

**PLATFORM DEVELOPMENT FOR CHARACTERIZATION
OF CRITICAL QUALITY ATTRIBUTES OF
BIOPHARMACEUTICAL PRODUCTS**

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by

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Submitted

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Certificate

This is to certify that the thesis entitled “**PLATFORM DEVELOPMENT FOR CHARACTERIZATION OF CRITICAL QUALITY ATTRIBUTES OF BIOPHARMACEUTICAL PRODUCTS**” being submitted by **HIMANSHU MALANI** to the Indian Institute of Technology Delhi for the award of the degree of **DOCTOR OF PHILOSOPHY** is a record of the original bonafide research work carried out by him under my guidance and supervision. The results contained in this thesis have not been submitted in part or in full to any other University or Institute for the award of any degree or diploma.

I certify that he has pursued the prescribed course of research.

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HIMANSHU

Abstract

The present thesis focuses on characterization of critical quality attributes (CQA) of biopharmaceutical products. Assessment of factors affecting process intermediates and final product quality is a penultimate requirement for designing robust products in the Quality by Design (QbD) paradigm.

Production of recombinant proteins in microbial systems leads to formation of inactive aggregated protein deposits known as inclusion bodies (IBs). This phenomenon occurs as bacterial cells lack the requisite machinery for protein folding. These IBs need to be refolded to get the bioactive protein. Understanding how the microenvironment, in particular fermentation growth medium, affects the quality and quantity of the IBs is desirable for optimal production of proteins. Chapter 3 explores the effect of various chemically defined media components on IBs using a variety of analytical techniques to establish them as a marker of final product titre and host cell protein (HCP) content. Expression of a Fab biotherapeutic, ranibizumab, has been chosen as a case study. A repertoire of nutrients, including but not limited to carbon and nitrogen sources, vitamins, cofactors, buffering agents and minerals, were examined. Sucrose, biotin, pantothenate, glutathione, K_2HPO_4 , K_2SO_4 , cyanocobalamin and $CaSO_4$ were shortlisted based on experimentation. A design of experiments (DOE) study was performed to identify optimal concentrations of and interactions between these nutrients. Results reveal that higher concentrations of sucrose, biotin and pantothenate, and lower concentrations of cyanocobalamin, calcium sulfate ($CaSO_4$) and glutathione, resulted in larger IBs and greater product expression. Further, dipotassium phosphate (K_2HPO_4) and potassium sulfate (K_2SO_4) did not seem to have a significant impact on product titre. Medium composition also greatly influences IB size and corresponding quality of final product. The size of IBs exhibits a positive correlation with the quantity of product expressed, as well as the purity of the product, but does not correlate with the total amount of host cell proteins produced. IB size could be used as a key process attribute during bioreactor optimization, and the otherwise difficult task of measuring product concentration in the fermentation broth could be avoided. Chapter 4 focuses on heterogeneity assessment and stability characterization of monoclonal antibody (mAb) products. Of late, the focus of mAb stability studies has largely been on the combined evaluation of both size and charge heterogeneity rather than solely focusing on size heterogeneity. Changes in charge variant profile are known to affect mAb stability and vice versa. In this chapter we explore the effects of magnesium metal (0.5 mM Mg^{2+}) on stability

of trastuzumab (IgG1 antibody). Mg^{2+} is often used as an excipient (50-100 mM) and lubricant (5-10% w/w) in biopharmaceutical formulations. Trastuzumab biosimilar was incubated at 37°C with and without magnesium sulphate. Analytical size-exclusion chromatography (SEC) and cation-exchange chromatography (CEX) coupled with mass spectrometry (MS) were used to evaluate the size and charge heterogeneity in the thermal and metal stressed samples and compared to the control sample (room temperature). The present study unveils that presence of Mg^{2+} significantly increases the rate of aggregation with 9% aggregation observed in Mg^{2+} stressed samples as compared to that from thermal stress (~2%) or control sample (<1%). Similarly, a 2-fold elevation in acidic variants was observed both in the presence of Mg^{2+} and thermal stress, when contrasted with the control sample. Application of stress also led to the formation of 17 additional chemical modifications (7 due to thermal stress and 10 due to Mg^{2+} stress) which were not identified in control, predominantly involving deamidation, isomerization of aspartic acid, oxidation, and succinimide modifications. The results indicate the need for a detailed analysis of the impact of presence of metals in biotherapeutic formulations.

