

**AGGREGATION & STABILITY ANALYSIS OF  
THERAPEUTIC MONOCLONAL ANTIBODIES:  
ROLE OF IMAGE PROCESSING, STRESS  
CONDITIONS, IN-VITRO MODELS & NOVEL  
STABILIZERS**

**SHRAVAN SREENIVASAN**



**DEPARTMENT OF CHEMICAL ENGINEERING  
INDIAN INSTITUTE OF TECHNOLOGY DELHI  
AUGUST 2024**

© Indian Institute of Technology Delhi (IITD), New Delhi, 2024

**AGGREGATION & STABILITY ANALYSIS OF  
THERAPEUTIC MONOCLONAL ANTIBODIES:  
ROLE OF IMAGE PROCESSING, STRESS  
CONDITIONS, IN-VITRO MODELS & NOVEL  
STABILIZERS**

by

**SHRAVAN SREENIVASAN**

**DEPARTMENT OF CHEMICAL ENGINEERING**

Submitted

In fulfilment of the requirements of the degree of

**DOCTOR OF PHILOSOPHY**

to the



**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

**AUGUST 2024**

**Certificate**

This is to certify that the thesis entitled “**Aggregation & Stability Analysis of Therapeutic Monoclonal Antibodies: Role of Image Processing, Stress Conditions, In-Vitro Models & Novel Stabilizers**” submitted by Mr. **Shravan Sreenivasan** 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. He has worked under my supervision and has fulfilled the requirements, which to my knowledge, has reached the requisite standard for the submission of this thesis. The results contained in this thesis have not been submitted in part or full to any University or Institute for the award of any degree or diploma.

**Prof. Anurag S. Rathore**

Department of Chemical Engineering

Indian Institute of Technology Delhi

## Acknowledgements

Here I stand, at the end of a journey, which was really long and arduous, but at the same time very much enriching and transformative. My doctoral expedition would be incomplete without the encouragement, guidance, and support of numerous incredible individuals, both within and outside IIT Delhi. As I write this part of my thesis, I am filled with gratitude for everyone who has been a part of my doctoral journey. Hence, I would like to thank all of you and dedicate these words as a token of appreciation for your support that led to relentless pursuit of knowledge.

First and foremost, I would like to express my deepest gratitude to my advisor, Prof. Anurag S. Rathore, whose unwavering support, insightful feedback, and constant encouragement have been the guiding light of my research. Your expertise, patience, support, perspectives, ideas, determination, and mentorship have been the most valuable in this journey and has helped me in shaping both my scientific acumen and my character. In the realm of scientific collaboration, I would also like to express my gratitude to Prof. Wim Jiskoot, University of Leiden, Netherlands for providing me with insightful feedback, especially on data analysis and manuscript drafting which enabled me to dive deeper into my area of research. Though I worked only for a very short time span of a little more than a year with you, those times were really insightful and helped in paving a direction in my PhD. I would also like to express my gratitude to Prof. Kedar Khare, Department of Physics, IIT Delhi for his dedicated mentoring to develop the fluorescence microscope image analysis algorithm for analyzing protein aggregates.

I would like to thank the SRC members, Prof. Anupam Shukla, Prof. James Gomes, and Prof. Manojkumar Ramteke for their valuable suggestions and insights that helped to improve the quality of research. I am also grateful to Department of Chemical Engineering, IIT Delhi for providing me a suitable environment for conducting the research and knowledge on various chemical engineering subjects, which were new to me during my initial stages of PhD. I am indebted to CSIR, New Delhi for providing the funding as part of direct CSIR-SRF fellowship. The financial support was really helpful and enabled me to focus entirely on my research goals.

I would like to express my gratitude to Dr. Srishti Joshi, co-ordinator of analytical section of our lab. I am highly thankful to you for the insightful discussions, mentorship, and belief in my

abilities, especially during the tough times. I am also thankful to Dr. Rozaleen Dash, coordinator of bioassay section of our lab. I would like to thank Dr. Shyampada Mandal for working with me in formulating the image analysis algorithm. I would also like to express my gratitude to Deepak Sonawat, an undergraduate student at IIT Delhi for collaborating with me for various projects and providing in-depth contributions. Deepak, I am yet to see a student of your age with better coding skills. I would now like to express my heartfelt thanks to all the lab mates who have contributed in some way or other for the progress of research. In this regard, I would especially like to thank Vineela Peruri, Sanjeet Patil, Deepika Sarin, Kratika Upadhyay, Preeti Saroha, Snehal Desai, Dr. Wajihul Hassan, Dr. Sunil Kumar, and Dr. Sharad Narnaware for helping me whenever there was a requirement.

I would like to express my gratitude to the support staffs of my lab, Surinder Ji, Mohit Ji, Nitin Ji, Ranjeet Ji, and Naresh Ji. Your efforts for the smooth functioning of lab will always be remembered. I would also like to state that, though indirectly, you guys have contributed a lot to the outcomes of this research.

I am grateful to my friends and family for their support and encouragement throughout this journey. I would like to dedicate a special thanks to my partner Dr. Pooja Madhavan, whose love, support, and understanding helped me to navigate the ups and downs of this journey. Your belief in me kept me going even in the face of challenges. I would also like to thank my parents. Your belief in education, and unconditional love have been very important for me.

Finally, I thank God, the Almighty, who has provided me countless blessings, knowledge, and opportunities. I would like to end this section of thesis by acknowledging again the privilege that I had at IIT Delhi for undertaking this research. I hope that my work contributes meaningfully to the field of therapeutic proteins.

Shravan Sreenivasan

**ABSTRACT**

Therapeutic proteins, such as monoclonal antibodies (mAbs) are used for the treatment of various diseases. Aggregation of mAb is a major stability issue and occurs during various stages of the product life cycle such as cell culture, downstream processing, formulation, transportation, storage, handling, and administration.

Microscopy based techniques are used for the analysis of aggregates. First objective was to develop a novel image processing algorithm to characterize micron sized aggregates of mAb using brightfield images. Initial steps of image analysis algorithm involved conversion of images to grayscale followed by pixel-based and size-based thresholding. Further steps involved morphological operations and calculation of size distribution. Size distribution output obtained from brightfield image processing was validated using images of liquid chromatography resins. Aggregate size distribution of mAb was also compared with various experimental techniques. Overall, it was concluded that analysis of IgG aggregates using image processing strategy could serve as a rapid orthogonal methodology to the existing approaches.

Combined occurrence of air/liquid interfacial stress and agitation is known to be highly detrimental to the stability of mAbs and hence requires deeper investigation. Second objective consisted of aggregation analysis of therapeutic mAb induced by rapid air/liquid interfacial agitation stress through air bubbling using an in-house set-up. Samples containing mAb subjected to stress were characterized using a wide array of analytical techniques. Stressed samples showed increasing turbidity with time, with mAb1 showing a protein loss of more than 50% after 240 minutes. Aggregate rich samples exhibited altered secondary structure and higher hydrophobicity with 40% reduction in activity. It was also found that the extent of aggregation was affected by protein concentration, sample volume, presence of surfactants, temperature, air flow rate, and presence of silicone oil. In conclusion, exposure to air/liquid interfacial stress through bubbling into liquid mAb samples effectively generated sub-visible and visible aggregates, making air bubbling an attractive approach for interfacial stress testing of mAbs.

