U.S. FOOD & DRUG ADMINISTRATION

# RESEARCH PROJECT PROGRESS REPORT

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# Contents

# Work carried out at the Indian Institute of Technology, New Delhi

## Overview

The foundation of a biosimilar manufacturer's regulatory filing is the demonstration of an analytical and functional similarity between the biosimilar product and the appropriate originator product. The interference that the excipients in the formulation cause during the standard range of analytical and functional techniques is one of the most difficult challenges that must be overcome while performing these operations. As a consequence, producers of biosimilar products resort to a variety of approaches to isolate the biotherapeutic protein from the drug product formulation. However, there is an element of uncertainty regarding the impact that this isolation has on product deterioration and the findings that are produced afterward. In light of these obstacles, the purpose of this project is to develop an analytical platform that will enable us to carry out trustworthy characterization and evaluation of the comparability of biosimilar medicine products in lyophilized and liquid formulations.

## Aims & Objectives

An excipient extraction study was conducted with the following objectives:

- To establish the impact of excipient extraction on mAb stability during the buffer exchange process
- Establishing destabilization/stabilization process of protein during excipient addition/removal and buffer exchange cycles
- To establish whether destabilization is reversible
- To investigate mAb loss during the extraction process

## Abbreviations

**mAb:** Monoclonal antibody
**DP:** Drug Product
**API:** Active Pharmaceutical Ingredient
**DV:** Diavolume
**CEX:** Cation Exchange Chromatography
**DSP:** Downstream processing
**SEC:** Size Exclusion Chromatography
**DLS:** Dynamic Light Scattering
**CD:** Circular Dichroism
**FTIR:** Fourier Transform Infrared Spectroscopy
**FLR:** Fluorescence Spectroscopy
**UV:** Ultraviolet Spectroscopy

## Project Progress Summary

The proposed project consists of three specific aims and studies are in progress in the following areas:

- Assess the similarity of products made of a single active pharmaceutical ingredient (API) but formulated as a variety of commercially available biosimilars.
- To establish the impact of excipient extraction on mAb stability: (a) during buffer exchange process and (b) using different buffers.
- To establish the impact of each excipient on mAb stability during the buffer exchange process.

# Material & Equipment

## Reagent & Consumables

### Reagents:

Sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium azide, isopropanol, acetic acid, sodium hydroxide, L-histidine, histidine HCl, trehalose dihydrate, polysorbate 20, sodium chloride, orthophosphoric acid, MilliQ,

### Column:

BioMab, NP5, PK column (4.6 x 250 mm, 5 µm, Agilent)

TSKgel G3000SWxl (7.8 mm× 300 mm, Tosoh)

### Consumables:

Quartz glass cuvette (1mm), black polystyrene 96-well microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany), 0.22 µm membrane filter (Pall Corporation), 96-well UV microplates (Corning, US), Centricons 30 kDa (Pall Corporation)

### Sample:

Trastuzumab was the chosen API for the excipient extraction study. One batch of innovator Herclon (Roche) 440 mg (Batch Number N3970B04 B3536, Expiry: 02/2025) and one batch of Indian biosimilar Eleftha (Intas Pharmaceuticals) 150 mg (Batch Number 26020038, Expiry: 11/2025) were used for the study. Control run was done using only Eleftha biosimilar.

## Procedures

Extraction of API from one innovator and one biosimilar product through buffer exchange cycles (1 cycle=6 diavolumes (DV))

- The API is extracted and exposed to alternate cycles of formulation buffer and cation exchange chromatography (CEX) elution buffer.
- CEX elution buffer is chosen as it forms the polishing step in downstream processing (DSP) before final formulation of API.
- mAb to be aliquoted after each step of buffer exchange cycle for stability analysis.
- Control of the experiment: Repeated extraction of API in only formulation buffer

CEX Elution buffer composition was 50mM sodium phosphate and 150 mM NaCl, pH 6. Formulation buffer composition was as in marketed DP with L-histidine, histidine HCl, trehalose dihydrate and polysorbate 20, pH 6.

**Table 1.** List of analytical tools used for stability analysis

| S.No | Tool | Aim | Sets |
|---|---|---|---|
| 1 | SEC | Size and aggregation | 3 |
| 2 | CEX | Charge variant | 3 |
| 3 | DLS | Aggregation | 3 |
| 4 | CD (Near and Far) | Secondary and tertiary structure | 3 |
| 5 | FLR | Tertiary Structure | 3 |
| 6 | FTIR | Secondary Structure | 3 |
| 7 | UV | Mass balance | 3 |

## Size Exclusion Chromatography (SEC)

SE-HPLC was performed using Dionex Ultimate 3000 HPLC (Thermo Scientific, USA) on TSKgel G3000SWxl Tosoh (7.8 mm× 300 mm) column from Tosoh, Tokyo. Chromatography was performed in isocratic mode at 0.500 ml/min flow rate with aqueous buffer composing 50 mM phosphate pH 6.5, 300 mM sodium chloride and 0.02% sodium azide. The column temperature was maintained at 25 °C. All the buffers were filtered through 0.22 µm filter.

## Cation Exchange Chromatography (CEX)

CEX-HPLC was performed to quantify charge variants on BioMab, NP5, PK column (4.6 x 250 mm, 5 µm, Agilent) operated at 25°C using Dionex Ultimate 3000 RSLC system (Thermo Scientific). Prior to injection, the column was saturated with 65% mobile phase A (15 mM sodium phosphate buffer and 0.05% $NaN_3$ at pH 6.2) and 35% mobile phase B (150 mM sodium phosphate buffer and 0.05% $NaN_3$ at pH 6.2). All buffers were filtered with a 0.22 µm cut-off nylon membrane filter and degassed prior to use. 30 µg of sample was loaded and differently charged species were separated using a 23 min linear gradient from 35% – 65% B at a flow rate of 0.8 ml/min.

## Dynamic Light Scattering (DLS)

The hydrodynamic radii of the mAb monomer and in the solution were determined using particle size analyser, Zetasizer Nano ZS 90, from Malvern Instruments, UK, which is equipped with 633 nm He-Ne laser and a temperature controller. ZEN0118, a low volume disposable cuvette with 50 µL sample volume, was used to record the intensities of the scattered light from the mAb solution at a fixed angle of 90°. The samples were diluted to 1mg/ml in corresponding extraction buffers that were also used as blank measurements.

## Circular Dichroism (CD)

Far-UV CD experiment was performed using Jasco-1500 spectrometer equipped with a pelltier temperature controller at 20 °C. Quartz glass cuvette with optical path length of 0.10 cm was used. 0.3 mg/ml concentration (diluted in MilliQ) of each sample were scanned in the wavelength ranging from 190nm-250nm. Corresponding extraction buffer was measured as a blank and was subsequently subtracted. The spectra obtained were baseline corrected using Savitzky-Golay filter of device software. Similarly, near-UV CD experiment was done on each sample diluted to 5mg/ml in MilliQ in a wavelength range of 250-340 nm.

## Fluorescence Spectroscopy (FLR)

Fluorescence measurements were performed using a Cary Eclipse Fluorescence Spectrophotometer by Agilent Technologies, Santa Clara, USA. In each well, 200 µl samples with protein concentration of 0.3 mg/ml was analysed using black polystyrene 96-well microtiter plates (Greiner Bio-One GmbH, Germany). The spectrum for intrinsic fluorescence was recorded at 25 °C, from 300 nm to 450 nm with step size of 1 nm and at an excitation wavelength of 280 nm.

