

AGGREGATION OF MONOCLONAL ANTIBODY BASED THERAPEUTIC PROTEINS

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by

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Dedicated to my family

Certificate

This is to certify that the thesis entitled “**AGGREGATION OF MONOCLONAL ANTIBODY BASED THERAPEUTIC PROTEINS**” being submitted by **ROHIT BANSAL** 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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Abstract

Protein biotherapeutics are becoming an important part of human health and are being used for treatment of wide number of severe diseases like various cancer forms, diabetes and ebola. Due to such high demand of these molecules, there is such a surge in the production and growth of these therapeutics that they are overtaking the net production and demand of small drug molecules. Despite the success of these biotherapeutics, there are hurdles that need to be overcome for ensuring smooth growth of biotherapeutics industry. A major issue is that of the stability of these biotherapeutics (Chapter 1).

The source of instability in biotherapeutics is multifaceted, which could be from production or fermentation during upstream processing, from purification or separation methods used during downstream processing or during filling, storage and shipping. Care must be taken at these different stages of production of biotherapeutics by appropriate monitoring to ensure consistent quality of drug product. The instabilities which are common in protein therapeutics are protein aggregation and fragmentation. These instabilities can reduce the safety and efficacy of the drug product by enhancing the product's immunogenicity or toxicity. The level of these instabilities has to be controlled below certain level in the final product to ensure safe and effective drug product release in the market. Therapeutic proteins encounter a range of environmental conditions such as extreme pH, buffers, temperature ranges and exposure to air-liquid interfaces which could make the protein product more labile to these degradations. Removing aggregation from a batch protein therapeutics, especially mAbs is a challenging task and involves extra cost incurrence (Chapter 2).

Kinetic modelling of protein using the quantification data obtained from SE-HPLC could be helpful for understanding the mechanism of aggregation. Studying kinetic modelling could help in controlling the rates of aggregation in drug product in various buffer conditions *via* their early prediction. We have attempted to use kinetic modelling of aggregation to predict the rate of aggregation in conditions prevailing during downstream purification of mAb therapeutics (Chapter 3). Lumry Eyring model and its modifications (Extended Lumry Eyring and Nucleated Polymerization) and Finke-Watzky model have been used successfully to predict the aggregation rates in the buffer conditions of mAbs purification. The rate of aggregation and aggregate formation mechanism have been understood by subjecting the mAb therapeutics to various buffer types, temperature conditions, pH and salt concentrations. The results obtained from experimental data have been compared to the model predicted values to identify the best fit model.

Apart from aggregation, another product instability impacting protein therapeutics' quality is fragmentation. Fragmentation leads to breakage of protein therapeutics into smaller entities having either no or limited functionality as well as huge impact on immunogenicity if introduced into patient's blood stream. Fragmentation study of mAbs was targeted at gaining knowledge about non-enzymatic fragmentation under extreme temperature conditions as the time progresses and mechanism development for the same (Chapter 4). Kinetics of fragmentation degradation was observed by incubating IgG1 based mAb products at high temperature of 50°C in PBS at pH 6.5 and sampling at regular time intervals. Samples were analysed using range of characterization tools like SE-HPLC, DLS, MS and SDS-PAGE. Mass spectrometry was used to measure the exact molecular weights of the fragmented species during course of study. Results obtained from experiments were fitted with different fragmentation mechanisms for obtaining better fit and calculating corresponding kinetic rate constants. Apart from this the model was also applied for another phosphate buffer system with different ionic strength. Temperature dependency of the model was verified using Arrhenius law.

For monitoring protein degradation, especially when the degraded products vary in their sizes, different analytical characterization tools are available which are routinely used by pharmaceutical scientists working in the domain of protein formulation development and stability. Manufacturers of biotherapeutic products spend a considerable time and resources on ways to avoid product degradation, monitor aggregate levels at different process points and the final product, and design their processes so as to reduce or remove aggregates and thereby ensure that the final product meets the desired specifications. We have performed a detailed investigation involving the analysis of a complete spectrum of aggregate species, from few nanometres sized oligomers to millimetre sized multimeric precipitates (Chapter 5). This was achieved by using three analytical tools, namely Size Exclusion Chromatography (SE-HPLC) (1 nm - 25 nm), Dynamic Light Scattering (DLS) (10 nm - 5 µm), and Micro Flow Imaging (MFI) (2 µm - 300 µm). Since each characterization tool covers a particular size range, data from multiple tools was collected in the "handover" regions to demonstrate accuracy of the platform. The proposed platform would be of utility to those engaged in formulation development as well as other aspects related to stability of biotherapeutic products.