Once the sample containing mAb is administered in the human body, the stability of mAb in physiological fluids might vary with time, and hence, different in-vitro models are used for stability assessment. Phosphate-buffered saline (PBS) is a buffer, also used to mimic conditions

of physiology. Samples containing mAb encounters different matrix components of blood once they are administered. However, PBS is devoid of various molecules found in in-vitro models such as serum and HSF. Third objective was to compare the aggregate profile of mAb in PBS with in-vitro models such as serum and HSF. In this study, aggregation of mAb in PBS and models derived from body fluids seeded with mAb samples subjected to various stresses were compared. It was found that aggregation of mAb was different in PBS compared to HSF. It was also found that PBS and serum containing mAb subjected to stirring and interfacial agitation resulted in aggregates of  $>2 \mu\text{m}$  size, and average size and percentage number of particles having  $>10 \mu\text{m}$  size was higher in serum compared to PBS at all analysis time point. Overall, it was found that aggregation of mAb in PBS was different models derived from human body fluids.

Therapeutic mAbs, upon exposure to chemicals such as ferrous ions ( $\text{Fe}^{2+}$ ) or hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), are known to result in the degradation.  $\text{H}_2\text{O}_2$  and  $\text{Fe}^{2+}$  reacts by Fenton reaction to form hydroxyl radical ( $\bullet\text{OH}$ ), that are known to rapidly degrade proteins. Before intravenous administration, the mAb product is often diluted in normal saline. Increased levels of  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$  could be found in the human body during various diseases as well. Fourth objective was to investigate the stability of therapeutic mAb in the combined presence of  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$  in saline and in in-vitro models. Saline samples containing mAb having the combined presence of both  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$  exhibited more than 20% HMW, whereas samples having only  $\text{Fe}^{2+}$ ,  $\text{H}_2\text{O}_2$ , or neither resulted in less than 2.5% HMW, respectively. Aggregate-rich samples also exhibited altered protein structure and hydrophobicity. Aggregation increased upon increasing the time, temperature, and concentration of  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$ . It was also found that in the presence of both  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$ , in-vitro models such as artificially prepared extracellular saline, HSF, and serum resulted in enhanced aggregation of mAb. In conclusion, presence of both  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$  in samples containing mAb resulted in multifold mAb degradation in saline and in-vitro models.

Samples containing mAb are typically stored at  $2-8^\circ\text{C}$ . However, the product may get subjected to higher temperatures during various stages, and it might perturb the native structure of the mAb, eventually leading to product degradation. Taurine is a naturally occurring sulfur-containing amino acid, known to offer stability to proteins. The fifth objective was to evaluate the potential of taurine as a stabilizer to reduce mAb degradation. Forced degradation of mAb1 containing samples by thermal stress for 30 minutes resulted in HMW by more than 65% in

sample without taurine compared to sample with taurine. Samples containing mAb1 without taurine also resulted in higher changes in protein structure and hydrophobicity compared to samples with taurine. Stabilizing effect of taurine was retained at different mAb and taurine concentrations, time, temperatures, buffers, and presence of polysorbate 80 (PS80). Additionally, mAb in the presence of taurine showed enhanced stability upon subjecting to light stress, combination of light and H<sub>2</sub>O<sub>2</sub>, and combination of Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub>. In conclusion, presence of taurine in the samples containing mAb enhanced its stability, thereby indicating its possible use as a stabilizer.

## सार

चिकित्सीय प्रोटीन, जैसे मोनोक्लोनल एंटीबॉडी का उपयोग विभिन्न रोगों के उपचार के लिए किया जाता है। mAb का एकत्रीकरण एक प्रमुख स्थिरता मुद्दा है और यह उत्पाद जीवन चक्र के विभिन्न चरणों जैसे सेल संस्कृति, डाउनस्ट्रीम प्रसंस्करण, फॉर्मूलेशन, परिवहन, भंडारण, हैंडलिंग और प्रशासन के दौरान होता है।

समुच्चय के विश्लेषण के लिए माइक्रोस्कोपी आधारित तकनीकों का उपयोग किया जाता है। 1 माइक्रोन से अधिक आकार वाले mAb के समुच्चय का आकार वितरण विश्लेषण अत्यधिक महत्वपूर्ण है। 1 माइक्रोन से अधिक आकार के समुच्चय की ब्राइटफील्ड छवियां आसानी से प्राप्त की जा सकती हैं। समुच्चय की पारभासी प्रकृति, पृष्ठभूमि और समुच्चय के बीच कम अंतर, नमूने का यादृच्छिक अनियमित आकार, और पृष्ठभूमि से असंगत भटकती रोशनी सूक्ष्म छवियों से समुच्चय के आकार निर्धारण विश्लेषण को एक कठिन कार्य बना सकती है। पहला उद्देश्य ब्राइटफील्ड छवियों का उपयोग करके mAb के माइक्रोन आकार के समुच्चय को चिह्नित करने के लिए एक उपन्यास छवि प्रसंस्करण एल्गोरिदम विकसित करना था। छवि विश्लेषण एल्गोरिदम के शुरुआती चरणों में छवियों को ग्रेस्केल में परिवर्तित करना और उसके बाद पिक्सेल-आधारित और आकार-आधारित थ्रेशोल्डिंग शामिल थी। आगे के चरणों में रूपात्मक संचालन और आकार वितरण की गणना शामिल थी। ब्राइटफील्ड छवि प्रसंस्करण से प्राप्त आकार वितरण आउटपुट को तरल क्रोमेटोग्राफी रेजिन की छवियों का उपयोग करके मान्य किया गया था। विभिन्न प्रायोगिक तकनीकों के साथ mAb के समग्र आकार वितरण की तुलना भी की गई। कुल मिलाकर, यह निष्कर्ष निकाला गया कि छवि प्रसंस्करण रणनीति का उपयोग करके आईजीजी समुच्चय का विश्लेषण मौजूदा दृष्टिकोणों के लिए एक तीव्र ऑर्थोगोनल पद्धति के रूप में काम कर सकता है।

विभिन्न इंटरफेस पर mAbs का एक्सपोजर विभिन्न ठोस/तरल, तरल/तरल और वायु/तरल सतहों के रूप में होता है। वायु/तरल इंटरफेसियल तनाव और उत्तेजना की संयुक्त घटना को mAb की स्थिरता के लिए अत्यधिक हानिकारक माना जाता है और इसके लिए गहन जांच की आवश्यकता होती है। दूसरे उद्देश्य में इन-हाउस सेट-अप का उपयोग करके वायु बुलबुले के माध्यम से तेजी से वायु/तरल इंटरफेसियल आंदोलन तनाव से प्रेरित फॉस्फेट बफर्ड सलाइन (पीबीएस) में चिकित्सीय मोनोक्लोनल एंटीबॉडी का एकत्रीकरण विश्लेषण शामिल था। तनाव के अधीन mAb वाले नमूनों को विश्लेषणात्मक तकनीकों की एक विस्तृत श्रृंखला का उपयोग करके चित्रित किया गया था। प्रोटीन सांद्रता, नमूना मात्रा, तापमान, बुदबुदाहट दर और सहायक पदार्थों की उपस्थिति जैसे विभिन्न कारकों के प्रभाव का भी मूल्यांकन किया गया था। तनावग्रस्त नमूनों में समय के साथ मैलापन बढ़ता हुआ दिखा, जिसमें mAb1 में 240 मिनट के बाद 50% से अधिक प्रोटीन की हानि देखी गई। समग्र समृद्ध नमूनों ने गतिविधि में 40% की कमी के साथ परिवर्तित माध्यमिक संरचना और उच्च हाइड्रोफोबिसिटी का प्रदर्शन किया। यह भी पाया गया कि एकत्रीकरण की सीमा प्रोटीन सांद्रता, नमूना मात्रा, सर्फैक्टेंट की उपस्थिति, तापमान, वायु प्रवाह दर और सिलिकॉन तेल की उपस्थिति से प्रभावित थी। निष्कर्ष में, तरल mAb नमूनों में

बुलबुले के माध्यम से हवा/तरल इंटरफेशियल तनाव के संपर्क में आने से प्रभावी ढंग से उप-दृश्यमान और दृश्यमान समुच्चय उत्पन्न होते हैं, जिससे एयर बबलिंग mAb के इंटरफेशियल तनाव परीक्षण के लिए एक आकर्षक दृष्टिकोण बन जाता है।