## Fourier Transform Infrared Spectroscopy (FTIR)

FTIR measurements were performed using Nicolet iS50 FTIR from Thermo Scientific, USA running on Omnics software. Each sample was diluted to 6 mg/ml and 6-8 µL of the sample was loaded, corresponding extraction buffer was measured as a blank and was subsequently subtracted.

## UV analysis & mass balance

UV measurements were performed using Epoch microplate reader (BioTek Instruments running on Gen 5 1.11 software. Each sample was diluted 80 times and loaded on to Corning UV plates. Corresponding extraction buffer was measured as a blank and was subsequently subtracted and absorbance obtained was used to determine the concentration through reported molar extinction coefficient values. The concentration (mg/ml) is a direct indication of amount of protein (mg) depending on volume (µL), thus, UV measurements were used to establish mass balance after each cycle.

# Results & Discussion

The control for the experiment was performed only for Eleftha biosimilar using only formulation buffer at each cycle. Extraction with alternate cycle of formulation buffer and CEX elution buffer was done on both innovator Herclon and biosimilar Eleftha samples. The control run used an initial amount of 40 mg of protein and lasted up to 10 cycles of buffer exchange including protein loss at each stage. The alternate cycle run used an initial amount of 42 mg each for both innovator and biosimilar and lasted up to 8 cycles of buffer exchange including protein loss at each stage.

## Aggregation: SEC and DLS

No significant aggregation for Eleftha control and both Eleftha and Herclon samples were observed in SEC analysis. No formation of dimers and other HMWs was observed in both products (Figure1). Also, significant aggregation was not observed for control as well as for both Eleftha and Herclon samples in DLS analysis. The z-average values in formulation buffer showed slight difference to the z-average values in CEX elution buffer, suggesting interference of buffer in DLS analysis (Figure 2). Thus, in terms of size and aggregation the excipient addition/removal and the buffer exchange does not impact the stability of the trastuzumab molecule in both biosimilar and innovator.

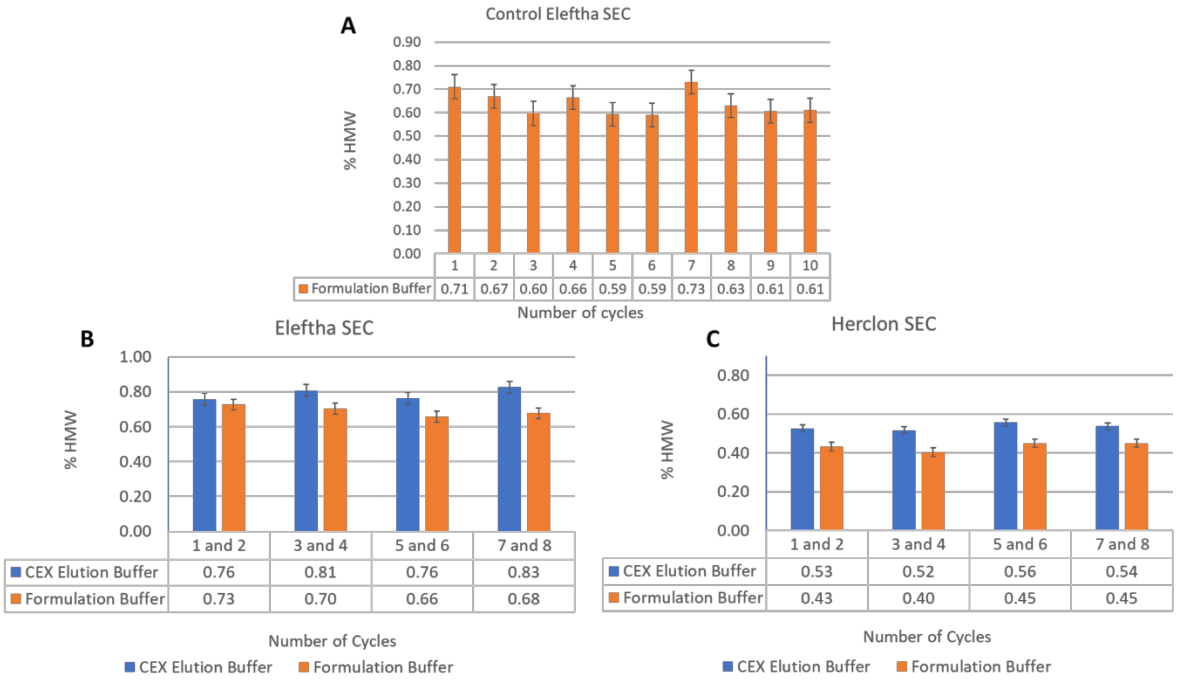



**Figure 1.** SEC results for innovator and biosimilar samples; A: Eleftha control sample; B: Eleftha SEC samples; C: Herclon SEC samples

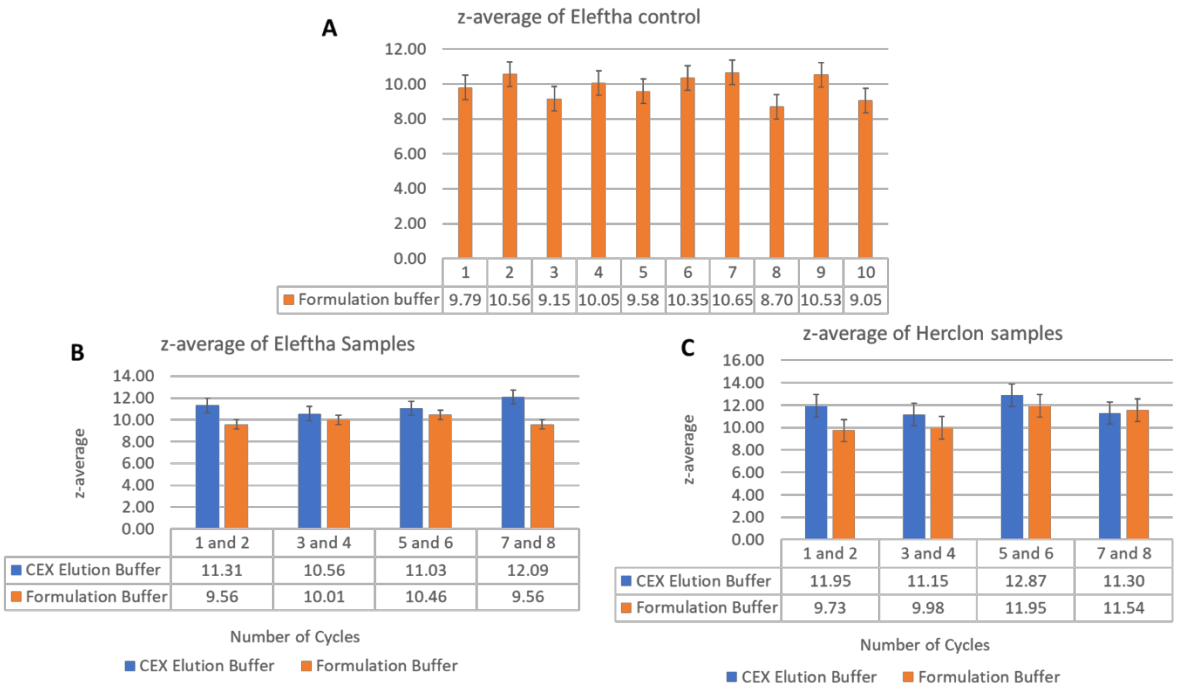



**Figure 2.** DLS results for innovator and biosimilar samples; A: Eleftha control sample; B: Eleftha DLS samples; C: Herclon DLS samples

## Charge variant profile: CEX

No significant changes were observed in charge variant composition of control Eleftha samples up to 10 cycles. Decrease in acidic variants and increase in basic variants were observed from cycle 1 to cycle 8 in Eleftha and Herclon samples (Figure 3). Thus, excipient addition and removal impacted charge variant composition in Eleftha and Herclon samples.