During processing of biotherapeutics, especially mAbs, protein degradation in terms of aggregation by forming particles of various sizes and morphologies when subjected to physical, mechanical, and/or chemical stresses during manufacturing, storage, filling, formulation

development, and shipping. While there is widespread consensus that protein aggregation can enhance immunogenicity and affect the activity and cytotoxicity of the concerned product, the underlying immunological and biological mechanisms are not completely understood. We have investigated how invisible and sub visible particles generated by a variety of mechanical, thermal and chemical stresses impact biological activity of the product (Chapter 6). Amongst the various stresses considered, those generated when exposing the sample to stirring and extreme pH or temperature were seen to significantly impact product stability and immunogenicity, as confirmed by detailed analytical and biological characterization of the samples. Increased aggregation resulted in enhanced immunogenic response, as confirmed by cytotoxicity assays (ADCC and CDC) and reduced activity of the drug product, as confirmed by antigen binding assays (SPR). It was observed that extreme pH (pH 3.5 and pH 11.0), stirring (1D and 3D stir), and oxidation via CuSO₄ have the most impact on aggregate formation as well as potency of the therapeutic. In contrast, stresses from pipetting, milder pH (4.3 and 8.5), oxidation via H₂O₂, freeze-thaw (-80 °C and LN₂) have relatively less effect on potency of the mAb biotherapeutic. The results affirm that understanding of the mechanism of aggregation is critical for achieving consistent product quality.

It is hoped that the insights presented in this thesis would assist with adequate planning for examining, monitoring and controlling product degradation during production and storage so as to increase the shelf life and maintaining the activity of the drug product.

सार

प्रोटीन बायोथेराप्यूटिक्स मानव स्वास्थ्य का एक महत्वपूर्ण हिस्सा बन रहा है और इसका उपयोग विभिन्न प्रकार के गंभीर रोगों जैसे विभिन्न कैंसर रूपों, मधुमेह और इबोला के इलाज के लिए किया जा रहा है। इन अणुओं की इतनी अधिक मांग के कारण, इन उपचारकों के उत्पादन में इतनी वृद्धि होती है कि वे छोटे दवा अणुओं के शुद्ध उत्पादन और मांग से आगे निकल जाते हैं। इन बायोथेराप्यूटिक्स की सफलता के बावजूद, ऐसी बाधाएं हैं जिन्हें बायोथेराप्यूटिक्स उद्योग के सुचारु विकास को सुनिश्चित करने के लिए दूर करने की आवश्यकता है। एक प्रमुख मुद्दा इन बायोथेरेप्यूटिक्स (अध्याय 1) की स्थिरता का है।

बायोथेराप्यूटिक्स में अस्थिरता का स्रोत बहुक्रियाशील है, जो अपस्ट्रीम प्रसंस्करण के दौरान उत्पादन या किण्वन से, डाउनस्ट्रीम प्रसंस्करण के दौरान या भरने, भंडारण और शिपिंग के दौरान उपयोग किए जाने वाले शुद्धिकरण या पृथक्करण विधियों से हो सकता है। दवा उत्पाद की निरंतर गुणवत्ता सुनिश्चित करने के लिए उचित निगरानी द्वारा जैव-तंत्र के उत्पादन के विभिन्न चरणों में देखभाल की जानी चाहिए। प्रोटीन थेरेपी में जो अस्थिरताएं हैं, वे प्रोटीन एकत्रीकरण और विखंडन हैं। ये अस्थिरता उत्पाद की प्रतिरक्षा या विषाक्तता को बढ़ाकर दवा उत्पाद की सुरक्षा और प्रभावकारिता को कम कर सकती हैं। बाजार में सुरक्षित और प्रभावी दवा उत्पाद जारी करने के लिए इन अस्थिरताओं के स्तर को अंतिम उत्पाद में निश्चित स्तर से नीचे नियंत्रित किया जाना चाहिए। उपचारात्मक प्रोटीन अत्यधिक पीएच, बफर्स, तापमान पर्वतमाला और वायु-तरल इंटरफेस के संपर्क में आने से पर्यावरणीय स्थितियों की एक श्रृंखला का सामना करते हैं जो प्रोटीन उत्पादों को इन गिरावटों के लिए अधिक प्रयोगशाला बना सकते हैं। बैच प्रोटीन थेरेपी से एकत्रीकरण को हटाना, विशेष रूप से मैबस एक चुनौतीपूर्ण कार्य है और इसमें अतिरिक्त लागत वृद्धि (अध्याय 2) शामिल है।

एसई-एचपीएलसी से प्राप्त मात्रात्मक डेटा को उपयोग करके प्रोटीन का गतिकी मॉडलिंग एकत्रीकरण के तंत्र को समझने में मददगार हो सकता है। गतिकी मॉडलिंग का अध्ययन उनके प्रारंभिक भविष्यवाणी