एक बार जब mAb युक्त नमूना मानव शरीर में डाला जाता है, तो शारीरिक तरल पदार्थों में mAb की स्थिरता समय के साथ भिन्न हो सकती है, और इसलिए, स्थिरता मूल्यांकन के लिए विभिन्न इन-विट्रो मॉडल का उपयोग किया जाता है। फॉस्फेट-बफर्ड सेलाइन (पीबीएस) एक बफर है, जिसका उपयोग शरीर विज्ञान की स्थितियों की नकल करने के लिए भी किया जाता है। mAb युक्त नमूनों को एक बार प्रशासित किए जाने पर रक्त के विभिन्न मैट्रिक्स घटकों का सामना करना पड़ता है। हालाँकि, पीबीएस सीरम और एचएसएफ जैसे इन-विट्रो मॉडल में पाए जाने वाले विभिन्न अणुओं से रहित है। तीसरा उद्देश्य पीबीएस में mAb के समग्र प्रोफाइल की तुलना सीरम और एचएसएफ जैसे इन-विट्रो मॉडल से करना था। इस अध्ययन में, पीबीएस में mAb के एकत्रीकरण और विभिन्न तनावों के अधीन mAb नमूनों के साथ शरीर के तरल पदार्थ से प्राप्त मॉडल की तुलना की गई। यह भी पाया गया कि पीबीएस और सीरम युक्त mAb को सरगर्मी और इंटरफेशियल उत्तेजना के अधीन किया गया, जिसके परिणामस्वरूप > 2 माइक्रोन आकार के समुच्चय थे, और औसत आकार और >10 माइक्रोन आकार वाले कणों की प्रतिशत संख्या सभी विश्लेषण समय बिंदु पर पीबीएस की तुलना में सीरम में अधिक थी। . कुल मिलाकर, यह पाया गया कि पीबीएस में mAb का एकत्रीकरण मानव शरीर के तरल पदार्थों से प्राप्त विभिन्न मॉडल थे।

चिकित्सीय प्रोटीन, जैसे कि mAbs, फेरस आयनों ( $Fe^{2+}$ ) या हाइड्रोजन पेरोक्साइड ( $H_2O_2$ ) जैसे रसायनों के संपर्क में आने पर क्षरण का कारण बनते हैं।  $H_2O_2$  और  $Fe^{2+}$  फेंटन प्रतिक्रिया द्वारा प्रतिक्रिया करके हाइड्रॉक्सिल रेडिकल ( $\bullet OH$ ) बनाते हैं, जो प्रोटीन को तेजी से नष्ट करने के लिए जाने जाते हैं। अंतःशिरा प्रशासन से पहले, mAb उत्पाद को अक्सर सामान्य खारा में पतला किया जाता है। विभिन्न रोगों के दौरान मानव शरीर में  $Fe^{2+}$  और  $H_2O_2$  का बढ़ा हुआ स्तर पाया जा सकता है। चौथा उद्देश्य खारा और इन-विट्रो मॉडल में  $Fe^{2+}$  और  $H_2O_2$  की संयुक्त उपस्थिति में चिकित्सीय mAb की स्थिरता की जांच करना था।  $Fe^{2+}$  और  $H_2O_2$  दोनों की संयुक्त उपस्थिति वाले mAb वाले खारे नमूनों में 20% से अधिक HMW प्रदर्शित हुआ, जबकि केवल  $Fe^{2+}$ ,  $H_2O_2$  या न होने वाले नमूनों में क्रमशः 2.5% से कम HMW प्रदर्शित हुआ। समग्र-समृद्ध नमूनों में परिवर्तित प्रोटीन संरचना और हाइड्रोफोबिसिटी भी प्रदर्शित हुई।  $Fe^{2+}$  और  $H_2O_2$  का समय, तापमान और सांद्रता बढ़ने पर एकत्रीकरण में वृद्धि हुई। यह भी पाया गया कि  $Fe^{2+}$  और  $H_2O_2$  दोनों की उपस्थिति में, इन-विट्रो मॉडल जैसे कृत्रिम रूप से तैयार बाह्यकोशिकीय खारा, HSF और सीरम के परिणामस्वरूप mAb का एकत्रीकरण बढ़ा। निष्कर्ष में, mAb युक्त नमूनों में  $Fe^{2+}$  और  $H_2O_2$  दोनों की उपस्थिति के परिणामस्वरूप हाइड्रॉक्सिल रेडिकल्स के गठन की तुलना में कई गुना mAb क्षरण हुआ।

mAb वाले नमूनों को आम तौर पर 2-8°C पर संग्रहित किया जाता है। हालाँकि, उत्पाद विभिन्न चरणों के दौरान उच्च तापमान के अधीन हो सकता है, और यह mAb की मूल संरचना को बिगाड़ सकता है, जिससे अंततः उत्पाद खराब हो सकता है। टॉरिन एक प्राकृतिक रूप से पाया जाने वाला सल्फर युक्त अमीनो एसिड है, जो प्रोटीन को स्थिरता प्रदान करने के लिए जाना जाता है। पाँचवाँ उद्देश्य mAb के क्षरण को कम करने के लिए एक सहायक पदार्थ के रूप में टॉरिन की क्षमता का मूल्यांकन करना था। इस कार्य में, तकनीकों और परखों की एक श्रृंखला का उपयोग करके mAb की स्थिरता की निगरानी की गई थी। 30 मिनट के लिए थर्मल तनाव द्वारा mAb1 युक्त नमूनों के जबरन क्षरण के परिणामस्वरूप टॉरिन वाले नमूने की तुलना में टॉरिन रहित नमूने में एचएमडब्ल्यू 65% से अधिक हो गया। टॉरिन के बिना mAb1 वाले नमूनों में टॉरिन वाले नमूनों की तुलना में परिवर्तित प्रोटीन संरचना, उच्च हाइड्रोफोबिसिटी और कम पिघलने का तापमान होता है। टॉरिन का स्थिरीकरण प्रभाव विभिन्न mAb और टॉरिन सांद्रता, समय, तापमान, बफर्स और पॉलीसोर्बेट 80 (PS80) की उपस्थिति पर बरकरार रखा गया था। इसके अतिरिक्त, टॉरिन की उपस्थिति में mAb ने हल्के तनाव, प्रकाश और H<sub>2</sub>O<sub>2</sub> के संयोजन, और Fe<sup>2+</sup> और H<sub>2</sub>O<sub>2</sub> के संयोजन के साथ तनाव के अधीन होने पर बढ़ी हुई स्थिरता दिखाई। निष्कर्ष में, mAb युक्त नमूनों में टॉरिन की उपस्थिति ने इसकी स्थिरता को बढ़ाया, जिससे एक स्थिर एजेंट के रूप में इसके संभावित उपयोग का संकेत मिलता है।