## Secondary Structure: Far-UV CD & FTIR

FTIR results show an interesting trend with respect to changes in the secondary structure as a function of number of excipient extraction cycles. In case of Eleftha control samples (Figure 4), transmittance for wave number 1624-1693 $cm^{-1}$ increases with increase in cycles, indicating degradation of secondary structures directly due to the buffer exchange stress. Since an increase in transmittance is observed, it is due to degradations other than aggregation like hydrolysis or changes in peptide bonds of protein. Extraction of Eleftha and Herclon samples with alternate buffers and FTIR analysis (Figures 5 and 6) show a trend of continuous increase and decrease of transmittance between formulation and CEX buffer for wave number 1624-1693 $cm^{-1}$. Transmittance increase is continuous as number of cycles progress and is greater for buffer exchange taking place from CEX elution buffer to formulation buffer. Further, the transmittance does not revert to initial levels at higher cycle numbers, especially for Eleftha samples, indicating destabilization to be irreversible and Herclon to be more stable than Eleftha.

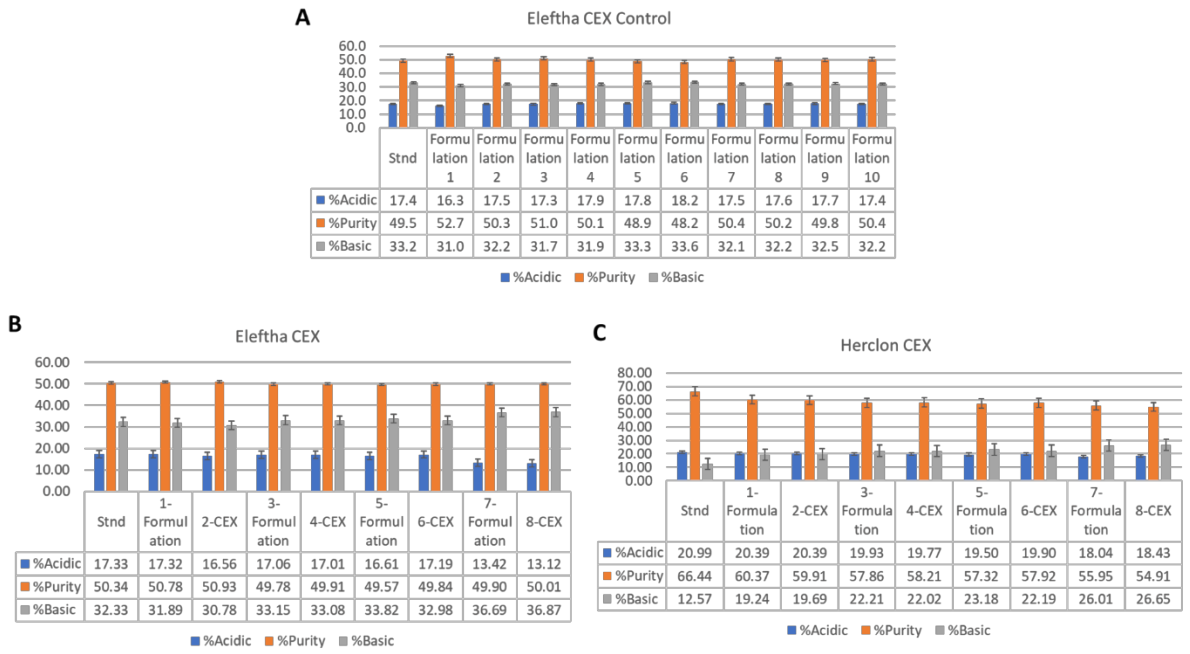



**Figure 3.** CEX results for innovator and biosimilar samples; A: Eleftha control sample; B: Eleftha CEX samples; C: Herclon CEX samples

