurities and process residuals**
11. **References and links to Regulatory guidance (unless this is incorporated into the general text)**

Ch6. Using QbD Principles in setting a control strategy for product quality attributes

Primary-Greg Flynn, Amgen
Gregg Nyberg, Amgen

1. QbD Principles and Product Quality Risk Assessment (PQRA)
    1.1. QbD in Biopharmaceutics/ICH guidelines/etc.
    1.2. PQRA Introduction
        1.2.1. Product Quality Attribute Assessment (PQAA) (Severity)
        1.2.2. Occurrence (Process Knowledge)
        1.2.3. Detection (Methodology)
    1.3. Lifecycle Application of PQRA
2. Example 1. Fc Deamidation on mAbs
    2.1. Severity
        2.1.1. General Knowledge
        2.1.2. Product specific knowledge/Gaps
        2.1.3. PQA Assessment
3. Occurrence
4. Detection
5. PQRA/Control
    5.1. Phase 1/2 clinical
    5.2. Phase 3 clinical
    5.3. Commercial
6. Example 2. Fc High Mannose Glycan
    6.1. Severity
        6.1.1. General Knowledge
        6.1.2. Product specific knowledge/Gaps
        6.1.3. PQA Assessment
    6.2. Occurrence
    6.3. Detection
    6.4. PQRA/Control

Book 2: Current State-of-the-Art Product and Process Testing

Current and State of the Art-Will be written mainly by industry researchers. The attempt will be to have triplicate results from 3 labs for each standard assay. Each chapters primary author/s will be responsible for determining which methods will be used and coordinating the results as well as writing the chapter.

Involving multiple authors from several companies to contribute to a same chapter is posing a potential risk of a "COMPETITIVE SUPERIORITY" of one method over another. This book should attempt to overcome this issue where possible; white papers and round table discussions at CASS meetings are a good example of such effort. Potential scenarios for such collaboration(s) are addressed in each chapter.

Ch7. The Protein Characterization Lab of Today

Darryl Davis, Janssen

Abstract

A typical lab within a biopharmaceutical organization is a mixed bag of instrumentation that reflects the toolbox approach used by most companies to address wide-ranging questions from various parts of the org. Each of the questions have levels of answers which depend on the stage gate at which the potential therapeutic is at. This chapter will detail the types of instruments and the biochemical or biophysical answer they provide. It will attempt to provide some context and background concerning why some labs no longer employ older instrumentation, such as N terminal sequencers, while others still do even though they have instrumentation that supplants it.

Outline

1. *Introduction*
2. *The questions that need to be answered*
   2.1 Methods to answer these questions in the 1970s
   2.2 Methods to answer these questions in the 1980s
   2.3 Methods to answer these questions in the 1990s
   2.4 Methods to answer these questions in the 2000s
3. *Current State of the Art*
   3.1 The importance of mass spectrometry
   3.2 Bridging mass spectrometry with other assays

Ch8. Determination of The Primary Sequence/Structure

Darryl Davis, Janssen
John Schiel, NIST
Trina Formolo, NIST
Lisa Kilpatrick, NIST
Karen Phinney, NIST
Lisa Marzilli, Pfizer
Jason Rouse, Pfizer
Thomas Porter, Pfizer
Amareth Lim, Eli Lilly
Bryan Harmon, Eli Lilly

Abstract
This is a critical parameter of any protein including mabs and as such a great deal of effort is applied at this stage. Many methodologies from many fields are employed when determining the fidelity of the predicted primary sequence. This chapter should also attempt to address the question of how much or how little characterization is needed to establish primary sequence (e.g. 100% sequence coverage by peptide mapping using multiple enzymes or a single peptide map supplemented by intact/reduced LC-MS or a combination of the above).
This chapter will give a historical review of peptide mapping, and move into the modern use and why peptide maps are important. Data from the NIST mAb will be used to demonstrate what it means to "confirm" a primary sequence. A discussion of what data is necessary and how/whether intact or middle down analysis can complement peptide mapping data for sequence verification. Data will be collected at a level expected for a typical BLA submission and beyond in some cases. A regulatory aspect may also be included in the chapter. The various methods from each contributor will be used to show different available technologies (column formats, data analysis software, and MS instruments). This will highlight that different perturbations can be used to achieve similar results. NIST reference standard with traceable quantification will be useful for internal comparison of methods to choose a platform as analytical technology evolves. Also useful to reviewer to see data to understand that there isn't one perfect peptide map, but that different instruments, columns, etc. can be used to obtain quality data.