## Table of Contents

<i>Title</i>	<i>Page No.</i>
<b>Certificate</b>	<b>i</b>
<b>Acknowledgements</b>	<b>ii</b>
<b>Abstract</b>	<b>iv</b>
<b>Table of Contents</b>	<b>x</b>
<b>List of Figures</b>	<b>xv</b>
<b>List of Tables</b>	<b>xxiii</b>
<b>List of Abbreviations</b>	<b>xxv</b>
<b>Chapter 1: Introduction</b>	<b>1-11</b>
1.1 Aggregation of therapeutic IgG	2
1.1.1 Permissible limit of aggregate content in therapeutic IgG samples	3
1.1.2 Adverse impact of therapeutic IgG aggregates	6
1.1.3 Stress factors responsible for aggregation of mAb	6
1.1.4 Techniques for analyzing aggregates of mAb	7
1.2 Stability assessment practices of sample containing mAb	7
1.2.1 Stability testing, accelerated testing, and forced degradation	8
1.2.2 Therapeutic mAb aggregate analysis in in-vitro models	10
1.3 Purpose of the current study	10
<b>Chapter 2: Review of Literature</b>	<b>11-40</b>
2.1. Image analysis algorithm to analyze aggregates of mAb	11
2.1.1 Steps of image processing	12
2.1.2 TEM image analysis algorithm	12
2.1.3 Brightfield and fluorescence microscope image processing	13
2.2 Air/liquid interfacial agitation stress	13
2.2.1 Occurrence of air/liquid interfacial agitation stress	14
2.2.2 Strategies to artificially induce air/liquid interfacial stress in IgG samples	15
2.2.3 Type of aggregates found in samples subjected to interfacial stress	16
2.2.4 Mechanism of IgG aggregation due to air/liquid interfacial agitation	17
2.2.5 Factors influencing aggregation due to air/liquid interfacial agitation	17
2.2.6 Strategies to mitigate aggregation due to air/liquid interfacial agitation	20
2.3 Aggregation of mAb in in-vitro models	22
2.4 Thermal stress	25

2.4.1 Occurrence of thermal stress in IgG containing samples	25
2.4.2 Strategies to artificially induce thermal stress in IgG containing samples	26
2.4.3 Type of aggregates formed in IgG samples subjected to thermal stress	28
2.4.4 Mechanism of IgG aggregation in samples subjected to thermal stress	28
2.4.5 Factors influencing aggregation of IgG due to thermal stress	30
2.5 Strategies to mitigate mAb degradation by thermal stress	34
2.6 Overall scope of the thesis	37
<b>Chapter 3: Image analysis algorithm for determining aggregate size distribution of therapeutic IgG using brightfield images</b>	<b>41-60</b>
3.1 Introduction	43
3.2 Approach for brightfield image processing	45
3.2.1 Importing image on MATLAB	45
3.2.2 Determination of pixel threshold	46
3.2.3 Removing hemocytometer lines	47
3.2.4 Data extraction	47
3.3 Materials and methods	48
3.3.1 Reagents and mAb	48
3.3.2 Preparation of aggregates	48
3.3.3 Analytical characterization	48
3.3.4 Imaging	50
3.3.5 Image processing	51
3.3.6 Imaging liquid chromatography (LC) resins	51
3.4 Results and discussion	51
3.4.1 Analytical characterization of aggregates	51
3.4.2 Acquisition of brightfield images	52
3.4.3 Aggregates size distribution by other experimental techniques	58
3.5 Conclusions	60
<b>Chapter 4: Rapid aggregation of therapeutic monoclonal antibodies by bubbling induced air/liquid interfacial and agitation stress at different conditions</b>	<b>61-93</b>
4.1 Introduction	63
4.2 Materials and methods	65
4.2.1 Reagents	65
4.2.2 IgG	65
4.2.3 Stressing of samples containing IgG	66

4.2.4 Visual observation	67
4.2.5 UV-Vis spectroscopy	67
4.2.6 Size-exclusion chromatography	68
4.2.7 Dynamic light scattering	68
4.2.8 Automated microscopic analysis	69
4.2.9 CD spectroscopy	69
4.2.10 Fluorescence spectroscopy	70
4.2.11 Cell-based assays	70
4.2.12 Impact of various parameters	71
4.3 Results	71
4.3.1 Visual observation	72
4.3.2 UV/Vis spectroscopy	73
4.3.3 Size-based characterization of stressed mAb samples	73
4.3.4 Structural characterization	78
4.3.5 Cell-based activity	82
4.3.6 Impact of various parameters on aggregation	83
4.4 Discussion	86
4.5 Conclusions	93
<b>Chapter 5: Does aggregation of therapeutic IgGs in PBS offer a true picture of what happens in models derived from human body fluids?</b>	<b>94-115</b>
5.1 Introduction	96
5.2 Materials and methods	98
5.2.1 Chemicals and reagents	98
5.2.2 IgG	98
5.2.3 Tagging IgG with FITC	99
5.2.4 Stressing of samples containing IgG	99
5.2.5 Human serum	100
5.2.6 HSF	100
5.2.7 Spiking studies	101
5.2.8 Analytical characterization	102
5.3 Results	105
5.3.1 Case study 1: Comparison of aggregate content in PBS vs. in HSF	108
5.3.2. Case study 2: Aggregate size distribution in PBS vs. serum	109
5.4 Discussion	111

5.4 Conclusions	115
<b>Chapter 6: Combined presence of ferrous ion and hydrogen peroxide in normal saline and in-vitro models induces enhanced aggregation of therapeutic IgG due to hydroxyl radicals</b>	<b>116-154</b>
6.1 Introduction	118
6.2 Materials and methods	120
6.2.1 Reagents	120
6.2.2 IgG	120
6.2.3 Isolation of serum	121
6.2.4 Isolation of macromolecule free fraction of serum	121
6.2.5 Tagging IgG with fluorescent dye	121
6.2.6 Stressing of IgG	122
6.2.7 UV-vis spectroscopy	122
6.2.8 Visual inspection	123
6.2.9 SEC	123
6.2.10 DLS	123
6.2.11 Fluorescence spectroscopy	123
6.2.12 Microscopy and image processing	124
6.2.13 Hemolytic assay	124
6.2.14 SPR	125
6.3 Results	125
6.3.1 Case study 1: Aggregation of IgG in saline	125
6.3.2 Case study 2: Aggregation in in-vitro models	139
6.3.3 Hemolysis due to Fe <sup>2+</sup> and H <sub>2</sub> O <sub>2</sub>	145
6.4 Discussion	146
6.5 Conclusions	153
<b>Chapter 7: Taurine, a naturally occurring amino acid, as a stability enhancer of therapeutic monoclonal antibodies</b>	<b>155-179</b>
7.1 Introduction	157
7.2 Materials and methods	159
7.2.1 Reagents	159
7.2.2 IgG	159
7.2.3 Subjecting IgG to different stress conditions	160
7.2.4 Analytical characterization	161

## Table of Contents

7.2.5 Hemolytic assay	162
7.3 Results	163
7.3.1 Forced degradation studies	163
7.3.2 IgG stability at 55°C	176
7.3.3 Taurine as IgG stabilizer against oxidative stress	177
7.3.4 Biocompatibility of taurine containing samples	179
7.4 Discussion	180
7.5 Conclusions	183
<b>Chapter 8: Conclusions &amp; future recommendations</b>	<b>184-186</b>
<b>References</b>	<b>187-210</b>
<b>List of publications</b>	<b>211-212</b>
<b>Curriculum vitae</b>	<b>213-215</b>

