

UNDERSTANDING AND IMPROVING STABILITY OF BIOTHERAPEUTICS

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by

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Dedicated to the memory of my father.

Certificate

This is to certify that the thesis entitled “**UNDERSTANDING AND IMPROVING STABILITY OF BIOTHERAPEUTICS**” being submitted by **SURBHI GUPTA** 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 her 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 she has pursued the prescribed course of research.

Prof. Anurag S. Rathore

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Surbhi Gupta

Abstract

Cancer is one of the leading causes of mortality in the world's population, accounting for millions of deaths annually. Notwithstanding notable advancements in cancer treatment, over half of all patients still die from a metastatic disease that spreads, does not react to the first medication, or relapses after receiving treatment. Various techniques, either separately or in combination, such as chemotherapy, targeted therapy, radiotherapy, surgery, immunotherapy, hormone therapy, and gene therapy, can be used to treat cancer. Recently, demand for biotherapeutic, including monoclonal antibodies (mAbs) is increasing due to their high specificity and low toxicity. A considerable amount of time and resources are invested first in mAb product development and then in clinical examination of the product. However, the major challenge with the biotherapeutics is that they are prone to degradation under various environmental conditions, including pH, temperature, metal ions, humidity, and light. This degradation may lead to structural, conformational, and biochemical changes that impact the stability of the product, reduce activity, or induce immunogenic responses.

Physical and chemical degradation are the two possible degradation mechanisms that could occur during manufacturing, processing, storage, handling, and administration of the mAbs. Physical degradation includes aggregation and denaturation, and aggregation is known to induce an immunogenic response in the patients. mAbs are susceptible to a myriad of chemical degradation pathways, including oxidation, deamidation, isomerization, hydrolysis, deglycosylation, racemization, disulfide bond breakage and formation, Maillard reaction, and β -elimination. Both physical and chemical instabilities lead to impacts on the safety and efficacy of the product. Physical instability may lead to chemical modifications or vice versa; hence, both physical and chemical instabilities are intertwined and interdependent. Oxidation and deamidation are the most common chemical degradation processes of mAbs, which may result in changes in physical properties, such as hydrophobicity, charge, secondary or/and tertiary structure, and may lower the thermodynamic or kinetic barrier to unfold. This may predispose the product to aggregation and other chemical modifications, which can alter the binding affinity, half-life, and efficacy of the product. Both degradation pathways are intertwined and interdependent. Chapter 2 summarizes major findings from the past decade on the impact of oxidation and deamidation on the stability, biological activity, and efficacy of mAb products. Mechanisms of action, influencing factors, characterization tools, clinical impact, and risk mitigation strategies have been addressed.

Oxidizing agents can be introduced unintentionally from various sources, such as H₂O₂, which is usually introduced during the surface sterilization of the bioreactor or is left in a trace amount or present in groundwater. Degradation of surfactants such as polysorbate (PS80, PS20) also produces peroxides. Metal ions are introduced unintentionally in the drug product as impurities from the formulation buffer components. The presence of these oxidizing agents induces oxidation of the mAb that could have an impact on stability, safety, and efficacy. Besides oxidation, the presence of these oxidizing agents also leads to the non-enzymatic fragmentation of the mAb. The first objective (Chapter 3) was to investigate the impact of the various factors on the kinetics of the non-enzymatic fragmentation. The effect of Cu²⁺ or hydrogen peroxide either singly or in combination was studied using size exclusion chromatography. A kinetic model is created using monomer degradation as a function of time. It was found that the fragmentation kinetics follow different orders with the change in the reaction conditions. It was hypothesized that this change in the order in various conditions is due to a change in the mechanism of hinge peptide bond cleavage. In the presence of Cu²⁺, fragmentation follows first-order kinetics and can occur by metal-catalyzed oxidative cleavage. On the other hand, in the presence of both Cu²⁺ and H₂O₂, reaction order changes to second-order kinetics and can occur by hydroxyl radical in the metal binding site of the hinge region. Also, in the presence of H₂O₂, hinge peptide bond cleavage follows zero-order reaction kinetics and can be a result of free-radical catalysis of peptide bonds. Also, the effect of temperature on the non-enzymatic fragmentation was studied. It was found that fragmentation kinetics shows temperature dependence and follows Arrhenius law. It was shown that trace amounts of these contaminants can have a major effect on a mAb's stability, hence it is advised to keep an eye on their level throughout the manufacturing process.