Outline

1. *Introduction*
   - 1.1 Brief description of primary sequence
   - 1.2 Historic Background
     - 1.2.1 Rational for using trypsin
     - 1.2.2 Limitations as practiced in the 70s and 80s
     - 1.2.3 Bridge to modern mass spectrometry
   - 1.3 Peptide mapping as currently practiced
     - 1.3.1 Types of digestions
       Enzymes; N-terminus, C-terminus, Partial Digests, Bottom-up, Middle-down, Top-down
     - 1.3.2 Types of LC-MS
       Instruments; Bottom-up, Middle-down, Top-down

        1.3.3 Limitations as currently practiced
    1.4 Data Analysis
        1.4.1 Manual analysis
        1.4.2 Automated analysis
        1.4.3 DB search
        1.4.4 Types of LC-MS experiments
        Fragmentation modes; IDA, DDA, multiple

2. *Experimental*

2.1 Tabulated sequence coverage
2.2 MS/MS sequence coverage
2.3 Gap analysis
2.4 Alternate outcomes and unknowns

3. *Discussion*

4. *Conclusion*

Ch9. Structural Elucidation of Co and Post-Translational Modifications

Sarina Benchaa, Amgen
Wenzhou Li, Amgen
James Kerwin, Amgen
Amareth Lim, Eli Lilly
Bryan Harmon, Eli Lilly
John Schiel, NIST
Catherine Formolo, NIST
Lisa Kilpatrick, NIST
Janssen

. **Abstract**
Therapeutic monoclonal antibodies (mAbs), a rapidly growing class of human therapeutic drugs, present a daunting challenge for structural characterization. Primary protein sequences are invariably modified in eukaryotic systems, and these modifications are essential for targeting or otherwise altering biological activity. Beginning with basic research and proceeding through all steps of the production and formulation environment, co- and post-translational modifications (PTMs) must be discovered, tracked, and evaluated for quality, safety, and efficacy. A brief review of PTMs commonly encountered is presented, with emphasis on those identified during mass spectrometric analyses of the reference mAb provided by the National Institute of Standards and Technology (NIST). Current and promising approaches for mass spectrometric and complementary techniques for structural characterization of mAbs are discussed in detail. Results following implementation of these techniques to characterize the NIST standard are summarized.

1. Background information leading up to NIST standard (experimental) section.

   1.1. Historical perspective on failure of initial attempts to use mAbs for therapeutic purposes.
   1.2. Serves to emphasize the need for rigorous analytical techniques.
2. Summaries of PTMs

   2.1. Overview of Post-Translational Modifications (PTMs). Glycosylation, oxidation, deamidation, thioether, C-termineal lysine, N-terminal pE, clips

3. Methods for Characterization of Monoclonal Antibody PTMs

   3.1. End with summary of advantages/disadvantages of various techniques.

4. Analysis of Native Material
   4.1. These experiments would be used to determine the PTM's present in the native material.
   4.2. PTM identification via Peptide mapping
      4.2.1. Perform analysis using two or three enzymes, and elucidate as many PTM's as possible.
         4.2.1.1. Each lab will perform their own method.
         4.2.1.2. Data will be compiled and returned in a template/tabular format
      4.2.2. LC-MS of reduced IgG and/or middle down analysis

            4.2.2.1. Discuss level of PTM currently identifiable with these techniques
    4.3. Use literature data to describe PTM's not present in the native sample.
5. Analysis of Stressed Materials
    5.1. This would elucidate "hot spots" for the occurrence of PTM's. Potential stressors include.
        5.1.1. Forced Oxidation
        5.1.2. pH stress
        5.1.3. Heat stress
        5.1.4. Photo Oxidation
    5.2. Perform same analytical characterization as on Native Material
6. Potential Discussion
    6.1. Method, instrument, and/or lab variability
    6.2. Relate findings to CQA assessment from previous chapter
    6.3. Discuss treatment of data for PTM quantification.

Ch10. Sequence Variant Analysis (SVA)

Primary-Oleg Borisov, Amgen
Victor Ling, Genentech
Melissa Alvarez, Genentech

1. Introduction
2. Review of literature on sequence variants in biotherapeutics
3. Types of sequence variants
   · Role of LC-MS for detection of low-level variants
   · Critical experimental parameters defining quality of analysis for sequence variants
4. Sample preparation – minimizing digestion artifacts,
5. Data acquisition and LC-MS conditions,
6. Data analysis – removing a bottleneck.
   · Desired Outcomes and Conclusions.

Ch11. Glycan Analysis

Justin Prien, Amgen
John Schiel, NIST
Catherine Formolo, NIST
Pauline Rudd, NIBRT
Barbara Adamczyk, NIBRT
Henning Stockmann, NIBRT
Amareth Lim, Eli Lilly
Bryan Harmon, Eli Lilly