## List of Figures

Figure No.	Figure Caption	Page No.
1.1	(a) Schematic representation of various stages and stress factors due to which sample containing mAb undergo stability issues leading formation of aggregates. (b) Different size ranges of aggregates of mAb and techniques used for their characterization. (c) Schematic picture of the experimental set-up for the generation and analysis of aggregates of mAb.	5
2.1	A fluorescence microscope image showing FITC tagged mAb subjected to stirring stress is shown in (a). (b) is the output image after image processing, and (c) is the size distribution graph of aggregates of mAb in $\mu\text{m}^2$ . From the area occupied by aggregates in the image, the circularity, radius, and diameter of aggregates can be calculated.	23
3.1	Gaussian like peaks obtained from a grayscale image separating the aggregate, boundary, and background region.	46
3.2	Series of steps used in processing brightfield images	48
3.3	Visual turbidity of samples subjected to stress and the control samples are shown in (a). (b) depicts the AI and protein loss, and (c) shows the loss in AUTC for SEC chromatogram.	52
3.4	A representative brightfield microscopic image of sample containing aggregates of mAb stressed by stirring acquired by Cytell is shown in (a). Sample containing 1mg/mL mAb in PBS not subjected to any stress did not show the presence of any particulate aggregate is shown in (b). Brightfield image of sample containing aggregate seen with C-chip is shown in (c), and a small part of (c) is further zoomed.	53
3.5	The output after each step of the brightfield image processing algorithm. Input image is shown in (a). Image after conversion to grayscale is shown in (b). Pixel distribution plot of grayscale image is shown in (c). (d) is the binary image. (e) is output images after size based upper and lower thresholding. The image in (f) is obtained after morphological operations. All the marked aggregates are shown in (g) and (h) is the aggregate size distribution plot.	55
3.6	Brightfield image of source 30S resin is shown in (a), (b) is the output of image processing and (c) is the size (radii) distribution of resin particles (in $\mu\text{m}$ ).	56
3.7	Plot showing the number of pixels corresponding to each color in grayscale image from 0 to 255 for a representative image of mAb containing samples stressed by stirring.	57

3.8	Aggregate size distribution (in diameter) of samples stressed by stirring obtained by MFI (a) and MS2000 (b).	58
4.1	Schematic picture of the experimental set-up for the generation of air/liquid interfacial agitation by introducing air bubbles into mAb samples.	67
4.2	Samples exposed to interfacial agitation through bubbling for up to 240 minutes with a sample volume of 500 $\mu$ L, mAb concentration of 1 mg/mL at 37°C and an air flow rate of 11.5 mL/min, as detected for mAb1 by (a) visual observation and turbidity, where 0 represents sample not subjected to any stress and 4 is the sample stressed for 240 minutes. The graph in (b) shows turbidity ( $A_{350}$ ) obtained at different time points and (c) shows aggregation index (AI, calculated from $A_{350}$ and $A_{280}$ ) and protein loss (%) (in the supernatant, cf. section 2.5), and (d) is the comparison of AI for mAb1, mAb2 and mAb3 samples.	72
4.3	(a) SEC chromatograms of supernatants of mAb1 sample obtained using Superdex 200 column. (b) The % loss of AUC in SEC chromatogram for mAb1 as function of bubbling time. (c) DLS results of mAb1 samples subjected to bubbling. The data was obtained by analyzing fractions of sample containing 500 $\mu$ L of 1 mg/mL mAb stressed at 37°C and 11.5 mL/min air flow rate for different time up to 240 minutes.	75
4.4	Digital microscopy images for mAb1 samples subjected to interfacial agitation stress: (a) A representative image showing aggregates and background of sample containing mAb (500 $\mu$ L of 1 mg/mL mAb in PBS stressed for 120 minutes at 37°C and 11.5 mL/min air flow rate). Images of samples subjected to bubbling (500 $\mu$ L of 1 mg/mL mAb in PBS at 37°C and 11.5 mL/min air flow rate) for (b) 30 minutes, (c) 60 minutes, (d) 120 minutes and (e) 240 minutes. The binary image obtained after the processing the images for 30 minutes is shown in (f), 60 minutes in (g), 120 minutes in (h) and 240 minutes in (i). The size distribution of aggregates in the set of acquired images for the samples subjected to stress for 30 minutes is shown in (j), 60 minutes in (k), 120 minutes (l) and 240 minutes (m).	77
4.5	Intrinsic fluorescence emission spectra of mAb1 samples exposed to bubbling for up to 240 minutes at 37°C is shown in (a). Extrinsic fluorescence emission spectra of mAb1 samples exposed to bubbling for up to 240 minutes at 37°C obtained with 10 $\mu$ M is shown in (b).	79
4.6	(a) Far-UV CD spectra and (b) near-UV CD spectra of supernatants of mAb1 samples exposed to bubbling for up to 240 minutes at 37°C.	80
4.7	SEC chromatograms of control samples containing mAb1 analyzed by Superdex 200 column.	81
4.8	Effect of mAb1 concentration on (a) turbidity and (b) protein loss (%) during air/liquid interfacial agitation through bubbling. 500 $\mu$ L of sample containing mAb of concentration ranging from 1 to 4 mg/mL in PBS was stressed for 240 minutes at 37°C and air flow rate of 11.5 mL/min.	84

4.9	Effect of (a) sample volume, (b) temperature, (c) presence of silicone oil or polysorbate and (d) bubbling rate on the stability of 1 mg/mL mAb1 in PBS exposed to bubbling for up to 240 minutes, as measured by turbidity ( $A_{350}$ ) and protein loss (%).	86
4.10	Scheme of the putative mechanism involved in the aggregation of mAb in the presence of air/liquid interfacial agitation through bubbling. The presence of air bubbles, headspace and monomers in the buffer is shown in (a). Exposure of hydrophobic patches and adsorption of mAb to air/liquid interface is shown in (b). The aggregates formed are shown in (c).	93
5.1	FITC tagged mAb1 is shown in (a). (b) and (c) are the SEC and DLS profiles of samples containing FITC tagged and untagged mAb.	99
5.2	SEC chromatogram of human serum filtrate (HSF) and serum (diluted 20 times) is shown in (a). Experimental workflow is shown in (b). Case 1: The samples containing mAb not tagged with any dye were spiked in HSF and PBS. Case 2: The samples containing FITC labeled mAb were spiked in serum and PBS. The percentage aggregates of mAb in the first case were determined by SEC, and the size distribution of aggregates in the second case was analyzed by fluorescence microscope image processing.	102
5.3	Overall procedure showing the different steps for processing the fluorescence microscope images.	105
5.4	Brightfield images showing dark-coloured aggregates against a bright background and sub-visible aggregate size distribution obtained by image processing of samples subjected to stirring are shown in (a) and (b), and interfacial agitation is shown in (c) and (d). Fluorescence microscope image of a FITC-tagged sample subjected to stirring stress is shown in (e). The binary image obtained after processing (e) is shown in (f) and the size distribution obtained after compiling data from numerous images with a bin size of 10 $\mu\text{m}$ is shown in (g). The fluorescence microscope image of a FITC tagged sample subjected to interfacial agitation stress is shown in (h). (i) is the binary image obtained by processing (h), and the size distribution obtained after compiling data from numerous images is shown in (j).	107
5.5	%HMW of mAb in HSF and PBS obtained by SEC containing unstressed mAb and mAb that were subjected to stirring, interfacial agitation, pH, temperature, and light stress at different time points are shown in (a)-(f).	108
5.6	A representative fluorescence microscope image captured at 24, 48, and 72 hours in PBS and serum containing mAb samples subjected to stirring stress and incubated at 37°C is shown in (a)-(f).	110
5.7	<b>PBS and serum containing aggregates of mAb formed by stirring stress.</b> The aggregate size distribution obtained by compiling all the images captured at 24, 48, and 72 hours of PBS and serum containing mAb sample subjected to stirring stress is shown in (a), (b), and (c). The average size of	110