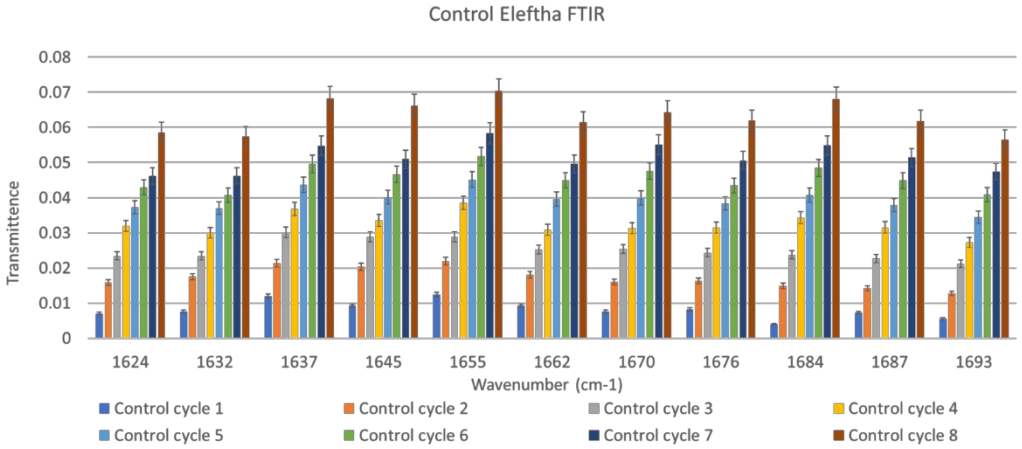



**Figure 4.** FTIR graph showing transmittance for Eleftha control samples

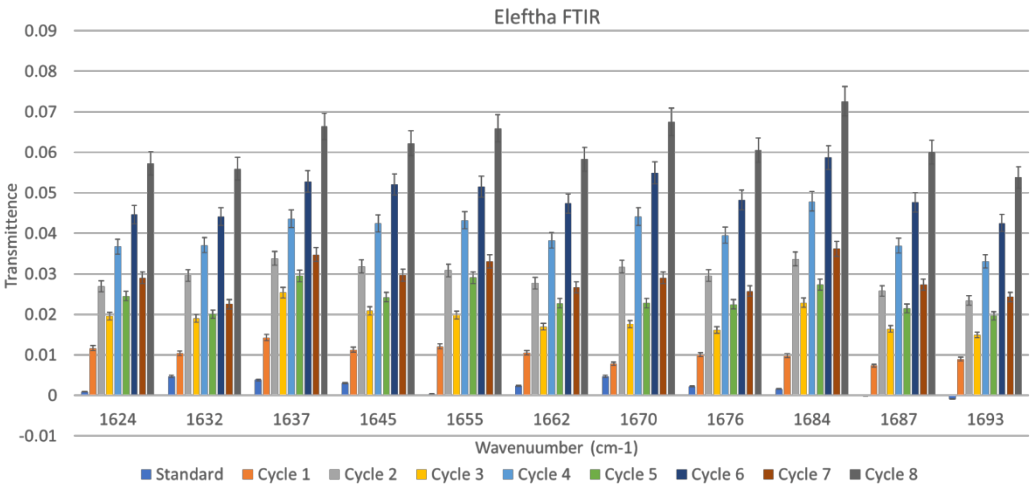



**Figure 5.** FTIR graph showing transmittance for Eleftha samples

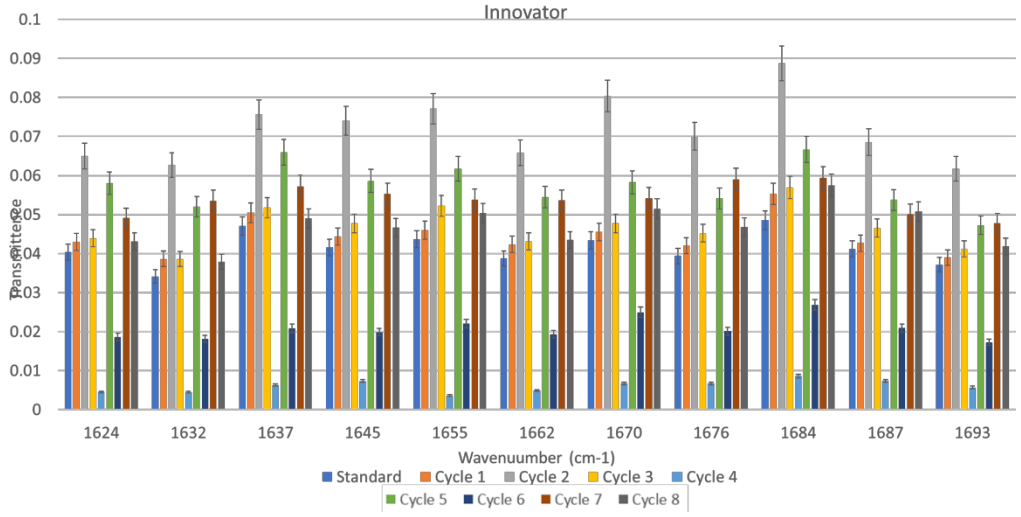



**Figure 6.** FTIR graph showing transmittance for Herclon samples

Far UV CD changes in the secondary structure are reflected by changes in the anti-parallel and parallel beta sheets of the protein. Figure 7 shows the percentages of parallel and anti-parallel sheets as the number of cycles increases in control and samples. In case of Figure 7A, destabilization is observed from cycle 3 to cycle 8 with respect to changes in antiparallel and parallel percentages, but a restoration in respective secondary structure is also observed as we reach cycles 9 and 10. Thus, changes to the secondary structure by buffer exchange cycles is reversible. Figure 7B represents changes in secondary structure for Eleftha and Herclon samples. Both Eleftha and Herclon samples show disruption in secondary structures relative to degradations in parallel and anti-parallel sheets, but only Herclon samples show re-stabilization of these structures like in the control sample. No such restoration is observed for Eleftha samples; hence, effect of excipient addition and removal is more disrupting for Eleftha than Herclon.

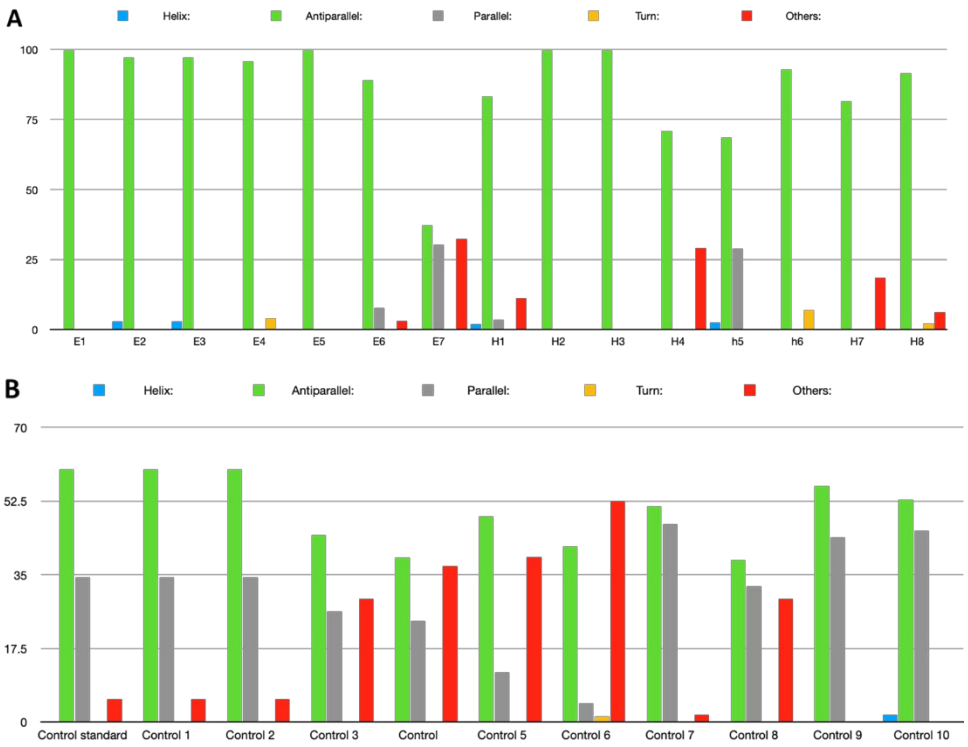



**Figure 7A.** Far UV graph for Eleftha control samples; B: Far UV graph for Eleftha and Herclon samples

## Tertiary Structure: Near-UV and FLR

Tertiary structure of a protein is depicted through changes in aromatic amino acid absorbance during near UV measurements. Figure 8 represents the near UV CD results. Changes in tryptophan and tyrosine absorbance were more when compared to the changes in absorbance of phenylalanine residue. The increase in absorbance is directly linked to disruption in tertiary structure exposing buried amino acid residues and increasing absorbance.

Redshift and blueshift observed in FLR analysis indicate folding/unfolding and environmental impact on protein structure. Figure 9A shows the trend for Eleftha control, when compared to the standard as number of cycles increase an increase in the redshift absorbance is observed suggesting effect of buffer exchange cycles on protein stability. Figure 9B highlights a similar trend for Eleftha samples with addition and removal of excipients, the redshift being higher than the control indicating that excipient extraction further effects protein stability leading to exposure of embedded residues. Herclon samples also show an increase in the redshift absorbance as cycles proceed, but interestingly the blueshift absorbance has been observed at the end. Again, Herclon samples seem to be more stable than Eleftha biosimilar and a re-stabilization is observed.