mAbs can undergo chemical modifications that alter their hydrophobicity, charge heterogeneity, and conformation, ultimately affecting their physical stability. Charge-based heterogeneity can impact the structure, stability, and biological function of the product. Therefore, to better understand the impact of individual charge variants on the physical stability and aggregation propensity under two different buffer conditions employed during downstream purification, a study was conducted, which is detailed in Chapter 4. The charge variants were separated using semi-preparative cation exchange chromatography and buffer exchanged in the two buffers with pH 6.0 and 3.8. Subsequently, each variant was analyzed for size heterogeneity using size exclusion chromatography and dynamic light scattering, conformational stability, colloidal stability, and aggregation behavior under accelerated stability conditions. Size

variants in each charge variant were similar in both pH conditions when analyzed without extended storage. However, conformational stability was lower at pH 3.8 than pH 6.0. All charge variants showed similar apparent melting temperatures at pH 6.0. In contrast, at pH 3.8, variants A3, A5, B2, B3, and B4 display lower T_m , suggesting reduced conformational stability. Further, A2, A3, and A5 exhibit reduced colloidal stability at pH 3.8. In general, acidic variants are more prone to aggregation than basic variants. Typical industry practice today is to examine in-process intermediate stability with acidic species and basic species taken as a single category each. As individual acidic or basic species exhibit varying levels of stability, we propose that stability evaluation be conducted at the species level. This knowledge may then be utilized to cleverly design the downstream process to produce a stable product.

In addition to altering the mAb's physical stability, chemical modifications may alter its biological activity and induce an immunogenic reaction. Both physical and chemical instabilities could occur during postproduction handling of the drugs and perhaps remain unnoticed. Since according to the current guidelines, stability studies implemented by the pharmaceutical industry are proposed to fulfill the licensing requirements. Therefore, this study (Chapter 5) was done to investigate the impact of elevated temperature and subtle shift in pH on the drug product post-dilution in saline. The mAb sample diluted in saline for administration was stressed at elevated temperature and slightly acidic pH condition. Extended stability studies were performed and monitored for size and charge heterogeneity. Size heterogeneity shows no significant changes, whereas charge heterogeneity shows an increase in basic variants and a reduction in main species. Further, basic variants were isolated and characterized to identify the type and site of chemical modification. Intact mass analysis and peptide mapping identify that the basic variants were attributed mainly to the isomerization of HC Asp102 into iso-Asp or its succinimide intermediate. Four basic variants were found to exhibit similar structural properties as the main and control samples. However, basic variants showed reduced binding affinity to the HER2 receptor, while there was no significant difference in FcRn binding. The results indicate that modification in the HC Asp102, which is present in the CDR, affects antigen binding and thus can influence the potency of the drug product. Hence, with the conventional stability studies required to license the drug product, including in-use or extended stability studies to mimic the postproduction handling would be desirable.

Insulin is another essential biotherapeutic drug used for the treatment of diabetes mellitus and is prone to aggregation under low pH and elevated temperature to form insoluble oligomers called amyloid fibrils. Insulin fibrillation also leads to the loss of active product and may induce

an immunogenic response. In this study (Chapter 6), we have investigated the effect of five FDA inactives obtained through virtual screening on the aggregation inhibition of insulin incubated under amyloidogenic conditions (high temperature, low pH). Aggregation kinetics for the formulations incubated under the aggregation-prone aqueous buffer condition were examined using size exclusion chromatography and the Thioflavin T fluorescence assay, and circular dichroism spectroscopy was used to determine the secondary structure. Under aggregation-prone conditions, riboflavin showed the best aggregation inhibition, with 85% native monomer retention after 48 h of incubation. In contrast, the no-ligand formulation showed complete monomer loss after 36 h. Benzoin, benzyl benzoate, and α -tocopherol acetate were found to be ineffective in inhibiting insulin aggregation. Furthermore, the CD spectra of insulin incubated with two of the screened inactives (riboflavin and aspartame) had the characteristic α -helical dip, whereas the no-ligand formulation changed to β -sheet-rich conformations. Riboflavin thus is a possible inhibitor that can be used in insulin formulation to prevent and reduce amyloid fibrils.