1. Introduction to glycosylation of mAb's
2. LC-Fluorescence and Glycobase and CE-LIF, rapid screening protocol
   - 2.1. NIBRT
     - 2.1.1. PNGAse, label
       - 2.1.1.1. HILIC-HPLC on TSK-amide
       - 2.1.1.2. Use GU units, Glycobase
       - 2.1.1.3. Exoglycosidase digests and additional LC runs for ID
   - 2.2. Eli Lilly
     - 2.2.1. CE-LIF
     - 2.2.2. Sialic acid monosaccharide analysis
     - 2.2.3. Possibly global monosaccharide analysis
3. Continue with full BLA characterization package
   - 3.1. Site occupancy, glycan composition, branching, and monosaccharide analysis.
   - 3.2. Amgen methods
     - 3.2.1. 2D LC-F-MS/MS (2-AA)
     - 3.2.2. LC-MSn (representative 2-AA glycan)
     - 3.2.3. Exoglycosidase
     - 3.2.4. Trypsin with and without Endo F's
   - 3.3. Complimentary techniques of NIST, NIBRT, and Eli Lilly.
     - 3.3.1. NIST
       - 3.3.1.1. LC-F-MS/MS (2-AB, 2-AA)
       - 3.3.1.2. MSn (permethylated pronase and/or 2-AA)
       - 3.3.1.3. Nano LC-MS/MS
       - 3.3.1.4. Enriched glycopeptide LC-MS
4. Present orthogonal techniques
   - 4.1. Additional methods can yield similar results
   - 4.2. Discuss utility of orthogonal techniques for BLA
   - 4.3. Discuss utility of Reference Material for method development, qualification, etc.
5. Conclusion

Ch12. Separation Assays and Orthogonal Methods

Karen Miller, Amgen
Tom Dillon, Amgen
David Michels, Genentech
Scott Lute, FDA
Kurt Brorson, FDA

1. Introduction to the utility of separations (chromatography and electrophoresis methods) for characterizing Mabs. Discussion of the importance of determining the quality attributes separated by these methods through peak isolation or other orthogonal methods.
2. Chromatography methods
   - 2.1. SEC – Brief discussion of the attributes separated and the recent introduction of UPLC. Show SEC-HPLC chromatogram and the features that indicate that it is a good separation, i.e. symmetric peak shape, resolution of higher molecular weight forms and clips (if present).
     - 2.1.1. SEC with MALS – Show MALS trace and discuss the value of this for characterizing the SEC profile.
   - 2.2. CEX – Discussion of how CEX methods can be developed and optimized using new software tools such as Fusion. Show final optimized chromatogram. Discuss the attributes that may be resolved by CEX and how to characterize the identity and potency of these attributes.
   - 2.3. RP-HPLC – Reference prior work in the literature on development of the RP-HPLC method. Show the chromatogram
     - 2.3.1. RP-HPLC with MS – Discuss peak ID's by mass spec and potential quality implications.
   - 2.4. HIC-HPLC – Show chromatogram. Discuss utility as an orthogonal method to RP-HPLC perhaps for collecting fractions for bioassay.
3. Electrophoresis Assays – David Michels to author
   - 3.1. Size Heterogeneity – Discussion about orthogonal methods
     - 3.1.1. SDS-PAGE completed in Kurt Brorson's lab.
     - 3.1.2. CE-SDS – Review of its intended purpose for characterization and lot release. Profiles for both UV and LIF detection to be presented with discussion about profile differences and sensitivity. Criticality of sample preparation is imperative to minimize artifacts related to denaturing conditions and reactive dye chemistry.
     - 3.1.3. MCE-SDS – Compare microchip profiles to those obtained by a capillary separation with discussion focused on desires for high-throughput applications
   - 3.2. Charge Heterogeneity – Discussion about orthogonal methods, applications for both identity (qualitative) and determination of charge distribution (quantitative).
     - 3.2.1. CZE – Free solution separation based on charge/mass ratio, provide electropherograms with intent to compare to orthogonal methods (CIEF).
     - 3.2.2. CIEF – Common two-step method on the Beckman platform combined with their commercialized kit requires both focusing and chemical mobilization (hydrodynamic mobilization will not be explored); determination of apparent pI value

3.2.3. ICIEF – Whole-column imaging detection eliminates the need for mobilization, compare charge distribution to CZE and CIEF; determination of apparent pI value

3.3. Characterization – discussion about common ways to characterize minor forms present in CE-SDS and CIEF; the need for indirect identification with various fractionation techniques.

Ch 13. Biophysical

Yatin Gokarn
William Weiss, Eli Lilly
Thomas Laue, U of NH
Sambit Kar, Pfizer
Nickolas Anastasiou, Pfizer
Kelly Arthur, Amgen
John Gabrielson, Amgen
Vladimir Razinkov, Amgen
Riki Stevenson, Amgen
David Hayes, Boehringer-Ingelheim
Dan Zarraga, Genentech

1. **Introduction**
    1.1. mAbs as the dominant therapeutic modality for treatment of disease
    1.2. maturation of mAb technology
        1.2.1. industrialization of mAbs – lower barrier for entry
    1.3. evolution of mAb based therapies (ADCs, bispecific, novel mAb based formats)
    1.4. The need for biophysical characterization (BC) for mAbs
    1.5. Complexity of higher order structure
    1.6. How does and how can BC help in comparability assessment across the development cycle
    1.7. Different techniques and structural information: overview cartoon (see next slide)
    1.8. Chapter objectives
        1.8.1. illustrate use of biophysical techniques for characterizing mAbs
        1.8.2. guide the reader on how to choose experimental conditions/instrument parameters – stress that this is a guide/starting point and not prescriptive protocol
        1.8.3. Understand the intra-lab and inter-lab variability from such methodologies across different labs
2. **Experimental (for each technique)**
        2.1.1. What information does the technique provide?
        2.1.2. How does it relate to the control system?
        2.1.3. How is it complementary/orthogonal to the other biophysical techniques
        2.1.4. Special experimental requirements/care that needs to be taken?
        2.1.5. What are some of the pitfalls to avoid in data interpretation?
        2.1.6. **Table with instrument parameters and rationale for each**
3. **Discussion**
        3.1.1. integrating the results to weave a more complete picture
        3.1.2. What did we learn from each technique
        3.1.3. What did we learn about the molecule
        3.1.4. How does this picture relate to one from other techniques
        3.1.5. precision (interlab and intralab)
4. **Conclusions**
    4.1. we'll get to it after integrating all the data