Next, we investigate a potential correlation between the two key CQAs that undergo simultaneous alterations. Aggregation stability of monoclonal antibody (mAb) therapeutics is influenced by many critical quality attributes (CQA) such as charge variants in addition to environmental factors. Chapter 5 investigates the potential correlation between charge heterogeneity and aggregation propensity of prominent mAbs (bevacizumab and trastuzumab) under a variety of conditions such as thermal stress at 40°C and 55°C, shaking (mechanical), and low pH stress. Size- and charge-based heterogeneities were monitored using analytical size exclusion chromatography and cation exchange chromatography, respectively while dynamic light scattering was used to assess changes in hydrodynamic size. CEX analysis revealed an increase in cumulative acidic content for all variants of both mAbs post-stress treatment attributed to increased deamidation. Higher charge heterogeneity was observed in variants eluting close to the main peak than the ones eluting further away (25-fold and 42-fold increase in acidic content for main and B1 of bevacizumab and 19-fold for main of trastuzumab, respectively, under thermal stress; 50-fold increase in acidic for main and B1 of bevacizumab and 10% rise in basic content of main of trastuzumab under pH stress). Conversely, variants eluting far away from the main exhibit greater aggregation as compared to close-eluting ones. Aggregation kinetics of variants followed different order for the different stresses for both mAbs (second order for thermal and pH stresses and zero order for shaking stress). Half-life of terminal charge variants of both mAbs was 2- to 8-fold less than main indicating increased

degradation propensity. Since charge variants are an important CQA for protein therapeutics, this methodology could be beneficial in assessing charge variant mediated stability of other popular modalities, including but not limited to, Fc-fusion proteins, bispecific antibodies and antibody-drug conjugates.

Having established charge variants as a key influencer of other product CQAs and overall mAb stability, we evaluated the biological effects of charge variants as an integral part of mAb characterization train. While aggregates are believed to be immunogenic, the impact of mAb charge variants has not received much attention. Chapter 6 aims to assess cytokine levels (IL-6, IL-10, IL-12, TNF- α , and IFN- γ) released by peripheral blood mononuclear cells (PBMC) and HER2 expressing target cancer cell line upon exposure to trastuzumab charge variants in an *in vitro* setting along with anti-proliferation action measurement against target cell line. A previously published mechanistical model has been utilized to elucidate cytokine expressions. The results reveal immediate elevation in pro-inflammatory cytokines (IL-6, IL-12, TNF- α) by PBMCs challenged with charge variants and slow increment in case of cancer cells. This also led to a corresponding increase in IL-10 levels which is a response to anti-inflammation. Dual natured IL-6 showed highest secretion of all cytokines suggesting an initial pro-inflammatory action with possible maintenance as anti-inflammatory agent. IL-6 also triggers release of TNF- α and IFN- γ . Acidic variants exhibited high levels of pro- and low levels of anti-inflammatory cytokines compared to basic and main variants along with reduced bioactivity (75% vs 100% of main). This suggests that acidic variants (asparagine deamidation) display higher immunogenic tendencies compared to basic variants (aspartate isomerization), likely due to more significant interactions between different basic-charged, highly glycosylated pattern recognition molecules. We hope that this investigation offers deeper understanding of how charge variants impact safety and efficacy of mAb therapeutics.

The results presented in this thesis provide a different characterization outlook of product CQAs so as to offer insights into structure-function relationship.