Overall, the studies presented in the thesis focus on the deeper understanding of the stability of the biotherapeutics and will help in improving stability during the early phase of development.

सार

कैंसर दुनिया की जनसंख्या में मृत्यु दर के प्रमुख कारणों में से एक है, जो हर साल लाखों मौतों के लिए जिम्मेदार है। कैंसर उपचार में उल्लेखनीय प्रगति के बावजूद, आधे से अधिक रोगी अभी भी मेटास्टैटिक रोग के कारण मर जाते हैं, जो फैलता है, पहली दवा का प्रतिक्रिया नहीं देता, या उपचार प्राप्त करने के बाद पुनः प्रकट होता है। कैंसर के उपचार के लिए विभिन्न तकनीकों का उपयोग किया जा सकता है, चाहे अलग-अलग रूप में या संयोजन में, जैसे कि कीमोथेरेपी, लक्षित चिकित्सा, रेडियोथेरेपी, सर्जरी, इम्यूनोथेरेपी, हार्मोन चिकित्सा, और जीन थेरेपी। हाल ही में, बायोथेरेप्यूटिक्स, विशेष रूप से मोनोक्लोनल एंटीबॉडीज (mAbs) की मांग बढ़ रही है, क्योंकि इनमें उच्च विशिष्टता और कम विषाक्तता होती है। mAbs उत्पाद विकास और उत्पाद के नैदानिक परीक्षण में काफी समय और संसाधनों का निवेश किया जाता है। हालांकि, बायोथेरेप्यूटिक्स के साथ सबसे बड़ी चुनौती यह है कि वे विभिन्न पर्यावरणीय परिस्थितियों जैसे कि pH, तापमान, धातु आयन, नमी, और प्रकाश के तहत खराब हो जाते हैं। यह खराबी उत्पाद की संरचना, स्वरूप, और जैव रासायनिक गुणों में परिवर्तन कर सकती है, जिससे उत्पाद की स्थिरता पर प्रभाव पड़ता है, इसकी गतिविधि कम हो जाती है, या प्रतिरक्षा प्रतिक्रियाएं उत्पन्न हो सकती हैं।

भौतिक और रासायनिक अपघटन दो संभावित अपघटन तंत्र हैं, जो mAbs के निर्माण, प्रसंस्करण, भंडारण, हैंडलिंग और प्रशासन के दौरान हो सकते हैं। भौतिक अपघटन में एग्रीगेशन (संचयन) और डीनैचुरेशन (रचना परिवर्तन) शामिल हैं, और एग्रीगेशन रोगियों में इम्यूनोजेनिक प्रतिक्रिया को प्रेरित कर सकता है। mAbs विभिन्न रासायनिक अपघटन मार्गों के प्रति संवेदनशील होते हैं, जिनमें ऑक्सीकरण, डीएमिडेशन, समावेशन, हाइड्रोलिसिस, डीग्लाइकोसिलेशन, रेसमाइजेशन, डिसल्फाइड बंध टूटना और बनना, मेलाई प्रतिक्रिया, और β -उन्मूलन शामिल हैं। भौतिक और रासायनिक अस्थिरताएं उत्पाद की सुरक्षा और प्रभावशीलता पर प्रभाव डालती हैं। भौतिक अस्थिरता रासायनिक संशोधनों को जन्म दे सकती है या इसके विपरीत; इसलिए, भौतिक और रासायनिक अस्थिरताएं आपस में जुड़ी और परस्पर निर्भर होती हैं। ऑक्सीकरण और डीएमिडेशन mAbs की सबसे सामान्य रासायनिक अपघटन प्रक्रियाएं हैं, जो भौतिक गुणों, जैसे कि हाइड्रोफोबिसिटी, चार्ज, माध्यमिक या/और तृतीयक संरचना में परिवर्तन कर सकती हैं और उत्पाद को अनफोल्ड करने के लिए थर्मोडायनामिक या काइनेटिक बाधा को कम कर सकती हैं। यह उत्पाद को एग्रीगेशन और अन्य रासायनिक संशोधनों के प्रति प्रवृत्त बना सकता है, जिससे बाइंडिंग एफिनिटी, आधा-जीवन, और उत्पाद की प्रभावशीलता बदल सकती है। दोनों अपघटन मार्ग आपस में जुड़े और परस्पर निर्भर होते हैं। अध्याय 2 पिछले दशक में mAb उत्पादों की स्थिरता, जैविक गतिविधि, और प्रभावशीलता पर ऑक्सीकरण और डीएमिडेशन के प्रभावों पर मुख्य निष्कर्षों को संक्षेप में प्रस्तुत करता है। इसमें क्रियाविधि, प्रभाव डालने वाले कारक, लक्षण वर्णन उपकरण, नैदानिक प्रभाव, और जोखिम न्यूनीकरण रणनीतियों पर चर्चा की गई है।