Techniques

- CD
  - Near-UV-CD
  - Far-UV-CD
- Intrinsic Fluorescence
- FTIR (
- Raman spectroscopy (Sathya)
- DSC
- DLS
  - Size
  - k_D*
- SLS
  - MW and size distribution (SEC)
  - B2 (bulk)*
- SV-AUC
  - Size & MW
  - Size Distribution
- Electrophoresis (effective charge)
  - MCE*
  - Cap-CE*

Ch 14. Formulation/Developability

Ch 15. Protein Particulates

Linda Narhi, Amgen
Dean Ripple, NIST

Outline

1.1. Discuss the concept that particle type and number depends on the combination of mAb properties, formulation, choice of container, and the applied stress.
1.2. Strategy and goals of counting/characterizing particles (optimize mAb/formulation/closure to minimize particle load and susceptibility to particle generation; characterize species of subpopulations; identify any anomalous behavior that could signal increased risk to safety or efficacy; establish CQAs)

2. Analysis techniques: Sample Handling
   - 2.1.1. 3.1. Introduction
   - 2.1.2. 3.2. Handling/resuspension
   - 2.2. Sample stability
   - 2.3. Degassing
   - 2.4. Interferences
3. Analysis techniques: general discussion
   - 3.1. Introduction
   - 3.2. Repeatability, linearity, accuracy, limit of quantification for single-particle methods
   - 3.3. Particle orientation
   - 3.4. Sampling: small sample sizes; esp. an issue with < 1 um methods
   - 3.5. Filtration for characterization
4. Metrology: light obscuration
   - 4.1.1. 5.1 Overview
   - 4.1.2. 5.2 Sample handling
   - 4.1.3. 5.3 Linearity, Repeatability, Accuracy, and Limit of Quantitation
   - 4.1.4. 5.4 Quality Assurance and Troubleshooting
5. Metrology: flow imaging
   - 5.1.1. 6.1 Overview
   - 5.1.2. 6.2 Sample handling and sample characteristics
   - 5.1.3. 6.3 Linearity, Repeatability, Accuracy, and Limit of Quantitation.
   - 5.1.4. 6.4 Quality Assurance and Troubleshooting
6. Advances in analytical methods: principle; size range; potential benefit
   - 6.1. Introduction
   - 6.2. Resonance mass for silicone droplets
   - 6.3. Electrical Sensing Zone
   - 6.4. Nanoparticle Tracking Analysis
   - 6.5. Flow cytometry: challenge on understanding fluorophore, calibration
   - 6.6. Atomic Force Microscopy, liquid state
   - 6.7. Asymmetric Field Fluid Flow Fractionation: separation without membrane clogging
7. Conclusions

Ch 16. Process Related impurities

Kesh Prakash, MedImmune
Weiben Chen, Waters

1. General introduction to HCPs and other process-related impurities
2. Regulatory Guidance (if any)
3. Immunogenicity pertaining to HCPs and other Quality issues related to HCPs
4. Some considerations for Manufacturing consistency and characterization
5. Technologies (immunological and non-immunological) for detection and quantitation of HCPs and other process-related impurities
6. Characterization and development of reagents with particular reference to HCP critical reagents
7. GMP testing
8. Life cycle management
9. Summary of data obtained
10. Conclusions

Book 3: Emerging Technologies for Product and Process Understanding
Emerging technologies- Will be written mainly by equipment manufacturers (OEMs) and academic researchers. Book 2 was organized along the same lines as characterization groups would be within a pharma org. Book 3 will have chapters focusing on unmet needs
Section 1. Emerging Product Characterization Techniques

Ch 17. The Protein Characterization Lab of Tomorrow
A vision of what characterization methodologies and practices are needed as the industry matures. This chapter will give an introduction to next generation analytics for product characterization.

Outline
1. Begin with echoing previous discussion on complexity of monoclonal antibodies and production landscape.
2. Discuss what drives the development of new technologies.
   2.1. Unmet characterization needs and improved product knowledge,
   2.2. Competition,
   2.3. Regulatory requirements and expectations,
   2.4. Cost effectiveness,
   2.5. Manufacturing needs (high throughput, PAT, etc)
   2.6. Give examples of method lifecycle and maturation.
      2.6.1. CE-SDS vs. SDS-PAGE
      2.6.2. Peptide mapping
3. Shifting paradigm from a method-centric to attribute-centric analytics
4. Discuss expectations from the lab-of-the-future based on the survey results.
5. Brief overview of emerging technologies covered in chapters of Book 3.