Abstract (Hindi)

वर्तमान थीसिस बायोफार्मास्युटिकल उत्पादों की महत्वपूर्ण गुणवत्ता विशेषताओं (CQA) के लक्षण वर्णन पर केंद्रित है। प्रक्रिया मध्यवर्ती और अंतिम उत्पाद गुणवत्ता को प्रभावित करने वाले कारकों का आकलन गुणवत्ता द्वारा डिजाइन (QbD) प्रतिमान में मजबूत उत्पादों को डिजाइन करने के लिए एक अंतिम आवश्यकता है।

माइक्रोबियल सिस्टम में पुनः संयोजक प्रोटीन के उत्पादन से निष्क्रिय एकत्रित प्रोटीन जमा का निर्माण होता है जिसे समावेशन निकाय (IB) के रूप में जाना जाता है। यह घटना तब होती है जब बैक्टीरिया कोशिकाओं में प्रोटीन फोल्डिंग के लिए आवश्यक मशीनरी की कमी होती है। बायोएक्टिव प्रोटीन प्राप्त करने के लिए इन IB को फिर से फोल्ड करने की आवश्यकता होती है। प्रोटीन के इष्टतम उत्पादन के लिए यह समझना वांछनीय है कि माइक्रोएनवायरनमेंट, विशेष रूप से किण्वन वृद्धि माध्यम, IB की गुणवत्ता और मात्रा को कैसे प्रभावित करता है। अध्याय 3 विभिन्न रासायनिक रूप से परिभाषित मीडिया घटकों के IB पर प्रभाव की खोज करता है ताकि उन्हें अंतिम उत्पाद टाइट्र और मेजबान सेल प्रोटीन (HCP) सामग्री के मार्कर के रूप में स्थापित किया जा सके। एक फैब बायोथेरेप्यूटिक, रैनिबिजुमाब की अभिव्यक्ति को एक केस स्टडी के रूप में चुना गया है। पोषक तत्वों की एक सूची, जिसमें कार्बन और नाइट्रोजन स्रोत, विटामिन, सहकारक, बफरिंग एजेंट और खनिज शामिल हैं, की जांच की गई। प्रयोग के आधार पर सुक्रोज, बायोटिन, पैन्थेनेट, ग्लूटाथियोन, K_2HPO_4 , K_2SO_4 , सायनोकोबालामिन और $CaSO_4$ को चुना गया। इन पोषक तत्वों की इष्टतम सांद्रता और उनके बीच परस्पर क्रिया की पहचान करने के लिए प्रयोगों का एक डिज़ाइन (DOE) अध्ययन किया गया। परिणाम बताते हैं कि सुक्रोज, बायोटिन और पैन्थेनेट की उच्च सांद्रता और सायनोकोबालामिन, कैल्शियम सल्फेट ($CaSO_4$) और ग्लूटाथियोन की कम सांद्रता के परिणामस्वरूप बड़े IB और अधिक उत्पाद अभिव्यक्ति हुई। इसके अलावा, डिपोटेशियम फॉस्फेट (K_2HPO_4) और पोटेसियम सल्फेट (K_2SO_4) का उत्पाद टाइट्र पर कोई महत्वपूर्ण प्रभाव नहीं पड़ा। माध्यम की संरचना भी IB आकार और अंतिम उत्पाद की संगत गुणवत्ता को बहुत प्रभावित करती है। आईबी का आकार व्यक्त उत्पाद की मात्रा के साथ-साथ उत्पाद की शुद्धता के साथ सकारात्मक सहसंबंध प्रदर्शित करता है, लेकिन उत्पादित मेजबान कोशिका प्रोटीन की कुल मात्रा के साथ सहसंबंध नहीं रखता है। बायोरिएक्टर अनुकूलन के दौरान आईबी आकार को एक प्रमुख प्रक्रिया विशेषता के रूप में इस्तेमाल किया जा सकता है, और किण्वन शोरबा में उत्पाद सांद्रता को मापने के अन्यथा कठिन कार्य से बचा जा सकता है।

अध्याय 4 मोनोक्लोनल एंटीबॉडी (mAb) उत्पादों की विषमता मूल्यांकन और स्थिरता लक्षण वर्णन पर