ऑक्सीकरण एजेंट विभिन्न स्रोतों से अनजाने में प्रवेश कर सकते हैं, जैसे कि H_2O_2 , जो आमतौर पर बायोरिएक्टर की सतह की स्टरलाइजेशन प्रक्रिया के दौरान उपयोग किया जाता है या ट्रेस मात्रा में बचा रह सकता है या भूजल में उपस्थित हो सकता है। पॉलिसोर्बेट (PS80, PS20) जैसे सर्फैक्टेंट्स के अपघटन से भी पेरोक्साइड्स उत्पन्न होते हैं। धातु आयन ड्रग उत्पाद में फार्मूलेशन बफर घटकों से अशुद्धियों के रूप में अनजाने में प्रवेश कर सकते हैं। इन ऑक्सीकरण एजेंटों की उपस्थिति mAb के ऑक्सीकरण को प्रेरित करती है, जिससे इसकी स्थिरता, सुरक्षा, और प्रभावशीलता पर प्रभाव पड़ सकता है। ऑक्सीकरण के अलावा, इन ऑक्सीकरण एजेंटों की उपस्थिति mAb के गैर-एंजाइमेटिक विखंडन (फ्रैगमेंटेशन) का कारण भी बनती है। पहला उद्देश्य (अध्याय 3) विभिन्न कारकों के गैर-एंजाइमेटिक विखंडन की गति पर प्रभाव का अध्ययन करना था। Cu^{2+} या हाइड्रोजन पेरोक्साइड के एकल या संयोजन में प्रभाव का अध्ययन साइज एक्सक्लूजन क्रोमैटोग्राफी का उपयोग करके किया गया। एक गतिज मॉडल (काइनेटिक मॉडल) तैयार किया गया जिसमें मोनोमर अपघटन को समय के साथ एक कार्य के रूप में दर्शाया गया। पाया गया कि विखंडन की गति विभिन्न अभिक्रिया परिस्थितियों के साथ अलग-अलग क्रम का पालन करती है। यह अनुमान लगाया गया कि विभिन्न परिस्थितियों में क्रम का परिवर्तन हिंग पेप्टाइड बॉन्ड के टूटने के तंत्र में बदलाव के कारण है। Cu^{2+} की उपस्थिति में, विखंडन प्रथम क्रम गतिज