	aggregates having more than 10 $\mu\text{m}$ size in serum and PBS at different time points is shown in (d).	
5.8	<b>PBS and serum containing aggregates of mAb formed by interfacial agitation stress.</b> The aggregate size distribution obtained by compiling all the images captured at 24, 48, and 72 hours of samples analyzed at least in duplicates in PBS and serum containing mAb sample subjected to interfacial agitation stress is shown in (a), (b), and (c). The average size of aggregates having more than 10 $\mu\text{m}$ size in serum and PBS and serum at different time points is shown in (d).	111
6.1	Experimental turbidity and normalized AI of mAb1 samples subjected to incubation at 55°C for 1 hour is shown in (a). The %HMW obtained by SEC and experimental turbidity of mAb2 samples subjected to stress is shown in (b). The SEC chromatogram of unstressed sample containing mAb1 is shown in (c), and the SEC chromatogram of stressed samples containing mAb1 is shown in (d). The DLS output graph of unstressed and stressed samples are shown in (e) and (f). In Figures (a) and (b), the x-axis shown as (A)-(D), is the sample containing mAb consisting of 0.2 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (A), 0.1% $\text{H}_2\text{O}_2$ (B), both 0.2 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1% $\text{H}_2\text{O}_2$ (C), and neither $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ nor $\text{H}_2\text{O}_2$ (D). The sample labeled as (E) is the mAb sample not subjected to any stress and not containing both $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{H}_2\text{O}_2$ .	127
6.2	(a) Intrinsic fluorescence spectra, (b) extrinsic fluorescence spectra, and (c) second derivative UV-vis spectra of mAb1 samples that were subjected to incubation at 55°C for 1 hour. In the Figures, samples (A)-(D) are samples containing mAb in saline consisting of 0.2 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (A), 0.1% $\text{H}_2\text{O}_2$ (B), both 0.2 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1% $\text{H}_2\text{O}_2$ (C), and neither $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ nor $\text{H}_2\text{O}_2$ (D). Sample labelled as (E) is the mAb sample not subjected to any stress and does not contain both $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{H}_2\text{O}_2$ .	130
6.3	%HMW obtained by SEC of mAb1 samples subjected to stress for different time points is shown in (a). The SEC chromatogram obtained every 30 minutes for mAb samples incubated at 55°C containing $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ is shown in (b), and for mAb samples containing both $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{H}_2\text{O}_2$ is shown in (c). The percentage of aggregates obtained by SEC in samples containing only either $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , or $\text{H}_2\text{O}_2$ , and neither $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ nor $\text{H}_2\text{O}_2$ is shown in (d).	133
6.4	%HMW in the samples of mAb1 subjected to stress at different temperatures for 1 hour is shown in (a). The SEC chromatogram of mAb1 samples containing 0.2 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and both 0.2 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1% $\text{H}_2\text{O}_2$ subjected to stress at 40, 55, and 70°C for 1 hour is shown in (b). The experimental turbidity of mAb1 samples incubated at different temperatures for 1 hour is shown in (c). The brightfield image and fluorescence microscope image that show aggregates of mAb1 in the	135

	sample containing both $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{H}_2\text{O}_2$ , incubated at $70^\circ\text{C}$ for 1 hour are shown in (d) and (e). (e) also shows the output image after processing the fluorescence image. The size distribution of aggregates of mAb1 obtained processing from a set of fluorescence microscope images of sample containing both $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{H}_2\text{O}_2$ , incubated at $70^\circ\text{C}$ for 1 hour is shown in (f).	
6.5	(a) SEC chromatogram of sample containing mAb1 (1mg/mL) in saline having 0.2 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (A), and both 0.2 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1% (C) incubated at $55^\circ\text{C}$ for 24 hours. (b) The percentage of aggregates obtained by SEC for samples of mAb1 subjected to stress at $40^\circ\text{C}$ till 72 hours and the SEC chromatogram of the mAb1 sample having $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and both $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{H}_2\text{O}_2$ subjected to stress at $40^\circ\text{C}$ for 72 hours. The %HMW for different samples of mAb1 incubated at $55^\circ\text{C}$ containing 0, 0.05, and 0.2 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ is shown in (c).	136
6.6	%HMW obtained by SEC for samples of mAb1 containing 0, 0.05, 0.1, 0.2, 0.4, and 0.8mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in the presence and absence of 0.1% $\text{H}_2\text{O}_2$ incubated for 1 hour at $55^\circ\text{C}$ is shown in (a), and the experimental turbidity is shown in (b). The %HMW for samples of mAb1 incubated for 1 hour at $55^\circ\text{C}$ containing 0, 0.025, 0.05, and 0.1% $\text{H}_2\text{O}_2$ in the presence and absence of 0.2mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ is shown in (c).	137
6.7	%HMW obtained by SEC of different samples of mAb1 incubated at $55^\circ\text{C}$ for 1 hour containing 0.2 mM $\text{CuCl}_2 \cdot \text{H}_2\text{O}$ or $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ in the presence and absence of 0.1% $\text{H}_2\text{O}_2$ is shown in (a), and the experimental turbidity of those samples are shown in (b). The percentage of monomer left in the samples after they were subjected to stress in the presence of different metals and $\text{H}_2\text{O}_2$ is shown in (c). Samples (A)-(D) are samples containing mAb consisting of 0.2 mM metal compound (A), 0.1% $\text{H}_2\text{O}_2$ (B), both metal compound and 0.1% $\text{H}_2\text{O}_2$ (C), and neither metal compound nor $\text{H}_2\text{O}_2$ (D).	139
6.8	(a) is the %HMW obtained by SEC and Z-average diameter obtained by DLS, and (b) is the experimental turbidity and AI of different samples of mAb1 incubated at $55^\circ\text{C}$ for 1 hour in extracellular saline. In the Figure, samples (A)-(D) are samples containing mAb consisting of 0.2 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (A), 0.1% $\text{H}_2\text{O}_2$ (B), both 0.2 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1% $\text{H}_2\text{O}_2$ (C), and neither $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ nor $\text{H}_2\text{O}_2$ (D). The unstressed sample is shown in (E).	140
6.9	%HMW obtained for different samples of HSF and extracellular saline containing mAb1 (1 mg/mL) having the presence of both 0.2mM $\text{Fe}^{2+}$ and 0.1% $\text{H}_2\text{O}_2$ incubated at $37^\circ\text{C}$ for 3 hours is shown in (a). %HMW obtained by SEC for different FBSF samples of mAb1 (0.2 mg/mL) incubated at $55^\circ\text{C}$ for 72 hours is shown in (b), and %HMW in different samples of mAb1 (0.2 mg/mL) incubated in FBSF at $40^\circ\text{C}$ for 72 hours is shown in (c).	143
6.10	%LMW obtained by SEC for different samples of mAb1 (0.2 mg/mL) incubated at $55^\circ\text{C}$ in FBSF for 72 hours is shown in (a) and, the %LMW for	144