Ch 18. Higher Order Structure

John Marino, NIST
Jeff Hudgens, NIST
Rob Brinson, NIST
Jane Langden, NIST
Travis Gallagher, NIST
Richard Huang, NIST

1. Higher-Order Structure:
    1.1. Protein Folding
        1.1.1. primary sequence to tertiary folds
        1.1.2. Post-translational Modification
        1.1.3. Energetics and Fold stability
    1.2. Protein Flexibility and Dynamics
    1.3. mAb architecture – domain structures
        1.3.1. Light chain
        1.3.2. Heavy chain
        1.3.3. CDRs, Fv, Fab and Fc Regions
        1.3.4. Immunoglobulin fold
        1.3.5. Epitopes
    1.4. The Structure-Function Relationship
    1.5. Quaternary and agglomerate structure

2. High-Resolution mAb Structure from X-ray Crystallography
    2.1. mAb structures
        2.1.1. full antibody
        2.1.2. Fc structures
        2.1.3. Fab structures
    2.2. mAb complexes
3. Current Low Resolution Methods for Higher Order Structure Assessment
    3.1. FT-IR, Fluorescence and CD Spectroscopies
    3.2. Thermal Stability – DSC
4. Emerging Technologies for Higher Resolution Structure Assessment
    4.1. High Resolution Solution NMR
        4.1.1. 1D and 2D NMR methods
        4.1.2. Isotope Editing
        4.1.3. Spectral ‘finger printing’ of protein biologics
        4.1.4. Glycan mapping
        4.1.5. Spectral ‘finger printing’ mAb domains
5. HDX Mass Spectrometry
        5.1.1. HDX description
        5.1.2. mAb structure/dynamics mapping
        5.1.3. Glycan mapping
        5.1.4. Epitope mapping
6. Current Technology Limits and Future Research Efforts

Ch 19. Aggregation
Allistair Kippen, MedImmune
Rick Remmele, MedImmune

1. Mechanism
    1.1. Common causes of recombinant protein aggregation [Jared B./Jifeng Zhang/Rick]
        1.1.1. Protein-protein self-association (Jifeng)
        1.1.2. Protein unfolding (Jifeng/Jared)
        1.1.3. Chemical modification(David Mo)
        1.1.4. Stress dependent properties (Jared)
            1.1.4.1. Temperature
            1.1.4.2. Shear
            1.1.4.3. Surface interfaces
            1.1.4.4. Concentration
2. Physico-Chemical Properties of mAbs affecting aggregation propensity
    2.1. pI [Jifeng]
    2.2. Electrostatic properties [Jifeng]
    2.3. Surface hydrophobicity [Rick/Jifeng]
    2.4. Post-translational modifications [Viv]
3. Conventional approaches to elucidate and characterize antibody aggregates
    3.1. Covalent vs non-covalent
        3.1.1. Isotype variants between IgG1, IgG2, and IgG4s [Dan Higazi/David Mo]
        3.1.2. Engineered Fc's (Dan Higazi/Jifeng Zhang)
    3.2. Separation and purification
        3.2.1. Whole antibody aggregates [David Mo]
        3.2.2. Proteolytic digests of aggregates [Dan Higazi]
        3.2.3. ? Downstream removal of aggregates [Alistair]
    3.3. Biophysical (low resolution structural techniques)
        3.3.1. FTIR[Jared]
        3.3.2. CD (near and Far-UV)[Alistair/Jared]
        3.3.3. Fluorescence[Alistair/Jared]
        3.3.4. AUC (aggregate ID with orthogonal SEC)[David/Jared]
        3.3.5. Dye-Tht reagent methods [Alistair]
        3.3.6. SEC detection of soluble aggregates? sections within BLA [David]
4. Emerging Technologies
    4.1. Imaging [/Rick]
        4.1.1. AFM
        4.1.2. TEM
        4.1.3. ?
    4.2. High resolution structural techniques
        4.2.1. HDX-MS, Cross-linking technologies (e.g. CovalX) [JJ/Viv]
        4.2.2. NMR, isotope mapping [Rick/David Mo]
        4.2.3. FPOP[JJ/Viv]
        4.2.4. Native & Ion Mobility Mass Spectrometry[Viv]
        4.2.5. SAX (Jared)
        4.2.6. Neutron Scattering (Jared)

5. Predictions based fundamentals derived from Protein Properties [Dan H.]

Ch 20. Intact Protein Mass Spectrometry

Patrick Bennett, Thermo
Zhiqi Hao, Thermo
Scott Lute, FDA
Kurt Brorson, FDA
Michael Boyne, FDA

Introduction

A review that covers

1. The importance of charactering antibody at intact protein level, from scientific and regulatory point of view
2. Currently used technology and challenges for intact antibody analysis
3. Orbitrap technology and recent improvements for intact protein analysis.
4. General roles for protein analysis using Orbitrap: desolvation, AGC control, resolution, spectral averaging, etc