(फर्स्ट-ऑर्डर काइनेटिक्स) का पालन करता है और धातु-उत्तेजित ऑक्सीडेटिव टूटने (मेटल-कैटलाइज़्ड ऑक्सीडेटिव क्लीवेज) से हो सकता है। दूसरी ओर, Cu^{2+} और H_2O_2 दोनों की उपस्थिति में, अभिक्रिया क्रम द्वितीय क्रम गतिज (सेकंड-ऑर्डर काइनेटिक्स) में बदल जाता है और हिंग क्षेत्र के धातु बाइंडिंग साइट में हाइड्रॉक्सिल रेडिकल द्वारा हो सकता है। H_2O_2 की उपस्थिति में, हिंग पेप्टाइड बॉन्ड का टूटना शून्य क्रम अभिक्रिया (ज़ीरो-ऑर्डर काइनेटिक्स) का पालन करता है और पेप्टाइड बॉन्ड्स के फ्री-रेडिकल उत्प्रेरण (कैटलिसिस) का परिणाम हो सकता है। इसके अलावा, गैर-एंजाइमेटिक विखंडन पर तापमान के प्रभाव का अध्ययन किया गया। पाया गया कि विखंडन की गति तापमान पर निर्भर करती है और यह Arrhenius नियम का पालन करती है। यह दिखाया गया कि इन संदूषकों की ट्रेस मात्रा भी mAb की स्थिरता पर महत्वपूर्ण प्रभाव डाल सकती है; इसलिए, निर्माण प्रक्रिया के दौरान इनके स्तर पर ध्यान रखना आवश्यक है।

मोनोक्लोनल एंटीबॉडी (mAbs) रासायनिक संशोधनों से गुजर सकते हैं जो उनकी हाइड्रोफोबिसिटी, चार्ज विविधता, और संरचना को बदल सकते हैं, जो अंततः उनकी भौतिक स्थिरता को प्रभावित करता है। चार्ज-आधारित विविधता उत्पाद की संरचना, स्थिरता, और जैविक कार्यप्रणाली पर प्रभाव डाल सकती है। इसलिए, डाउनस्ट्रीम शुद्धिकरण के दौरान उपयोग किए गए दो विभिन्न बफर स्थितियों में व्यक्तिगत चार्ज वेरिएंट्स के भौतिक स्थिरता और एग्रीगेशन प्रवृत्ति पर उनके प्रभाव को बेहतर ढंग से समझने के लिए एक अध्ययन किया गया, जिसका विवरण अध्याय 4 में दिया गया है। चार्ज वेरिएंट्स को सेमी-प्रिपरेटिव कैशन एक्सचेंज क्रोमैटोग्राफी का उपयोग करके अलग किया गया और इन्हें pH 6.0 और 3.8 वाले दो बफरों में बदला गया। इसके बाद, प्रत्येक वेरिएंट का साइज विविधता (size heterogeneity) साइज एक्सक्लूजन क्रोमैटोग्राफी और डायनामिक लाइट स्कैटरिंग के द्वारा विश्लेषण किया गया, साथ ही संरचनात्मक स्थिरता, कोलोइडल स्थिरता, और तेज स्थिरता स्थितियों (accelerated stability conditions) में एग्रीगेशन व्यवहार का अध्ययन किया गया। प्रत्येक चार्ज वेरिएंट में आकार विविधता दोनों pH स्थितियों में विस्तारित भंडारण के बिना समान पाई गई। हालांकि, pH 3.8 पर संरचनात्मक स्थिरता pH 6.0 की तुलना में कम पाई गई। सभी चार्ज वेरिएंट्स ने pH 6.0 पर समान स्पष्ट मेल्टिंग तापमान (T_m) दिखाए। इसके विपरीत, pH 3.8 पर वेरिएंट्स A3, A5, B2, B3, और B4 ने कम T_m दिखाया, जो कम संरचनात्मक स्थिरता को संकेत करता है। इसके अतिरिक्त, A2, A3, और A5 ने pH 3.8 पर कम कोलोइडल स्थिरता प्रदर्शित की। सामान्यतः, अम्लीय वेरिएंट्स क्षारीय वेरिएंट्स की तुलना में अधिक एग्रीगेशन के प्रति संवेदनशील होते हैं। वर्तमान औद्योगिक प्रथा के अनुसार, इन-प्रोसेस इंटरमीडिएट स्थिरता का परीक्षण अम्लीय और क्षारीय प्रजातियों को एक ही श्रेणी में लिया जाता है। चूंकि व्यक्तिगत अम्लीय या क्षारीय प्रजातियां विभिन्न स्तरों पर स्थिरता प्रदर्शित करती हैं, हम प्रस्तावित करते हैं कि स्थिरता का मूल्यांकन प्रजाति-स्तर (species level) पर किया जाए। इस ज्ञान का उपयोग फिर डाउनस्ट्रीम प्रक्रिया को कुशलतापूर्वक डिजाइन करने में किया जा सकता है ताकि स्थिर उत्पाद प्राप्त किया जा सके।