	different samples of mAb1 (0.2 mg/mL) incubated at 40°C in FBSF for 72 hours is shown in (b).	
6.11	Fluorescence microscope image showing aggregates of mAb1 (0.2 mg/mL) in serum containing 0.8 mM FeSO <sub>4</sub> ·7H <sub>2</sub> O and 0.375% H <sub>2</sub> O <sub>2</sub> incubated at 40°C for 72 hours is shown in (a), the output image after processing shown in (b), and the size distribution of aggregates obtained by compiling a set of images is shown in (c).	141
6.12	Fluorescence microscope image showing aggregates of mAb in serum containing both 0.2 mM FeSO <sub>4</sub> ·7H <sub>2</sub> O and 0.1% H <sub>2</sub> O <sub>2</sub> , incubated at 55°C for 72 hours is shown in (a) The output image after processing of the image (a) is shown in (b), and the size distribution of aggregates obtained after processing a set of fluorescence microscope images is shown in (c).	145
6.13	Hemolytic cytotoxicity of samples containing 1 mg/mL mAb1 in the presence of 0.2 mM FeSO <sub>4</sub> ·7H <sub>2</sub> O and/or 0.1% H <sub>2</sub> O <sub>2</sub> . Samples (A), (B), (C), and (D) are mAb sample in saline containing 0.2 mM FeSO <sub>4</sub> ·7H <sub>2</sub> O (A), 0.1% H <sub>2</sub> O <sub>2</sub> (B), both 0.2 mM FeSO <sub>4</sub> ·7H <sub>2</sub> O and 0.1% H <sub>2</sub> O <sub>2</sub> (C), and neither FeSO <sub>4</sub> ·7H <sub>2</sub> O nor H <sub>2</sub> O <sub>2</sub> (D).	145
6.14	SEC chromatogram obtained using Superdex 75 column for the sample containing mAb1 (1mg/mL) in saline having both 0.2 mM FeSO <sub>4</sub> ·7H <sub>2</sub> O and 0.1% (C) incubated at 55°C. The SEC chromatogram of marker proteins having a molecular weight of 66.5 kDa and 30 kDa are also shown.	146
6.15	Schematic figure showing the production of hydroxyl radical by Fenton reaction and degradation of therapeutic mAb.	152
7.1	Chemical structure of taurine is shown in (a). %HMW, %Monomer, and %LMW at obtained different time points in samples containing mAb1 with and without 250 mM taurine subjected to thermal stress of 70°C are shown in (b), (c), and (d). SEC chromatogram of samples containing mAb1 with and without 250 mM taurine subjected to thermal stress of 70°C for 30 minutes is depicted in (e).	164
7.2	Intrinsic fluorescence spectra of samples containing mAb1 with and without 250 mM taurine subjected to thermal stress of 70°C for 30 and 720 minutes are shown in (a) and (b). Far-UV CD spectra of samples containing mAb with and without 250 mM taurine subjected to thermal stress of 70°C for 30 and 720 minutes are shown in (c) and (d). Second derivative ATR-FTIR spectra of samples containing 10 mg/mL mAb with and without 250 mM taurine subjected to stress at 70°C for 120 minutes is shown in (e). Extrinsic fluorescence spectra of samples containing mAb with and without 250 mM taurine subjected to thermal stress at 70°C for 30 minutes is shown in (f).	167
7.3	%HMW and %Monomer obtained by SEC of samples C, T1, T2, and T3 containing 1mg/mL mAb1 in PBS subjected to thermal stress of 70°C for 30 minutes are shown in (a), and (b). Extrinsic fluorescence spectra of samples C, T1, T2, and T3 containing 1mg/mL mAb1 in PBS subjected to	170

	thermal stress of 70°C for 30 minutes are shown in (c). %Monomer obtained by SEC for samples with and without 250 mM taurine containing different concentrations of mAb1 subjected to thermal stress of 70°C for 30 minutes is shown in (d). %HMW and %Monomer obtained by SEC of samples containing 0.01% PS80, 250 mM ectoine, 250 mM taurine, and 250 mM trehalose is shown in (e) and (f). %HMW and %Monomer obtained by SEC of samples containing 1mg/mL mAb1 with and without 250 mM taurine in histidine buffer subjected to thermal stress of 70°C for 4 hours are shown in (g) and (h). Percentage of HMW and monomer obtained by SEC of samples containing 5mg/mL mAb1 in histidine buffer with 0 and 250 mM taurine subjected to thermal stress of 55°C for 12 days are shown in (i) and (j).	
7.4	Visual turbidity, OD at 350 nm, and extrinsic fluorescence spectra of samples C, T1, T2, and T3 containing 1 mg/mL mAb1 subjected to thermal stress at 70°C for 24 hours are shown in (a), (b), and (c). Samples containing mAb having different concentrations of taurine (C, T1, T2, and T3) incubated at 70°C for up to 150 minutes were not visually turbid. However, when samples T, T1, T2, and C were incubated for 24 hours at 70°C, it was found that samples T3 and T2 were visually the least turbid, and the visual turbidity increased with a decrease in taurine concentration, as sample C showed maximum visual turbidity. Samples containing 0, 10, 100, and 250 mM taurine are termed C, T1, T2, and T3. HMW obtained by SEC of samples with different concentrations of taurine (250 mM-700 mM) containing mAb1 subjected to thermal stress of 70°C for 30 minutes is shown in (d).	171
7.5	%HMW and %Monomer obtained by SEC for samples C, T1, T2, and T3 containing mAb1 subjected to thermal stress of 65°C for 150 minutes is shown in (a) and (b). Intensity at 505 nm obtained from extrinsic fluorescence spectra for samples containing 1 mg/mL mAb in PBS with 250 and 0 mM taurine subjected to thermal stress of 65°C for 150 minutes is shown in (c). Figures (d) and (e) shows the %HMW and %Monomer for samples containing mAb in PBS with 250 and 0 mM taurine subjected to thermal stress of 65°C for different time intervals of up to 24 hours. %HMW obtained by SEC for samples C, T1, T2, and T3 containing mAb1 subjected to thermal stress of 65°C for different time intervals is shown in (f).	172
7.6	HMW obtained by SEC of samples with 250 and 0 mM taurine containing different concentrations of mAb1 subjected to thermal stress of 70°C for 30 minutes is shown in (a). SEC output of samples containing 5 mg/mL mAb1 subjected to thermal stress of 70°C for 30 minutes is shown in (b). HMW obtained by SEC of samples C, T1, T2, and T3 containing 5 mg/mL mAb1 subjected to thermal stress is shown in (c). %HMW obtained by SEC for samples containing constant molar ratio (0.67) of mAb to taurine with	174

	different concentrations of mAb1 that were subjected to thermal stress at 70°C for 30 minutes is shown in (d).	
7.7	Monomer obtained by SEC of samples containing 1mg/mL mAb2 with and without 250 mM taurine in PBS subjected to thermal stress of 60°C are shown in (a). Monomer obtained by SEC of samples containing 1mg/mL mAb2 with and without 250 mM taurine in phosphate buffer subjected to thermal stress of 60°C are shown in (b).	176
7.8	Percentage of IgG monomer in samples containing 1 mg/mL mAb1 in PBS with and without 250 mM taurine subjected to light of 365 nm wavelength at 45°C are shown in (a). Percentage of monomer obtained by SEC of samples containing 1 mg/mL mAb1 with and without 250 mM taurine subjected to combined presence of light and 0.1% H <sub>2</sub> O <sub>2</sub> at 40°C is shown in (b). The amount of monomer obtained by SEC of samples containing 1 mg/mL mAb1 with and without 250 mM taurine subjected to stress in the combined presence of 0.2 mM Fe <sup>2+</sup> and 0.1% H <sub>2</sub> O <sub>2</sub> at 40°C is shown in (c). SEC chromatogram of samples containing 1 mg/mL mAb1 with and without 250 mM taurine analyzed after incubation in light for 15 minutes is shown in (d). SEC chromatogram of samples containing 1 mg/mL mAb1 with and without 250 mM taurine analyzed after incubation in the combined presence of Fe <sup>2+</sup> and H <sub>2</sub> O <sub>2</sub> for 60 minutes is shown in (e).	178
7.9	Supernatants after the final centrifugation step in hemolysis assay for different samples are shown in (a), and the hemolytic activities are shown in (b). In the figures, PBS is blank, and C, T1, T2, and T3 are mAb containing samples in the presence of 0, 10, 100, and 250 mM taurine.	179