1. **20.1 Intact antibody mass measurement**
    1.1. Mini review of intact antibody mass measurement.
2. **20.1.1. Measurement of average mass**
    2.1. Sample: mAb: NIST /Waters/DR2, glycosylated and deglycosylated
    2.2. LC-MS, E plus or/and QE
    2.3. Spectrum quality, accuracy and reproducibility of intact mass, glycoform relative abundance,
    2.4. Spectrum deconvolution using Protein Deconvolution SW
3. **20.1.2. Intact mass of ADC**
    3.1. Sample: Thermo ADC standard
    3.2. QE/QE plus or E plus
    3.3. LC-MS
    3.4. Spectrum quality, accuracy of measurement, deconvolution.
4. **20.1.3. Measurement of monoisotopic mass**
    4.1. Sample: NIST/Waters/DR2, Heavy and light chain
    4.2. LC-MS, QE plus and Elite/Fusion
    4.3. Spectrum quality, accuracy of measurement. Deconvolution
5. **20.1.4. Native MS of antibody**
    5.1. A mini review of why and when Native MS is necessary, critical instrument features, sample prep tips for native MS
    5.2. Sample: ADC, antibody mixture, antibody and antigen complex
    5.3. SEC LC-MS, E plus EMR
    5.4. Spectrum quality,
    5.5. Deconvolution
6. **20.2 Top-down and middle-down sequencing of antibody**
    6.1. Mini review of top-down sequencing
    6.2. 20.2.1. Top-down sequencing using ETD
    6.3. 20.2.2. Top-down sequencing using HCD
    6.4. Sample: Waters/NIST/DR2
    6.5. QE /QE plus, Elite/Fusion, ETD and HCD fragmentation,

6.6. Spectrum quality, sequence coverage, disulfide linkage confirmation complimentary coverage from ETD/HCD,

6.7. Data analysis using ProSightPC

7. 20.3. Applications (Applied discussion)

7.1. From crude cell extract to MS characterization

7.2. 2D LC-purification- LC/MS-data analysis

8. 20.4. Regulatory Perspective

9. ----

10. Conclusions

10.1. Summary

10.2. Trends of technology innovation for intact antibody characterization

10.3. Towards MS analysis into regulatory QC

Ch 21. Covalent Labeling Techniiques for Higher Order Structure

Mark Chance, Case Western University
Parminder Kaur, Case Western University

1. -Introduction:
    1.1. Mass spectrometry based methods for analyzing the higher order structure of proteins
    1.2. Covalent Labeling (CL) approaches: Hydroxyl radical based methods, chemical reagent based techniques
    1.3. Literature survey of CL labeling techniques applied to monoclonal antibodies (mAb)
    1.4. Structural characterization of reference Igg1 using hydroxyl radical footprinting via synchrotron radiation methodology
    1.5. Characterization of reference Igg1 using carboxyl group labeling techniques

2. -Methods
    2.1. Description of sample preparation, labeling, and mass spectrometry protocols
    2.2. Data analysis pipeline employed

3. -Results
    3.1. Evaluation of the authenticity of the sample using 1 D Gel
    3.2. Total sequence coverage analysis
    3.3. Results from hydroxyl radical footprinting
        3.3.1. Analysis of oxidized residues
        3.3.2. Quantitative characterization of oxidatively labeled peptides using dose response plots
        3.3.3. Summary table of oxidized tryptic peptides of Igg1
    3.4. Results from Carboxyl group labeling
        3.4.1. Analysis of labeled residues
        3.4.2. Quantitative characterization of labeled peptides using dose response plots
        3.4.3. Summary table of labeled peptides

    3.5. Mapping of labeled residues from the two approaches onto Igg1 homology models
    3.6. Comparison of results from the two techniques
    3.7. Applicability of the results obtained in various contexts
    3.8. Discussion of overall findings
4. Future directions

Ch 22. Ion mobility
Primary-open
Potl. assays-

Ch 23. Microfluidics

Gregory Staples, Agilent
Hongfeng Yin, Agilent

1. Introduction to Microluidic Analysis of mAbs and Biotherapeutics
2. Peptide Mapping
    - 2.1.1 Protein ID chip analysis of mAb peptides
    - 2.1.2 glycopeptide chip analysis of mAb glycopeptides
3. Released Glycans
    - 3.1.1 mAb-glyco-chip analysis of released glycans
    - 3.1.2 HILIC chip analysis of released glycans
4. Intact Protein
    - 4.1.1 2100 Bioanalyzer analysis of intact protein and fragments
    - 4.1.2 Protein Chip analysis of intact protein and fragments
5. Discussion

Ch 24. Automation

Darryl Davis, Janssen

Abstract

Many labs within biopharma are not automated however in fact every assay and analysis can be carried out by liquid handlers, robotics, and the analysis can be automated as well. This trend continues even while successful applications using automation are on the rise. The common rationale for not automating processes is due to a low to medium sample number. The number of samples necessary to process is however secondary to the needs and benefits of automation. This chapter will detail the types of automation that have been employed, the significance of the CVs from experiments using automated liquid handling techniques, the more involved experimental plans that can be undertaken by using automation and the types of analysis that can be automated.