मोनोक्लोनल एंटीबॉडी (mAb) की भौतिक स्थिरता को प्रभावित करने के अलावा, रासायनिक संशोधन उसकी जैविक क्रियाशीलता को बदल सकते हैं और इम्यूनोजेनिक प्रतिक्रिया को उत्पन्न कर सकते हैं। भौतिक और रासायनिक अस्थिरताएं उत्पादन के बाद दवाओं के हैंडलिंग के दौरान हो सकती हैं और शायद अनदेखी रह सकती हैं। चूंकि वर्तमान दिशा-निर्देशों के अनुसार, फार्मास्यूटिकल उद्योग द्वारा किए गए स्थिरता अध्ययन लाइसेंसिंग आवश्यकताओं को पूरा करने के लिए प्रस्तावित किए जाते हैं, इस अध्ययन (अध्याय 5) का उद्देश्य उच्च तापमान और pH में हल्के परिवर्तन का दवाइयों के उत्पाद पर प्रभाव का अध्ययन करना था। mAb के नमूने को प्रशासन के लिए सलाइन में पतला किया गया था और उच्च तापमान और थोड़ा अम्लीय pH स्थिति में तनावित किया गया। विस्तारित स्थिरता अध्ययन किए गए और आकार और चार्ज विविधता की निगरानी की गई। आकार विविधता में कोई महत्वपूर्ण परिवर्तन नहीं देखा गया, जबकि चार्ज विविधता में क्षारीय वेरिएंट्स में वृद्धि और मुख्य प्रजातियों में कमी पाई गई। इसके बाद, क्षारीय वेरिएंट्स को अलग किया गया और रासायनिक संशोधन के प्रकार और स्थान की पहचान करने के लिए उनका लक्षण वर्णन किया गया। इंटेक्ट मास विश्लेषण और पेप्टाइड मैपिंग से यह पता चला कि क्षारीय वेरिएंट्स मुख्य रूप से HC Asp102 के आइसोमेरिजेशन से उत्पन्न होते हैं, जो iso-Asp या उसके succinimide इंटरमीडिएट में परिवर्तित हो जाते हैं। चार क्षारीय वेरिएंट्स को पाया गया जो संरचनात्मक रूप से मुख्य और नियंत्रण नमूनों के समान थे। हालांकि, क्षारीय वेरिएंट्स ने HER2 रिसेप्टर से बाइंडिंग एफिनिटी में कमी दिखाई, जबकि FcRn बाइंडिंग में कोई महत्वपूर्ण अंतर नहीं पाया गया। इन परिणामों से यह

संकेत मिलता है कि HC Asp102 में संशोधन, जो CDR में स्थित है, एंटीजन बाइंडिंग को प्रभावित करता है और इस प्रकार दवाइयों के उत्पाद की प्रभावशीलता को प्रभावित कर सकता है। इसलिए, दवा उत्पाद को लाइसेंस देने के लिए आवश्यक पारंपरिक स्थिरता अध्ययन के अलावा, उपयोग के दौरान या विस्तारित स्थिरता अध्ययन भी करने की आवश्यकता हो सकती है, जो उत्पादन के बाद की हैंडलिंग की नकल करें।