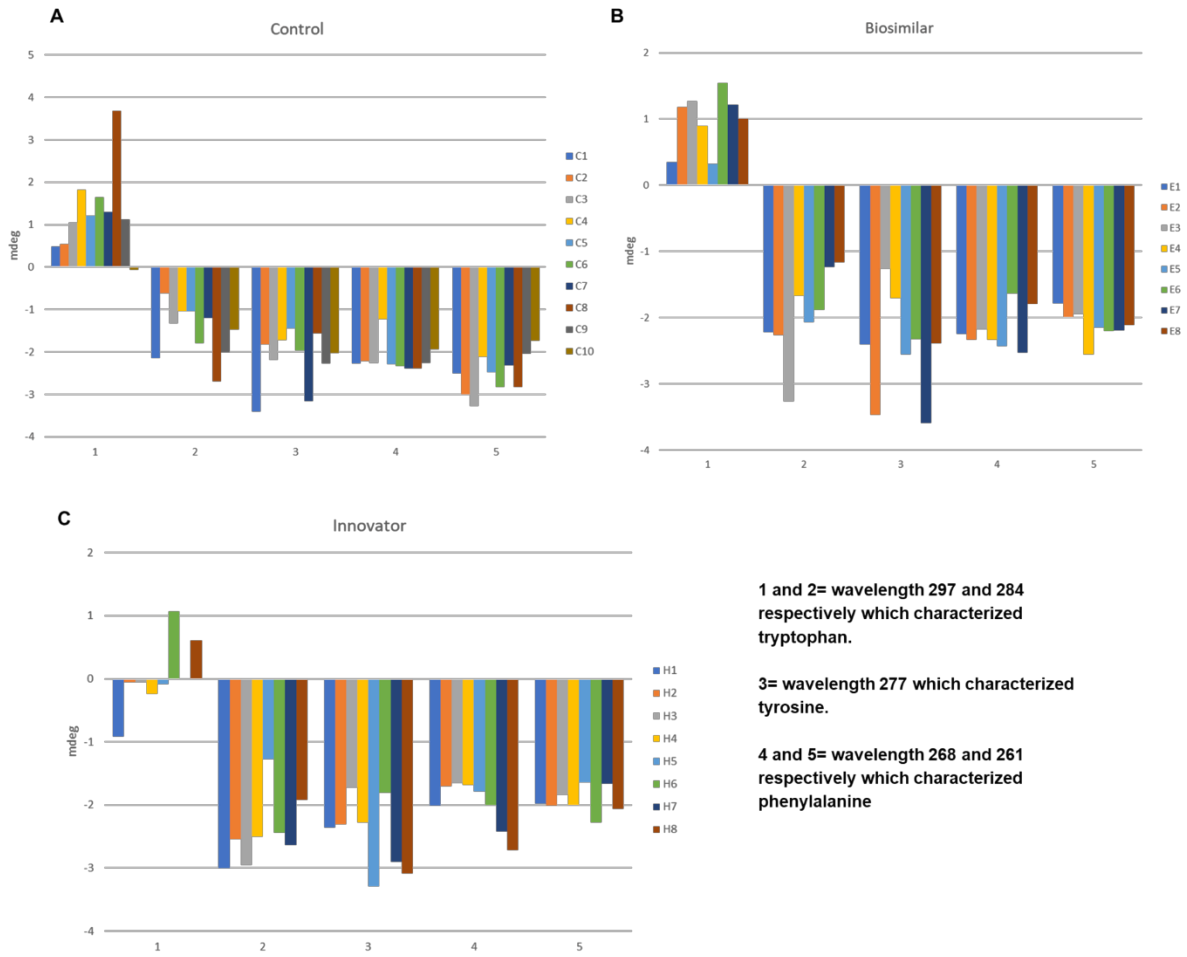



**Figure 8A.** Near UV graph for Eleftha control samples; B: Near UV graph for Eleftha samples; C: Near UV graph for Herclon samples

## Mass balance

Mass balance was performed on basis of UV measurements. Protein loss was more during buffer exchange from CEX elution buffer than in formulation buffer (Figures 9B and 9C). Protein loss in control samples was less since no CEX elution buffer was used, allowing up to 10 cycles of buffer exchange (Figure 9A).