## List of Tables

Table No.	Table Caption	Page No.
3.1	Comparison of image analysis output and manufacturer's specification of LC resin sizes.	56
4.1	Percentage of aggregates and monomers in supernatant containing mAb1 obtained by SEC using Superdex 200 column, and percentage of fragments determined by Superdex 75 column.	75
4.2	The details of aggregates in the control samples incubated at 37°C obtained by SEC and DLS.	81
4.3	(a) ADCC and (b) CDC of stressed samples with aggregates and control samples containing mAb2.	82
5.1	Conditions used for generating aggregates using samples containing 1mg/mL mAb.	100
5.2	%HMW obtained by SEC, experimental turbidity (OD at 350 nm), and visual turbidity of samples containing mAb1 subjected to different stresses such as stirring, interfacial agitation, light, pH, and temperature.	106
5.3	%HMW, and visual turbidity of samples containing mAb1 tagged with FITC subjected to different stresses such as stirring, interfacial agitation, light, pH, and temperature.	106
6.1	%HMW obtained from SEC, Z-average diameter obtained by DLS, and visual turbidity observed by separate users for samples containing mAb1 (1 mg/mL) in saline incubated at 55°C for 1 hour.	129
6.2	Visual turbidity for different samples of mAb1 subjected to stress for different temperatures for 24 hours. In the Table, sample (A), (C), and (D) are mAb samples containing 0.2 mM FeSO <sub>4</sub> .7H <sub>2</sub> O (A), both 0.2 mM FeSO <sub>4</sub> .7H <sub>2</sub> O and 0.1% H <sub>2</sub> O <sub>2</sub> (C), and neither FeSO <sub>4</sub> .7H <sub>2</sub> O nor H <sub>2</sub> O <sub>2</sub> (D).	136
6.3	%LMW obtained from SEC chromatogram of samples containing mAb1 (1 mg/mL) in saline incubated at 55°C for 1 hour in presence of different metal compounds with and without H <sub>2</sub> O <sub>2</sub> .	138
6.4	(i) %HMW and %LMW obtained by SEC for sample containing 1 mg/mL mAb1 in FBSF consisting of both 0.2 mM FeSO <sub>4</sub> .7H <sub>2</sub> O and 0.1% H <sub>2</sub> O <sub>2</sub> , and neither 0.1% H <sub>2</sub> O <sub>2</sub> nor FeSO <sub>4</sub> .7H <sub>2</sub> O incubated at 55°C for 1 hour. (ii) The %HMW and %LMW obtained by SEC for sample containing 1 mg/mL mAb1 in HSF consisting of both 0.2 mM FeSO <sub>4</sub> .7H <sub>2</sub> O and 0.1% H <sub>2</sub> O <sub>2</sub> , and neither 0.1% H <sub>2</sub> O <sub>2</sub> nor FeSO <sub>4</sub> .7H <sub>2</sub> O incubated at 55°C for 1 hour. (iii) The %HMW and %LMW obtained by SEC for sample containing 0.2 mg/mL mAb1 consisting of both 0.2 mM FeSO <sub>4</sub> .7H <sub>2</sub> O and 0.1% H <sub>2</sub> O <sub>2</sub> , and neither 0.1% H <sub>2</sub> O <sub>2</sub> nor FeSO <sub>4</sub> .7H <sub>2</sub> O incubated at 55°C for 1 hour FBSF. (iv) The %HMW obtained by SEC for the sample containing mAb1 consisting of both 0.2 mM FeSO <sub>4</sub> .7H <sub>2</sub> O and 0.1% H <sub>2</sub> O <sub>2</sub> , and neither H <sub>2</sub> O <sub>2</sub> nor FeSO <sub>4</sub> .7H <sub>2</sub> O incubated at 55°C for 72 hours in HSF. (v) The %HMW obtained by SEC for the sample containing mAb1	140

	consisting of both 0.2 mM FeSO <sub>4</sub> .7H <sub>2</sub> O and 0.1% H <sub>2</sub> O <sub>2</sub> , and neither H <sub>2</sub> O <sub>2</sub> nor FeSO <sub>4</sub> .7H <sub>2</sub> O incubated at 40°C for 72 hours in HSF. (vi) and (vii) are the %HMW obtained by SEC for the sample containing mAb1 consisting of both 0.05 mM FeSO <sub>4</sub> .7H <sub>2</sub> O and 0.025% H <sub>2</sub> O <sub>2</sub> , and neither H <sub>2</sub> O <sub>2</sub> nor FeSO <sub>4</sub> .7H <sub>2</sub> O incubated at 37°C in HSF.	
6.5	%HMW and %LMW obtained by SEC for different samples of mAb1 (0.2 mg/mL) incubated at 40°C in FBSF for 72 hours containing different amounts of FeSO <sub>4</sub> .7H <sub>2</sub> O and H <sub>2</sub> O <sub>2</sub> .	144
6.6	Hemolytic cytotoxicity of samples (A)-(D) containing 1 mg/mL mAb in the presence of 0.2 mM FeSO <sub>4</sub> .7H <sub>2</sub> O and/or 0.1% H <sub>2</sub> O <sub>2</sub> obtained after 7.5 hours. In the Table, samples (A)-(D) are mAb1 samples containing 0.2 mM FeSO <sub>4</sub> .7H <sub>2</sub> O (A), 0.1% H <sub>2</sub> O <sub>2</sub> (B), both 0.2 mM FeSO <sub>4</sub> .7H <sub>2</sub> O and 0.1% H <sub>2</sub> O <sub>2</sub> (C), and neither FeSO <sub>4</sub> .7H <sub>2</sub> O nor H <sub>2</sub> O <sub>2</sub> (D).	146
6.7	K <sub>D</sub> value obtained from the SPR analysis for different samples of mAb1 is shown in the table. The binding of mAb1 samples with recombinant human FcRn receptor was determined by SPR. The equilibrium constant (K <sub>D</sub> ) is the ratio of the antibody dissociation rate (k <sub>off</sub> ), i.e., how quickly it dissociates from its antigen to the antibody association rate (k <sub>on</sub> ) i.e, and how quickly it binds to its antigen. In the Table, samples (C), (E) are mAb1 samples containing both 0.2 mM FeSO <sub>4</sub> .7H <sub>2</sub> O and 0.1% H <sub>2</sub> O <sub>2</sub> (C), and unstressed sample not containing any Fe <sup>2+</sup> and H <sub>2</sub> O <sub>2</sub> (E).	148
7.1	%Monomer, Z-average diameter, and visual turbidity of samples containing mAb1 with and without 250 mM taurine subjected to thermal stress at 70°C for different time intervals.	165

**List of Abbreviations**

ADCC, Antibody dependent cell-mediated cytotoxicity  
AF4, Asymmetrical flow field-flow fractionation  
AFM, Atomic force microscope  
AI, Aggregation index  
ANS, 1-anilino-8 naphthalenesulfonate  
ATR-FTIR, Attenuated total reflectance-Fourier transform infrared  
AUC Analytical ultracentrifugation  
AUTC, Area under the curve  
BSA, Bovine serum albumin  
CD, Circular dichroism  
CDC, Complement dependent cytotoxicity  
CDR, Complementarity determining regions  
CLSM, Confocal Laser Scanning Microscope  
CQA, Critical quality attributes  
DLS, Dynamic light scattering  
DSC, Differential scanning calorimetry  
FBSF, Fetal bovine serum filtrate  
FITC, Fluorescein isothiocyanate  
HCP, Host cell proteins  
HCD, Host cell DNA  
HMW, High molecular weight species  
HPLC, High performance liquid chromatography  
HSA, Human serum albumin  
HSF, Human serum filtrate  
ICH, International Conference of Harmonization  
IgG, Immunoglobulins  
kDa, kilo Dalton  
IL, Ionic liquid  
LDH, Lactate dehydrogenase  
LMW, Low molecular weight species  
LO, Light obscuration  
mAb, Monoclonal antibodies

MFI, Micro flow imaging

MS2000; Mastersizer 2000

MTS, 3-(4,5-dimethylthiazol-2-yl)5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium salt

NTA, Nanoparticle tracking analysis

OD, Optical density

PBMC, Peripheral blood mononuclear cells

PBS, Phosphate buffered saline

Ph. Eur., European Pharmacopeia

PMS, Phenazine methosulfate

PS, Polysorbate

QSFF, Q Sepharose Fast Flow

RBC, Red blood cell

RI, Refractive index

ROS, Reactive oxygen species

SEC, Size exclusion chromatography

SPR, Surface plasmon resonance

U, Unstressed sample

USP, United States Pharmacopeia

$\lambda_{\max}$ , Maximum emission intensity wavelength