इंसुलिन एक और महत्वपूर्ण जैव चिकित्सा दवा है, जो मधुमेह मेलिटस के उपचार में उपयोग होती है और यह कम pH और उच्च तापमान पर अस्थिर हो सकती है, जिससे यह अपक्षारी ऑलिगोमर्स, जिन्हें एमीलॉयड फाइब्रिल्स कहा जाता है, का निर्माण कर सकती है। इंसुलिन का फाइब्रिलेशन सक्रिय उत्पाद की हानि का कारण बनता है और इम्यूनोजेनिक प्रतिक्रिया उत्पन्न कर सकता है। इस अध्ययन (अध्याय 6) में, हमने पांच FDA इनएक्टिव्स का प्रभाव जांचा, जिन्हें वर्चुअल स्क्रीनिंग के माध्यम से प्राप्त किया गया था, और यह अध्ययन किया कि ये इंसुलिन के एमीलॉयडोजेनिक स्थितियों (उच्च तापमान, कम pH) में संकलन को रोकने में कितने प्रभावी हैं। संकलन की गतिशीलता का अध्ययन किए गए सूत्रों पर किया गया, जो संकलन-प्रवृत्त जलयुक्त बफर स्थितियों में इनक्यूबेट किए गए थे। आकार बहिष्करण क्रोमैटोग्राफी और थायोफ्लेविन T फ्लोरेसेंस परीक्षण का उपयोग करके इनका परीक्षण किया गया, और गोलाकार डाइक्रोइजम स्पेक्ट्रोस्कोपी का उपयोग करके द्वितीयक संरचना का निर्धारण किया गया। संकलन-प्रवृत्त स्थितियों में, रिबोफ्लेविन ने सबसे अच्छा संकलन रोधी प्रभाव दिखाया, जिसमें 48 घंटे की इनक्यूबेशन के बाद 85% मूल मोनोमर संरक्षित रहा। इसके विपरीत, बिना लिगेंड वाले सूत्र में 36 घंटे के बाद पूर्ण मोनोमर हानि देखी गई। बेंजोइन, बेंजाइल बेंजोएट, और α -टोकोफेरॉल एसोटेट इंसुलिन संकलन को रोकने में अप्रभावी पाए गए। इसके अतिरिक्त, रिबोफ्लेविन और अस्पार्टेम से इनक्यूबेट किए गए इंसुलिन के CD स्पेक्ट्रा में विशिष्ट α -हेलिकल डिप देखा गया, जबकि बिना लिगेंड वाले सूत्र ने β -शीट-समृद्ध संरचनाओं में परिवर्तन किया। इस प्रकार, रिबोफ्लेविन एक संभावित रोधी है जिसे इंसुलिन सूत्र में उपयोग किया जा सकता है ताकि एमीलॉयड फाइब्रिल्स को रोका जा सके और उनका आकार कम किया जा सके।

कुल मिलाकर, इस शोध में प्रस्तुत अध्ययन जैव चिकित्सा उत्पादों की स्थिरता को बेहतर समझने पर केंद्रित हैं और विकास के प्रारंभिक चरणों में स्थिरता में सुधार लाने में मदद करेंगे।

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List of Abbreviations

D_o	Self-Diffusion Coefficient
k_D^*	Apparent Diffusion Interaction Parameters
[M]	Insulin Monomer Concentration
[M0]	Concentration of Insulin at Time 0
[Mt]	Concentration of Insulin at Time t,
3-OH-Kyn	3- Hydroxykynuerinine
AAPH	2,2-Azobis(2-Amidinopropane) Dihydrochloride
ADA	Antidrug Antibody
ADCC	Antibody-Dependent Cellular Cytotoxicity
ADCP	Antibody Dependent Cellular Phagocytosis
Ala	Alanine
Asn	Asparagine
Asp	Aspartic acid
Aspt	Aspartame
AUC	Area Under the Curve
B1	Basic 1
B2	Basic 2
B3	Basic 3
B4	Basic 4,
BeB	Benzyl Benzoate
Bez	Benzoin