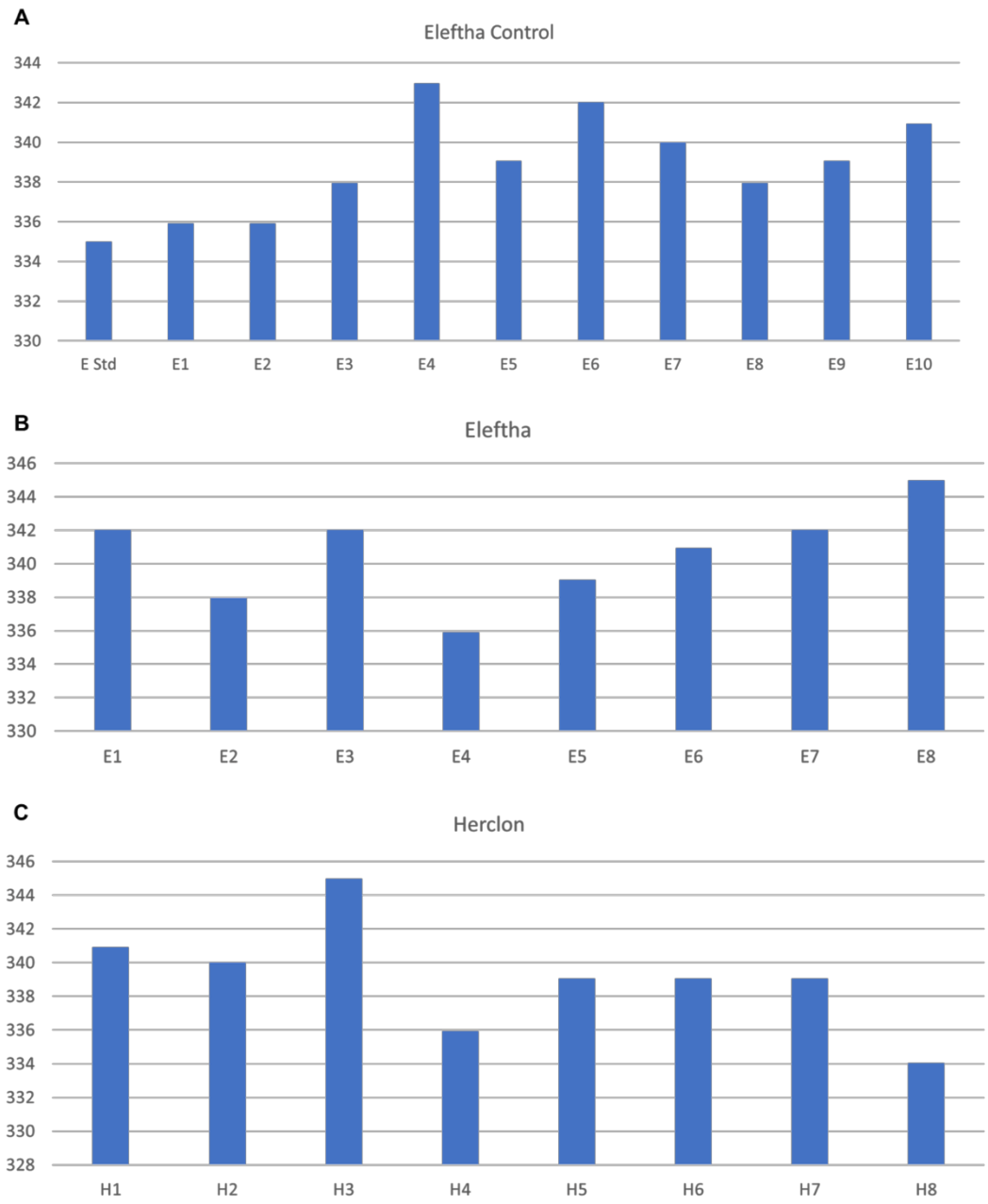



**Figure 9A.** FLR of Eleftha control samples; B: FLR of Eleftha samples; C: FLR of Herclon samples

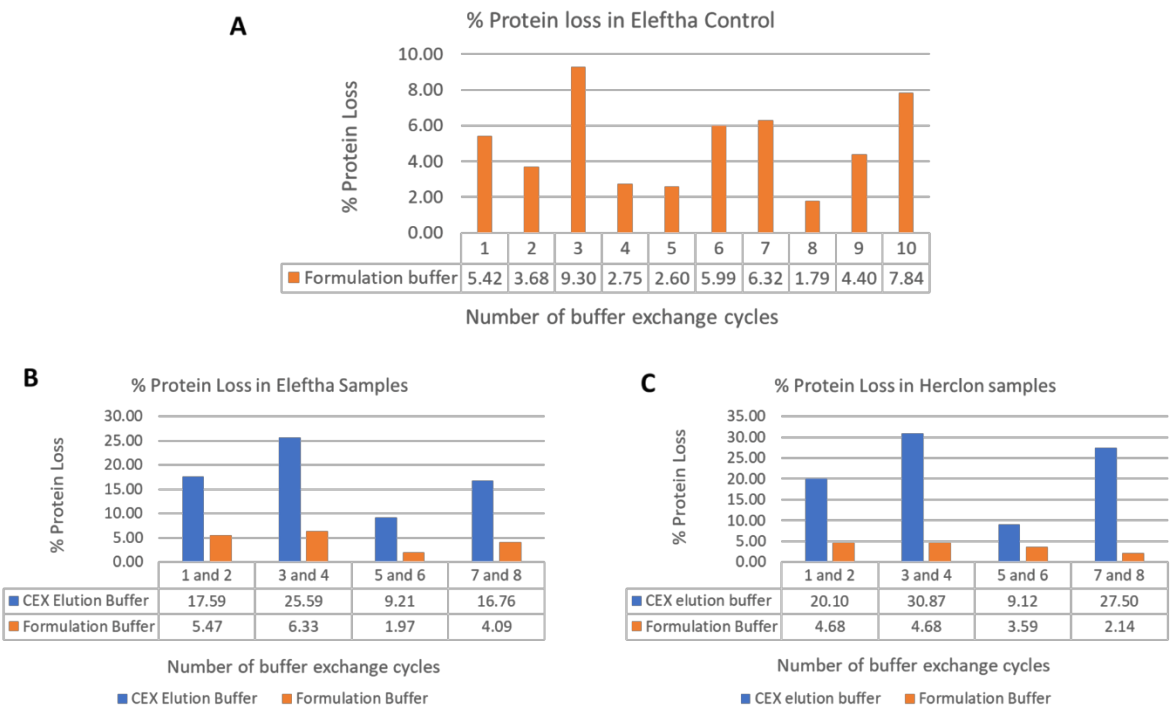



**Figure 10A.** Mass balance for Eleftha control samples; B: Mass balance for Eleftha samples; C: Mass balance for Herclon samples

# Work carried out at the University of Minnesota (UMN) and the University of Iowa (UI)

**Investigators:** Raj Suryanarayanan (UMN) and Reza Nejadnik (UI)

## Overview

It is addressed in the first part of the report. Therefore, this report will deal with the rest of the sections.

## Aims & Objectives

- Study the effect multiple Freeze thaw cycles on the stability of Trastuzumab (Tmab)
- Optmizing of the freeze drying cycle without  protein
- Optimizing the freeze-drying cycle with a model protein (BSA)
- First batch of freeze drying cycle of Tmab (Herzuma)
- Chararcterization of Tmab lyophile formulated at UMN (same composition as Herzuma)
- Chararcterization of Tmab lyophile formulated at UMN (same composition as Herzuma) – reconstituted at UI

## Abbreviations

**Tmab:** Trastuzumab –therapeutic monoclonal antibody
**BSA:** Bovine serum albumin
**DSC:** Differential scanning calorimetry
**KFT:** Karl-Fischer titrimetry
**TGA:** Thermogravimetric analysis
**SEC:** Size exclusion chromatography
**DLS:** Dynamic light scattering
**XRD:** X-ray diffraction (also X-ray diffractometry and powder X-ray diffractometry)
**$T_g$:** Glass transition temperature
**$T'_g$:** Glass transition temperature of freeze-concentrate
**RT:** Retention time

## Project progress summary

A commerical Tmab formulation (Herzuma®) was characterized. The Tmab from this formulation was extracted and reformulated and freeze-dried (at UMN). The freeze-dried formulation was characterized.

## Materials

### Chemicals and consumables

#### Chemicals

Trehalose dihydrate, polysorbate 20, L-histidine, histidine hydrochoride, MiliQ, bovine serum albumin (BSA), Herzuma®, centrifugal concentrators (30 KDa).

### Consumables

Microcentrifuges tubes (Abdos), detergent removal resin column kit, 0.2 µm filters, syringes, glass vials (DWK wheaton)  stoppers (Gry butyl stil, Wheaton)  falcon tubes and reagent bottles.

### Samples

Trastuzumab (20.7 mg/ml) in histidine buffer (pH 6) was used for the preliminary studies**.** The transtuzumab was extracted from a commercial formulation - Herzuma®.

# Procedure

- Preparation of histidine buffer in MiliQ
- Removal of polysorbate 20 by detergent removal resin column kit.
- Removal of Trehalose by centrifugal concentrators.
- Recovered/reformulated Tmabs were exposed to 5 freeze thaw cycles.
- Samples were analyzed after F/T Cycles 1 and 5 Vs control Tmabs using dynamic light scattering, Flowcam, HP-SEC and UV.
- Preparation of excipient solution
- Lyophilization of placebo formulation (only excipients)
- Lyophilization of excipient and BSA formulation
- Lyophilization  of first batch of Tmab (Herzuma®)
- Characterization of Tmab lyophile
- Characterization of reconstituted Tmab.

## Buffer preparation

Histidine buffer pH 6.0 was prepared by mixing the L-histidine and histidine-HCl as follows:

L-Histidine (MW 155.15 g/mol) = 6.1 mg (20 ml)

Histidine HCl (MW 209.63 g/mol) = 9.5 mg (20 ml)

In order to prepare 200 ml of buffer solution,  61 mg of L-histidine and and 95 mg of histidine HCl were dissolved in 180 ml of MiliQ and mixed using a magnetic stirrer and the resultant pH was 6.0. The solution volume was adjusted to 200 ml, and the solution was filtred through 0.2 µm filter.

## Removal of polysorbate 20

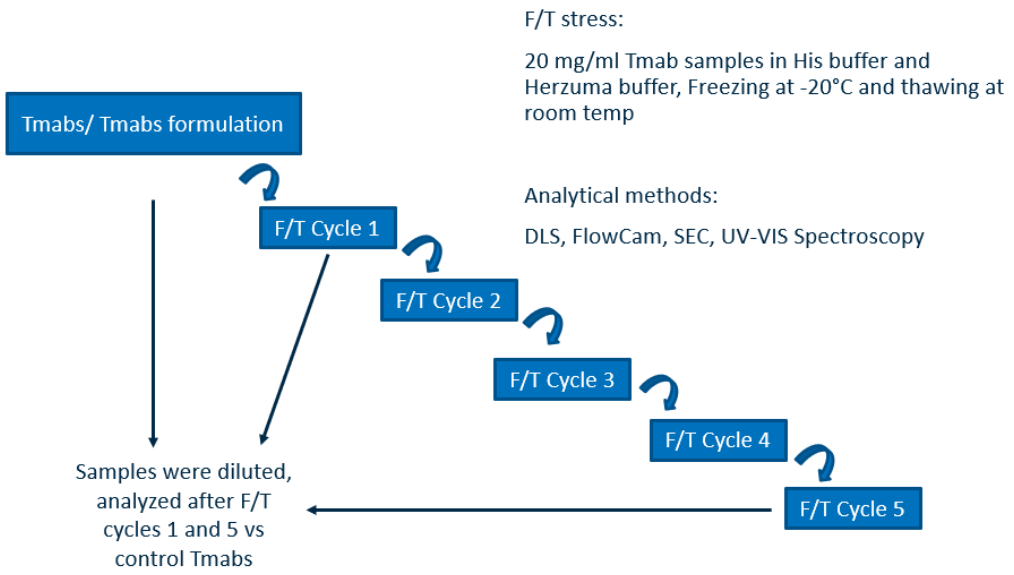
Reconstituted Herzuma® Tmab (21 mg/ml) was incubated with detergent removal resin for 30 minutes to allow the resin to adsorb the polysorbate 20. The yield is Tmabs and trehalose.