के माध्यम से विभिन्न बफर स्थितियों में दवा उत्पाद में एकत्रीकरण की दरों को नियंत्रित करने में मदद कर सकता है। हमने मैब थैरेप्यूटिक्स (अध्याय 3) के डाउनस्ट्रीम शुद्धि के दौरान प्रचलित परिस्थितियों में एकत्रीकरण की दर का अनुमान लगाने के लिए एकत्रीकरण के गतिज मॉडलिंग का उपयोग करने का प्रयास किया है। लुमरी ईयरिंग मॉडल और इसके संशोधनों (एक्सटेंडेड लुमरी ईयरिंग एंड न्यूक्लियेटेड पॉलिमराइजेशन) और फिंक-वेजेटकी मॉडल का उपयोग सफलतापूर्वक मैबस शोधन की बफर स्थितियों में एकत्रीकरण दर का अनुमान लगाने के लिए किया गया है। विभिन्न बफर प्रकार, तापमान की स्थिति, पीएच और नमक सांद्रता के लिए मैब चिकित्सीय के अधीन द्वारा एकत्रीकरण और कुल गठन तंत्र की दर को समझा गया है। प्रयोगात्मक आंकड़ों से प्राप्त परिणामों की तुलना सबसे अच्छे फिट मॉडल की पहचान करने के लिए अनुमानित मॉडल की तुलना में की गई है।

एकत्रीकरण के अलावा, प्रोटीन थैरेप्यूटिक्स की गुणवत्ता को प्रभावित करने वाला एक अन्य उत्पाद अस्थिरता है। विखंडन से रोगी की रक्त प्रवाह में शुरू होने पर या तो सीमित या सीमित कार्यक्षमता नहीं होने के कारण प्रोटीन थैरेप्यूटिक्स का टूटना होता है और प्रतिरक्षाजनकता (इम्युनोजेनेसिटी) पर भारी प्रभाव पड़ता है। समय की प्रगति और तंत्र विकास के समान (अध्याय 4) के तहत मैबस के विखंडन अध्ययन को अत्यधिक तापमान स्थितियों में गैर-एंजाइमी विखंडन के बारे में ज्ञान प्राप्त करने के लिए लक्षित किया गया था। विखंडन गिरावट के कैनेटीक्स को पीएच 6.5 में खारा फास्फेट बफर में 50 डिग्री सेल्सियस के उच्च तापमान पर आयीजीजी 1 आधारित मैब उत्पादों को ऊष्मायन और नियमित समय अंतराल पर नमूने द्वारा देखा गया था। नमूने का विश्लेषण एसई-एचपीएलसी, डीएलएस, एमएस और एसडीएस-पेज जैसे लक्षण वर्णन उपकरणों की रेंज का उपयोग करके किया गया था। मास स्पेक्ट्रोमेट्री का उपयोग अध्ययन के दौरान खंडित प्रजातियों के सटीक आणविक भार को मापने के लिए किया गया था। प्रयोगों से प्राप्त परिणाम बेहतर फिट प्राप्त करने और संबंधित गतिज दर स्थिरांक की गणना के लिए अलग-अलग विखंडन तंत्र के साथ फिट किए गए थे। इसके अलावा यह मॉडल अलग आयनिक ताकत के साथ एक और फॉस्फेट बफर सिस्टम के लिए भी

लागू किया गया था। मॉडल का तापमान निर्भरता अर्हनीस कानून का उपयोग करके सत्यापित किया गया था।