केंद्रित है। हाल ही में, mAb स्थिरता अध्ययनों का ध्यान मुख्य रूप से आकार और आवेश विषमता दोनों के संयुक्त मूल्यांकन पर रहा है, न कि केवल आकार विषमता पर ध्यान केंद्रित करने पर। आवेश भिन्न प्रोफ़ाइल में परिवर्तन mAb स्थिरता को प्रभावित करने के लिए जाने जाते हैं और इसके विपरीत। इस अध्याय में हम ट्रेस्टुजुमैब (IgG1 एंटीबॉडी) की स्थिरता पर मैग्नीशियम धातु (0.5 mM Mg^{2+}) के प्रभावों का पता लगाते हैं। Mg^{2+} का उपयोग अक्सर बायोफार्मास्युटिकल फॉर्मूलेशन में एक एक्सीपिएंट ($50\text{--}100 \text{ mM}$) और स्नेहक ($5\text{--}10\% \text{ w/w}$) के रूप में किया जाता है। ट्रेस्टुजुमैब बायोसिमिलर को मैग्नीशियम सल्फेट के साथ और उसके बिना 37°C पर इनक्यूबेट किया गया था। थर्मल और धातु तनाव वाले नमूनों में आकार और चार्ज विषमता का मूल्यांकन करने और नियंत्रण नमूने (कमरे के तापमान) से तुलना करने के लिए मास स्पेक्ट्रोमेट्री (एमएस) के साथ मिलकर विश्लेषणात्मक आकार-बहिष्करण क्रोमैटोग्राफी (एसईसी) और कैशन-एक्सचेंज क्रोमैटोग्राफी (सीईएक्स) का उपयोग किया गया था। वर्तमान अध्ययन से पता चलता है कि Mg^{2+} की उपस्थिति थर्मल तनाव ($\sim 2\%$) या नियंत्रण नमूने ($<1\%$) की तुलना में Mg^{2+} तनाव वाले नमूनों में 9% एकत्रीकरण के साथ एकत्रीकरण की दर को महत्वपूर्ण रूप से बढ़ाती है। इसी तरह, नियंत्रण नमूने के साथ तुलना करने पर, Mg^{2+} और थर्मल तनाव दोनों की उपस्थिति में अम्लीय वेरिएंट में 2 गुना वृद्धि देखी गई। तनाव के आवेदन से 17 अतिरिक्त रासायनिक संशोधनों (थर्मल तनाव के कारण 7 और Mg^{2+} तनाव के कारण 10) का निर्माण हुआ, परिणामों से जैव-चिकित्सीय योगों में धातुओं की उपस्थिति के प्रभाव के विस्तृत विश्लेषण की आवश्यकता का संकेत मिलता है।

इसके बाद, हम दो प्रमुख CQAs के बीच संभावित सहसंबंध की जांच करते हैं जो एक साथ परिवर्तनों से गुजरते हैं। मोनोक्लोनल एंटीबॉडी (mAb) चिकित्सा विज्ञान की एकत्रीकरण स्थिरता पर्यावरणीय कारकों के अलावा कई महत्वपूर्ण गुणवत्ता विशेषताओं (CQA) जैसे चार्ज वेरिएंट से प्रभावित होती है। अध्याय 5 विभिन्न स्थितियों जैसे 40 डिग्री सेल्सियस और 55 डिग्री सेल्सियस पर थर्मल तनाव, कंपन (यांत्रिक), और कम पीएच तनाव के तहत प्रमुख mAbs (बेवाकिजुमैब और ट्रेस्टुजुमैब) की चार्ज विषमता और एकत्रीकरण प्रवृत्ति के बीच संभावित सहसंबंध की जांच करता है। आकार और चार्ज-आधारित विषमताओं की निगरानी क्रमशः विश्लेषणात्मक आकार बहिष्करण क्रोमैटोग्राफी और धनायन विनिमय क्रोमैटोग्राफी का उपयोग करके की गई, जबकि हाइड्रोडायनामिक आकार में परिवर्तनों का आकलन करने के लिए गतिशील प्रकाश बिखराव का उपयोग किया गया। CEX विश्लेषण ने तनाव उपचार के बाद दोनों mAbs के सभी वेरिएंट के लिए संचयी अम्लीय सामग्री में वृद्धि का खुलासा किया, जो कि बढ़े हुए डिएमिडेशन के कारण था। मुख्य शिखर के करीब निकलने वाले वेरिएंट में दूर निकलने वाले वेरिएंट की तुलना में अधिक चार्ज विषमता देखी गई (थर्मल तनाव के तहत, बेवाकिजुमैब के मुख्य और बी1 के लिए अम्लीय सामग्री में क्रमशः 25 गुना और 42 गुना वृद्धि और ट्रेस्टुजुमैब के मुख्य के लिए 19 गुना