## Removal of trehalose

Trehalose was removed through buffer exchange using centrifugal concentrators (30 KDa) for minimum 4 cycles of buffer addition and centrifugation. The yield is Tmabs only in histidine buffer. Tmabs were also formulated as Herzuma through buffer exchange using Herzuma excipients solution (trehalose and histidine buffer) and then polysorbate 20 were added. The yield is reformulated Herzuma Tmabs.

## Freeze thaw cycles

(See schematic below): Tmab (20 mg/ml) samples in histidine buffer and Herzuma buffer, freezing at -20°C and thawing at room temp.




## Dynamic light scattering (DLS)

DLS is a technique used to measure the average size of particulate matter in the suspension in the nanometer size range. By analyzing the intensity fluctuations of scattered light, it provides information about the hydrodynamic radius and polydispersity of the protein population, aiding in assessing protein stability and aggregation propensity.

## Flow Imaging microscopy (FlowCam®)

FlowCam® is a flow imaging microscopic technique to analyze individual particles in a protein sample. It enables the visualization and characterization of protein aggregates, including their size, shape, and morphology in the micrometer size range. FlowCam® helps in monitoring stability under different conditions.

## Size exclusion chromatography (SEC)

SEC separates protein monomers from oligomers based on their size using a porous column. By measuring the elution profile, SEC can determine the presence and relative abundance of different aggregate (dimer, trimer up to small oligomer) species. It is a commonly used technique for monitoring protein aggregation and stability, enabling the assessment of changes in monomer content and quantification of small aggregates.

## UV-Visible (UV-VIS) Spectroscopy

UV-VIS spectroscopy measures the absorption and scattering of light by protein samples at different wavelengths. It provides information about the secondary and tertiary structure of proteins and can detect changes associated with protein aggregation and stability. UV-VIS spectroscopy is often employed to monitor protein conformational changes and identify spectral signatures indicative of aggregation events.

## Excipient solution preparation

The stock solution of excipients, trehalose and polysorbate 20 were prepared in histidine buffer (pH 6.0). The concentrations of trehalose and polysorbate 20 in the Herzuma® formulation are 42.0 and 0.085 mg/ml respectively. Therefore, we have prepared a stock solutions of trehalose (420 mg/ml) and polysorbate 20 (0.85 mg/ml) which were filtered (0.2 µm membrane filter).

## Lyophilization of placebo formulation (only excipients)

The excipient solution containing trehalose and polysorbates 20 in histidine buffer was filled in 10 ml glass vials (DWK wheaton) and partially capped with 20 mm stoppers (Gry butyl stil, Wheaton). The

fill voloume was 2 ml/vial. The lyophilization cycle (details in Table 2) was carried out on a benchtop freeze-drier (VirTis Advantage, Gardiner, NY). After completion of cycle, vials were stoppered and stored at -20 °C until used.

**Table 1.** Composition of prelyophilization excipient solution

| Excipient | Volume (µl) | Weight (mg/vial) |
|---|---|---|
| Trehalose dihydrate | 200 | 84.0 |
| Polysorbate 20 | 200 | 0.17 |
| Histidine buffer | 1600 | 0.815 |
| **Total volume** | **2000 µl** | - |

In order to set the parameters for lyophilization cycle, DSC scan of prelyophilization solution was collected prior to the start of lyophilization. Our lyo-cycle consists of three steps: freezing, primary drying and secondary drying. Further details have been provided in Table 2.

**Table 2.** Freeze drying (lyophilization) cycle conditions

| Parameters | Freezing | Primary drying | Secondary drying |
|---|---|---|---|
| Temperarure (°C) | -45 | -30 | 25 |
| Rate (°C) | 1 | 1 | 1 |
| Time (Hours) | 3 | 48 | 5 |
| Pressure (mTorr) | 200 | 200 | 200 |

## Lyophilization of BSA formulation

Herein, composition of prelyophilization solution is the same as in Table 1, but with the addition of BSA (composition in Table 3). All the freeze-drying conditions were the same as in Table 2. As in the previous cycle, DSC was conducted prior to lyophilization.

**Table 3.** Composition of prelyophilization BSA formulation

| Excipient/Protein | Volume (µl) | Weight (mg/vial) |
|---|---|---|
| Trehalose dihydrate | 200 | 84 |
| Polysorbate 20 | 200 | 0.17 |
| Histidine buffer | 1600 | 0.815 |
| BSA | - | 42 |
| **Total volume** | **2000 µl** | - |

## Lyophilization of Tmab formulation (the composition matches that of Herzuma)

The stock concentration of Tmab in Herzuma was 20.7 mg/ml. Therefore, to keep the same ratio of excipients to protein as in the commercial formulation, we have appropriately adjusted the excipient and protein concentrations. While the prelyophilization solution volume was 2 ml, it is designed to be reconstituted to 1 ml.

**Table 4.** Composition of lyophilization cycle of Tmab formulation (2 ml)

| Excipient/Protein | Volume (µl) | Weight (mg/vial) |
|---|---|---|
| Trehalose dihydrate | 100 | 42 |
| Polysorbate 20 | 100 | 0.085 |
| Tmab | 1000 | 20.7 |
| Histidine buffer | 800 | 0.815 |
| **Total volume** | **2000 µl** | - |

## Characterization of Tmab formulation

The lyophiles were analyzed by a number of techniques:

- Karl Fischer titrimetry and TGA – volatile (specifically water) content
- DSC – thermal behavior, specifically $T_g$
- XRD – determining the crystallinity

### Karl-Fischer titrimetery

The sample was weighed (10-20 mg) into the Karl-Fischer titrimetry cell, and the water content was calculated coulometrically. The experiments were performed in triplicate and the average values were reported.

### Thermogravimetric analysis (TGA)

The lyophile was subjected to controlled temperature program in a TGA (TA Instruments). About 1 mg was heated from 20 to 300 °C at 10 °C/min. The experiments were performed under a constant nitrogen purge.

### Differential scanning calorimetry (DSC)

A differential scanning calorimeter (Q2000, TA Instruments, New Castle, DE) equipped with a refrigerated cooling accessory (RCS90) was used. Samples were weighed (2 to 5 mg) in Tzero aluminum pans, hermetically sealed, and heated under a dry nitrogen purge of 50 mL/min.

### X-ray Diffractometry (XRD)

The lyophiles were gently crushed and put in a sample holder and leveled with the aid of a glass slide. The XRD scans were carried out over an angular range of 10 to 60° 2theta (X'Pert Pro; PANalytical Inc., West Borough, MA).

# Results and discussion

## DLS

DLS measurements were performed at 25 °C. Ten acquisitions were measured for each sample and 3 replicates were measured, and the average and standard deviations (SD) were calculated and plotted. Normalized intensity values were considered to check the quality of the measurements. Results showed that Tmabs, whether formulated or not, are not affected by freeze/thaw stress up to 5 cycles.