bis-ANS	4,4'-Dianilino-1,1'-Binaphthyl-5,5'-Disulfonic Acid
BSA	Body Surface Area
BSA	Bovine Serum Albumin
BSPOTPE	1,2-Bis[4-(3- Sulfonatopropoxyl) Phenyl]-1,2-Diphenylethene Salt
c	Antibody Concentration
CD	Circular Dichroism
CDC	Complement Dependent Cytotoxicity
CDR	Complementary Determined Region
CDRs	Complementarity Determining Regions
CE	Capillary electrophoresis
CEX	Cation exchange chromatography
CID	Collision-induced dissociation
cIEF	Capillary Isoelectric Focusing
CM	Monomer Concentration at Time t
CM0	Initial Monomer Concentration
CQA	Critical Quality Attributes
CV	Column Volume
Cys	Cysteine
CZE	Capillary Zone Electrophoresis
D	Diffusion coefficients
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulfoxide
DP	Drug Product

DS	Drug Substance
DTPA	Diethylenetriaminepentaacetic Acid
EAD	Electron-Activated Dissociation
ECD	Electrons Capture Dissociation
EDTA	Ethylenediaminetetraacetic acid
ERLIC	Electrostatic Repulsion Hydrophilic Interaction Chromatography
ETD	Electron Transfer Dissociation
Fab	Antigen Binding Fragment
FcRn	Neonatal Fc Receptor
FcγRIIa	Fc-Gamma Receptor IIa
FTIR	Fourier-Transform Infrared Spectroscopy
Gln	Glutamine
H ₂ O ₂	Hydrogen Peroxide
HC	Heavy Chain
HDX-MS	Hydrogen Deuterium Exchange Mass Spectrometry
HER2	Human Epidermal Growth Factor Receptor 2
HIC	Hydrophobic Interaction Chromatography
HILIC	Hydrophilic Interaction Liquid Chromatography
His	Histidine
HMW	High Molecular Weight
HPLC	High-Performance Liquid Chromatography
HTP	High Throughput Assays

ICH	International Council for Harmonisation of Technical Requirements For Pharmaceuticals for Human Use
IEF	Isoelectric Focusing
IEX	Ion Exchange Chromatography
IgG	Immunoglobulin G
Iso-Asp	Iso-Aspartic Acid
K	Rate constant
k_D	Diffusion coefficient parameter
Kyn	Kynurenine
LC	Light Chain
LC-MS	Liquid Chromatography Coupled With Mass Spectrometry
LMW	Low Molecular Weight
M	Main
mAb	Monoclonal Antibodies
MCO	Metal Catalyzed Oxidation
Met	Methionine
MetSO	Methionine Sulfoxide
MRE	Mean Residual Ellipticities
Na ₂ EDTA	Disodium Ethylenediaminetetraacetic Acid
NEM	N-ethylmaleimide
NFK	N-formylkynurenine
OH-Trp	Hydroxytryptophan
OH [•]	Hydroxyl Radical

Phe	Phenylalanine
PIMT	L-Isoaspartyl Methyltransferase
PK	Pharmacokinetics
PS20	Polysorbate 20
PS80	Polysorbate 80
PTMs	Posttranslational Modifications
RDD	Radical-Based Dissociation
Rib	Riboflavin
ROS	Reactive Oxygen Species
RP-HPLC	Reverse-Phase Chromatography
RP-HPLC	Reverse-Phase High Pressure Liquid Chromatography
SAH	S-Adenosyl-L-Homocysteine
SDS	Sodium Dodecyl Sulfate
SEC	Size Exclusion Chromatography
SEC-MALS	SEC Coupled With Multi-Angle Light Scattering
Ser	Serine
SOD	Superoxide Dismutase
SPR	Surface Plasmon Resonance
t-BHP	Tertiary Butyl Hydroperoxide
$t_{1/2}$	Half-Life
TCEP	Tris (2-Carboxyethyl) Phosphine-Hcl
Tg	Transgenic
Thr	Threonine

ThT	Thioflavin T
T _m	Apparent Melting Temperature
T _{m1}	Melting Temperature Of CH ₂
T _{m2}	Melting Temperature of Fab
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultraviolet
V	Volume of the injection.
VWD	Variable Wavelength Detector
Z _{avg}	z-Average
α-tA	A-Tocopherol Acetate