Outline

1. *Introduction*
   1.1 Liquid handler background
   1.2 Robotics background
   1.3 Conversion of manual methods to automated ones
2. *Experimental*
   2.1 Process automation
      2.1.1 Chromatography
      2.1.2 Modeling
   2.2 Analytical assay automation
      2.2.1 Peptide mapping
      2.2.2 Intact molecular weight
      2.2.3 Stability indicating assays
      2.2.4 Impurity
   2.3 Formulation automation
   2.4 Automation of the automation
3. *Discussion*
4. *Conclusion*

Ch 25. Informatics

Wenzhou Li, Amgen
Oleg Borizov, Amgen
Hua Xu, Pepsico

1. Introduction
2. Informatics for metabolomics
3. Informatics for Proteomics (Hua Xu)
4. Informatics for Peptide Mapping (Oleg Borisov and Wenzhou Li)
   4.1 Goal of peptide mapping
      4.1.1 Sequence confirmation (SVA)
      4.1.2 Modification monitoring (oxidation, deamidation, etc.)
      4.1.3 Impurity Monitoring (HCP)
   4.2 Data Processing
      4.2.1 Peak detection / alignment
      4.2.2 Identification
      4.2.3 Quantification
   4.3 Software and Data Analysis
      4.3.1 Difference between proteomics and peptide mapping
      4.3.2 Limitations of proteomics software (mascot, seqeust) for product characterization
      4.3.3 Software specifically designed for mAb peptide mapping (mass analyzer, mass hunter, biopharmaLynx): specifically designed modification library, better peak alignment and quantification.
      4.3.4 False positive control
      4.3.5 Common misidentifications
      4.3.6 Search strategies (Peptide map, HCP, SVA)
   4.4 The value of retention time data in peptide identification
5. Informatics for Global Data Integration

Section 2. Emerging Process-Related Technologies
Ch 26. In-process, Process Development, PAT and QbD
Steve Mehrman, Janssen
John Cunningham, Janssen

Ch 27. Proteomics

Yazen Jmeian, Janssen

Proteomics has lagged behind genomics due to the inherent complexity of the proteome arising from (i) the wide dynamic protein concentration range extending over 10 orders of magnitude; (ii) the very large numbers of proteins and their diversity due to post-translational modifications; (iii) the lack of powerful methods to amplify low level proteins analogous to the polymerase chain reaction (PCR). In recent years, major advances in proteomics have been made. The first part of this chapter is concerned with the samples preparation and prefractionation, separation of tryptic peptides and mass spectrometric (MS) identification. The second part of this chapter will focus on the emerging proteomic technologies applied in the drug discovery process. For example, the application of proteomics approaches for the identification of monoclonal antibodies and the use of activity based protein profiling in drug discovery will be discussed. In addition, recent advances in affinity purification-MS the application of this technology in the identification of protein-protein interactions will be highlighted.

Ch 28. Metabolomics

Primary- Open

Ch 29. Recent developments in analytical methods for host cell protein analysis

Weiben Chen, Waters
Kesh Prakash, MedImmune

1. Introduction - why HCP analysis and regulation requirement
2. Analytical challenges for host cell protein analysis
3. Overview on the common methodologies for HCP analysis and the limitations
4. LC/MS analysis of HCPs – methods & performance
    4.1. 1D LC-MS
    4.2. 2D LC-MS
5. Case studies – Application of 2D-LC/MS methods for HCP analysis
    5.1. Reference samples – Waters mAb standard and NIST samples studies & comparison against ELISA assay
    5.2. Process development
    5.3. Tracking individual HCP across purification stages
    5.4. Understand the HCP nature of drug substance (?)

6. Summary and Future development

Chapter 30. Adventitious Agent Testing of Biologicals; Changing to a new frontier of technology.

Dieter Schmalzing, Genentech
Ivar Kljavin, Roche-Genentech
Kevin McCarthy, Roche-Genentech

1. Testing should only augment several viral risk mitigation strategies.

2. In Vitro Viral Detection, Design & Consideration.
   - 2.1.1.1. Where testing occurs in the process - most likely to detect adventitious agents.
   - 2.1.1.2. On each lot of a production run.
   - 2.1.1.3. Bulk harvest: Best chance of amplification and detection before further manipulation of the sample.
   - 2.1.1.4. Raw materials: On a sub-aliquot of the material, i.e., serum or other RM, i.e., peptone batch.
   - 2.1.1.5. Classical virology & cell culture practices by examination of a viral effect at an end-point.
   - 2.1.1.6. Basic design of viral detection
   - 2.2. End points of indicator cell morphological changes, or hemadsorption/hemagglutination of erythrocytes.
   - 2.3. Presumptive identification of virus isolates can usually be made on the basis of the type of CPE, haemadsorption, and selective cell culture susceptibility.
3. Methods can not definitely identify the virus – use of immunofluorescence antibody staining, neutralization, electronmicroscopy, nucleic acid based detection.
4. When CPE is detected: Infectious nature is determined by sub-passage onto a fresh preparation of effected indicator cell to demonstrate propagation.
5. Inherent Issues with Cell based Viral Detection.