प्रोटीन के क्षरण की निगरानी के लिए, विशेष रूप से जब अपमानित उत्पाद अपने आकार में भिन्न होते हैं, तो विभिन्न विश्लेषणात्मक लक्षण वर्णन उपकरण उपलब्ध होते हैं, जो कि प्रोटीन निर्माण और स्थिरता के क्षेत्र में काम करने वाले फार्मास्यूटिकल वैज्ञानिकों द्वारा नियमित रूप से उपयोग किए जाते हैं। जैव-चिकित्सीय उत्पादों के निर्माता उत्पाद के क्षरण से बचने के तरीकों पर काफी समय और संसाधन खर्च करते हैं, विभिन्न प्रक्रिया बिंदुओं और अंतिम उत्पाद पर कुल स्तरों की निगरानी करते हैं, और अपनी प्रक्रियाओं को डिजाइन करते हैं ताकि समुच्चय को कम या हटा दिया जा सके और इस तरह यह सुनिश्चित हो सके कि अंतिम उत्पाद वांछित को पूरा करता है विशेष विवरण। हमने कुल प्रजातियों के एक पूर्ण स्पेक्ट्रम के विश्लेषण से संबंधित एक विस्तृत जांच की है, जिसमें कुछ नैनोमीटर के आकार के ओलिगोमर्स से लेकर मिलीमीटर के आकार के मल्टीमेरिक अवक्षेप (अध्याय 5) शामिल हैं। यह तीन विश्लेषणात्मक उपकरणों का उपयोग करके हासिल किया गया था, अर्थात् आकार बहिष्करण क्रोमैटोग्राफी (एसई-एचपीएलसी) (1 एनएम - 25 एनएम), डायनेमिक लाइट स्कैटरिंग (डीएलएस) (10 एनएम - 5 माइक्रोन), और माइक्रो फ्लो इमेजिंग (एमएफआई) (2 माइक्रोन - 300 माइक्रोन)। चूंकि प्रत्येक लक्षण वर्णन उपकरण एक विशेष आकार की सीमा को कवर करता है, मंच की सटीकता प्रदर्शित करने के लिए कई उपकरणों का डेटा "हैंडओवर" क्षेत्रों में एकत्र किया गया था। प्रस्तावित प्लेटफॉर्म उन लोगों के लिए उपयोगी होगा जो फॉर्मूलेशन के विकास में लगे हैं और साथ ही जैव-चिकित्सीय उत्पादों की स्थिरता से संबंधित अन्य पहलू भी हैं।

बायोथेराप्यूटिक्स के प्रसंस्करण के दौरान, विशेष रूप से मैबस, विभिन्न आकारों और आकारिकी के कणों का निर्माण करके एकत्रीकरण के संदर्भ में प्रोटीन का क्षरण जब निर्माण, भंडारण, भरने, निर्माण विकास और शिपिंग के दौरान भौतिक, यांत्रिक और रासायनिक तनावों के अधीन होता है। जबकि व्यापक सहमति है कि प्रोटीन एकत्रीकरण प्रतिरक्षा को बढ़ा सकता है और संबंधित उत्पाद की गतिविधि और

कोशिका आविषता को प्रभावित कर सकता है, अंतर्निहित प्रतिरक्षाविज्ञानी और जैविक तंत्र पूरी तरह से समझ में नहीं आते हैं। हमने जांच की है कि विभिन्न प्रकार के यांत्रिक, थर्मल और रासायनिक तनावों से उत्पन्न अदृश्य और उप-दृश्य कण उत्पाद की जैविक गतिविधि (अध्याय 6) को कैसे प्रभावित करते हैं। जिन विभिन्न तनावों पर विचार किया गया है, उनमें सरगर्मी और चरम पीएच या तापमान के नमूने को उजागर करते समय उत्पन्न होने वाले उत्पादों की स्थिरता और प्रतिरक्षात्मकता पर काफी प्रभाव देखा गया, जैसा कि नमूनों के विस्तृत विश्लेषणात्मक और जैविक लक्षण वर्णन द्वारा पुष्टि की गई है। एंटीजन बाइंडिंग एस्से (एसपीआर) द्वारा पुष्टि की गई कोशिका आविषता (साइटोटॉक्सिसिटी) परख (एडीसीसी और सीडीसी) और दवा उत्पाद की गतिविधि में कमी के रूप में वृद्धि हुई एकत्रीकरण से प्रतिरक्षात्मक प्रतिक्रिया में वृद्धि हुई। यह देखा गया कि चरम पीएच (पीएच 3.5 और पीएच 11.0), सरगर्मी (1 दिन और 3 दिन हलचल), और कॉपर सल्फेट के माध्यम से ऑक्सीकरण कुल गठन के साथ-साथ चिकित्सीय क्षमता पर सबसे अधिक प्रभाव पड़ता है। इसके विपरीत, पिपेटिंग, माइल्ड पीएच (4.3 और 8.5) से तनाव, हाइड्रोजन पेरोक्साइड के माध्यम से ऑक्सीकरण, फ्रीज-पिघल (-80 डिग्री सेल्सियस और लिक्विड नाइट्रोजन) मैब बायोथेराप्यूटिक की शक्ति पर अपेक्षाकृत कम प्रभाव डालते हैं। परिणाम पुष्टि करते हैं कि निरंतर उत्पाद की गुणवत्ता प्राप्त करने के लिए एकत्रीकरण के तंत्र की समझ महत्वपूर्ण है।

यह आशा की जाती है कि इस थीसिस में प्रस्तुत अंतर्दृष्टि उत्पादन और भंडारण के दौरान उत्पाद पतन की जांच, निगरानी और नियंत्रण के लिए पर्याप्त योजना बनाने में मदद करेगी ताकि शेल्फ जीवन को बढ़ाया जा सके और दवा उत्पाद की गतिविधि को बनाए रखा जा सके।

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