वृद्धि; पीएच तनाव के तहत बेवाकिजुमैब के मुख्य और बी1 के लिए अम्लीय में 50 गुना वृद्धि और ट्रैस्टुजुमैब के मुख्य की बुनियादी सामग्री में 10% वृद्धि। इसके विपरीत, मुख्य से बहुत दूर निकलने वाले वेरिएंट करीब निकलने वाले की तुलना में अधिक एकत्रीकरण प्रदर्शित करते हैं। वेरिएंट के एकत्रीकरण कीनेटिक्स ने दोनों mAbs के लिए अलग-अलग तनावों के लिए अलग-अलग क्रम का पालन किया (थर्मल और pH तनावों के लिए दूसरा क्रम और कंपन तनाव के लिए शून्य क्रम)। चूंकि प्रोटीन चिकित्सा के लिए चार्ज वेरिएंट एक महत्वपूर्ण सीक्यूए है, इसलिए यह पद्धति अन्य लोकप्रिय तौर-तरीकों की चार्ज वेरिएंट मध्यस्थता स्थिरता का आकलन करने में फायदेमंद हो सकती है, जिसमें एफसी-प्पूजन प्रोटीन, बाइस्पेसिफिक एंटीबॉडी और एंटीबॉडी-ड्रग कंजुगेट शामिल हैं, लेकिन इन्हीं तक सीमित नहीं हैं।

चार्ज वेरिएंट को अन्य उत्पाद CQAs और समग्र mAb स्थिरता के एक प्रमुख प्रभावक के रूप में स्थापित करने के बाद, हमने mAb लक्षण वर्णन ट्रेन के अभिन्न अंग के रूप में चार्ज वेरिएंट के जैविक प्रभावों का मूल्यांकन किया। जबकि समुच्चय को प्रतिरक्षात्मक माना जाता है, mAb चार्ज वेरिएंट के प्रभाव पर अधिक ध्यान नहीं दिया गया है। अध्याय 6 का उद्देश्य परिधीय रक्त मोनोन्यूक्लियर कोशिकाओं (PBMC) और HER2 व्यक्त लक्ष्य कैंसर सेल लाइन द्वारा इन विट्रो सेटिंग में ट्रैस्टुजुमाब चार्ज वेरिएंट के संपर्क में आने पर जारी साइटोकाइन स्तरों (IL-6, IL-10, IL-12, TNF- α , और IFN- γ) का आकलन करना है, साथ ही लक्ष्य सेल लाइन के खिलाफ प्रसार-रोधी क्रिया मापन करना है। साइटोकाइन अभिव्यक्तियों को स्पष्ट करने के लिए पहले से प्रकाशित एक यांत्रिक मॉडल का उपयोग किया गया है। इससे IL-10 के स्तर में भी इसी तरह की वृद्धि हुई, जो सूजन-रोधी की प्रतिक्रिया है। दोहरे स्वभाव वाले IL-6 ने सभी साइटोकिन्स का उच्चतम स्त्राव दिखाया, जो सूजन-रोधी एजेंट के रूप में संभावित रखरखाव के साथ एक प्रारंभिक प्रो-इंफ्लेमेटरी क्रिया का सुझाव देता है। IL-6 TNF- α और IFN- γ के स्त्राव को भी ट्रिगर करता है। अम्लीय वेरिएंट ने बुनियादी और मुख्य वेरिएंट की तुलना में एंटी-इंफ्लेमेटरी साइटोकिन्स के प्रो- और निम्न स्तरों के उच्च स्तरों को प्रदर्शित किया, साथ ही कम जैव सक्रियता (मुख्य का 75% बनाम 100%)।

यह बताता है कि अम्लीय वेरिएंट (एस्पेरिगिन डीएमिडेशन) बुनियादी वेरिएंट (एस्पार्टेट आइसोमेराइजेशन) की तुलना में उच्च इम्युनोजेनिक प्रवृत्ति प्रदर्शित करते हैं, जो संभवतः विभिन्न बुनियादी-चार्ज, अत्यधिक ग्लाइकोसिलेटेड पैटर्न पहचान अणुओं के बीच अधिक महत्वपूर्ण अंतःक्रियाओं के कारण होता है।

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