   5.1.1.1.1. Table of cell based viral/adventitious agent testing

6. •**Lengthy testing time period**
7. *Potential business and supply chain impacting*
8. •**Methods require an element of several levels of expertise**
9. *Quality, Cell culture, Cell Biology, Virology – adventitious agent.*
10. •**Prove the negative**
11. *Importance of sample handling, cell culture, etc.*
12. •**Scope of viral agent detection has a limit**
13. *Impractical to screen for all theoretical viruses.*

14. •**Not all viruses can induce microscopically noticeable cytopathic effect (CPE) and hemadsorption.**
15. *Not all virus may replicate in test method systems, or maybe replicate without any visible effect on the indicator cells*.

16. •**Demonstrating infectivity/further propagation may not always be possible with an infectious agent.**
17. *Not all isolates remain infectious with subpassage.*

17.1.1.1.1. Table of cell based viral/adventitious agent testing - “Industry direct experience”

18. •**False “negatives”:**
    - 18.1.1.1. •Inactivation of the virus itself.
    - 18.1.1.2. •Decrease infectivity potential of the indicator cells.
    - 18.1.1.3. •Effects from the sample matrix.
    - 18.1.1.4. •Specific impact from the biological activity of the product.
    - 18.1.1.5. •Variations in cell culture maintenance.

19. •**False “positives”:**
    - 19.1.1.1. •Sample matrix may induce morphological changes.
    - 19.1.1.2. •Product or process matrix biological activity.

20. PCR detection of adventitious agents, design & considerations

    20.1. Brief background about PCR discovery/development

    20.2. Target DNA or RNA sequence selection

    - 20.2.1. Conserved DNA sequences to detect different bacteria, i.e. 16s rDNA gene
    - 20.2.2. Specific DNA sequences for more targeted detection, i.e. surface protein genes
    - 20.2.3. mRNA or tRNA targets (require a short reverse transcriptase step)
    - 20.2.4. Organisms with RNA genomes (require a short reverse transcriptase step)

    20.3. Design and selection of primers (and probes)

    - 20.3.1. Selection of a target sequence with an appropriate length
    - 20.3.2. Commercially available primer and probe sets
    - 20.3.3. Many options for self-designing primers and probes
        - 20.3.3.1. Software available for primers and probes selection based on target sequence input (i.e. Primer Express®)
        - 20.3.3.2. Consideration given to: Melting Temperature, G-C content, primer dimers, hairpin structures

    20.4. Discussion of a basic PCR setup

    - 20.4.1. Cycle parameters
        - 20.4.1.1. Adjusting annealing temperature allows for optimization of specificity, i.e. general detection for many different species or highly specific detection for one or two species
    - 20.4.2. Master mix design and optimization

    20.5. Options for Automated DNA extraction

    - 20.5.1. Automated extractions is more efficient and consistent
    - 20.5.2. Automation provides for a higher throughput then Manual extractions

    20.6. Wide array of available amplification/detection platforms (partial list):

    - 20.6.1. Normal PCR, uses gel based detection
    - 20.6.2. Touch Down PCR, uses gel based detection

20.6.3. Real Time PCR (i.e. TaqMan® based), uses instrument based detection
20.6.4. Real Time PCR (i.e. Hoechst dye based) , uses instrument based detection
20.6.5. Reverse Transcriptase Real Time PCR, uses instrument based detection
20.6.6. Multi Plex Real Time PCR (TaqMan® based) , uses instrument based detection

20.7. Assay control selection
20.7.1. Internal (extraction) Control: "model Organism" spiked into sample
20.7.2. Negative extraction Control with Internal Control spike
20.7.3. PCR negative control: water
20.7.4. PCR positive Control: plasmid

20.8. Generic Real Time PCR test method example

21. Inherent issues with PCR methods
21.1. No delineation of viable versus non-viable organisms
21.2. Non-specific amplification and amplification artifacts: primer dimers, probe degeneration (can be mitigated through proper primer design and PCR optimization)
21.3. Requires expensive capital equipment

21.4. Requires equipment IQ/OQ and PQ services as well as regular PM services
21.5. Possible equipment compliance issues (i.e. 21CFR part 11)
21.6. Requires specific PCR suite design to minimize cross contamination of possibly contaminated samples, especially into the master mix room

22. Advantages of the PCR based over the Cell based technology
22.1. Able to design the desired specificity (through primer probe design)
22.2. Faster and more consistent results, <1day
22.3. Little analyst manipulation required when using automated systems
22.4. High throughput using automated systems, up 96 detection at one time depending on the system used
22.5. Incorporation of Uracil-N-Glycosylase in the master mix in conjunction with replacing deoxythymidine triphosphate with deoxyuridine triphosphate eliminates possible cross contamination from amplification products

Ch 31. Summary

This chapter will serve to discuss the findings of this collaborative and extensive effort regarding characterization of a mAb. This section will serve to make an observation of the current state of the art technologies in book 2 and highlight how the emerging technologies of book 3 may lead to more in-depth product understanding.