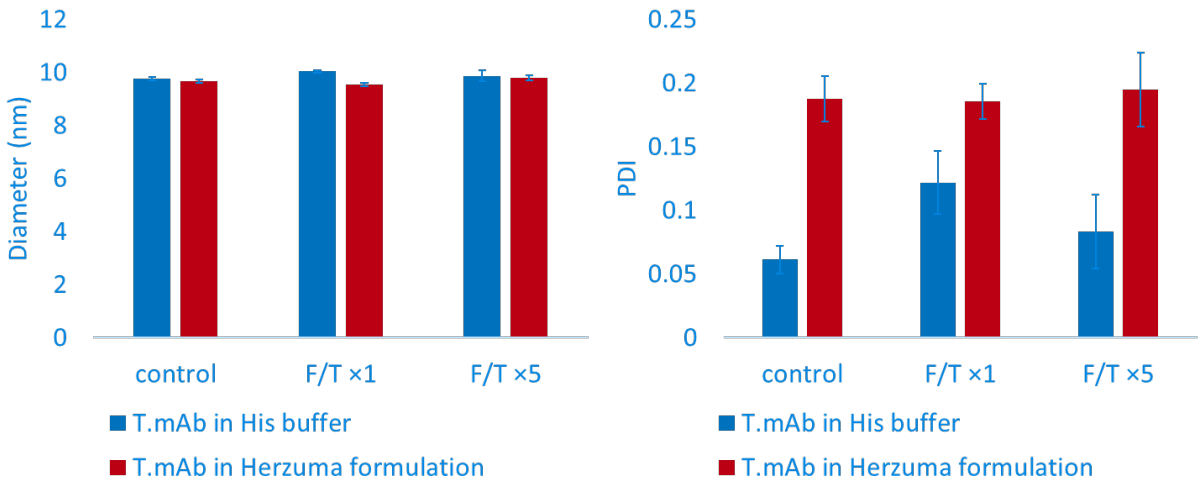



**Figure 1.** DLS of Tmab formulation showing the particle diameter and PDI in the commercial (Herzuma®) formulation and in the recovered Tmab (in histidine buffer). The freeze-thaw (F/T) cycling was conducted once (F/T x 1) and five (F/T x 5) times.

## Flowcam®

The sample volume was 500 µl and the concentration was 1 mg/ml. Samples do not show signs of aggregation due to F/T stress as shown in the figure. Almost no change in the number of particles in different size ranges were observed which indicates the stability of the samples exposed to F/T stress.

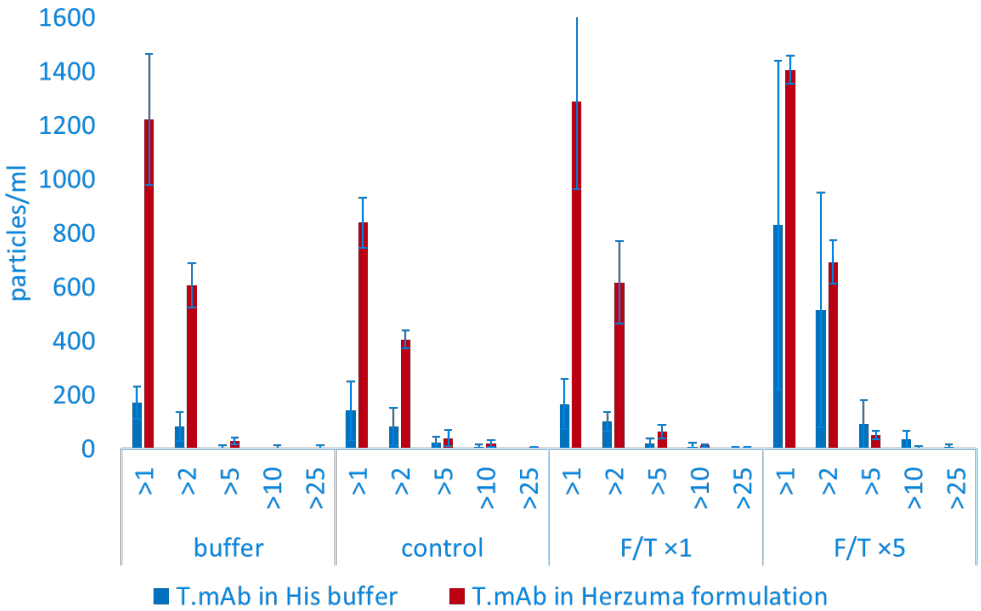



**Figure 2.** Flowcam graph showing the particle counts in the commercial (Herzuma®) formulation and in the recovered Tmab (in histidine buffer). The freeze-thaw (F/T) cycling was conducted once (F/T x 1) and five (F/T x 5) times.

## SEC

Twenty µl of 1 mg/ml Trastuzumab was injected in the SEC column. Fifty mM phosphate buffer, 300 mM sodium chloride, and 0.05 sodium azide were used as mobile phase with 0.2 ml flow rate. No high molecular weight species (HMWs) were observed and there was no significant difference in the AUC of the monomer peak (Figures 3a and 3b).

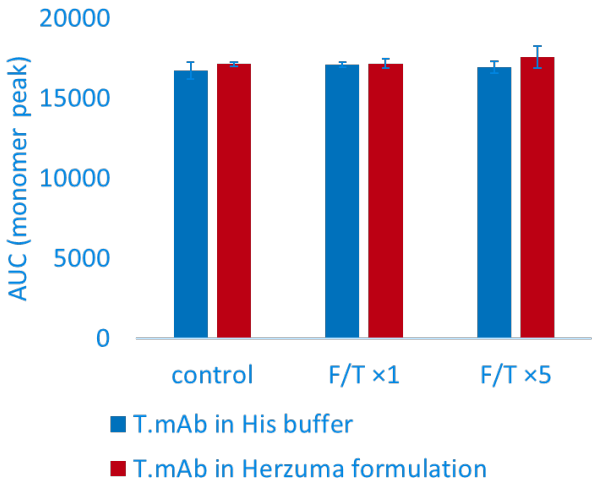



**Figure 3A.** Size exclusion chromatography. The AUC in the commercial (Herzuma®) formulation and in the recovered Tmab (in histidine buffer). The freeze-thaw (F/T) cycling was conducted once (F/T x 1) and five (F/T x 5) times.

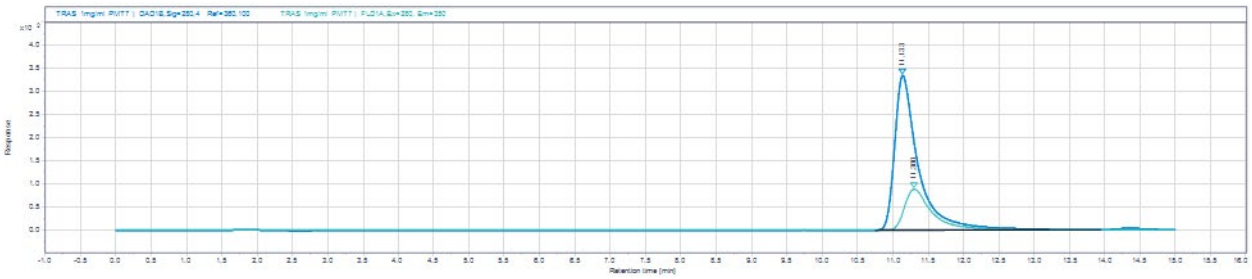


**Figure 3B.** SEC chromatogram of Tmab samples.

UV-VIS spectroscopy. Samples, at a concentration 1 mg/ml, were scanned from 200 to 800 nm to evaluate the spectrum and the amount of aggregates by means of optical density at 350 nm (OD350). In addition, the OD350 was the same for both pre and post F/T samples, suggesting no major aggregation in the samples (Figure 4).

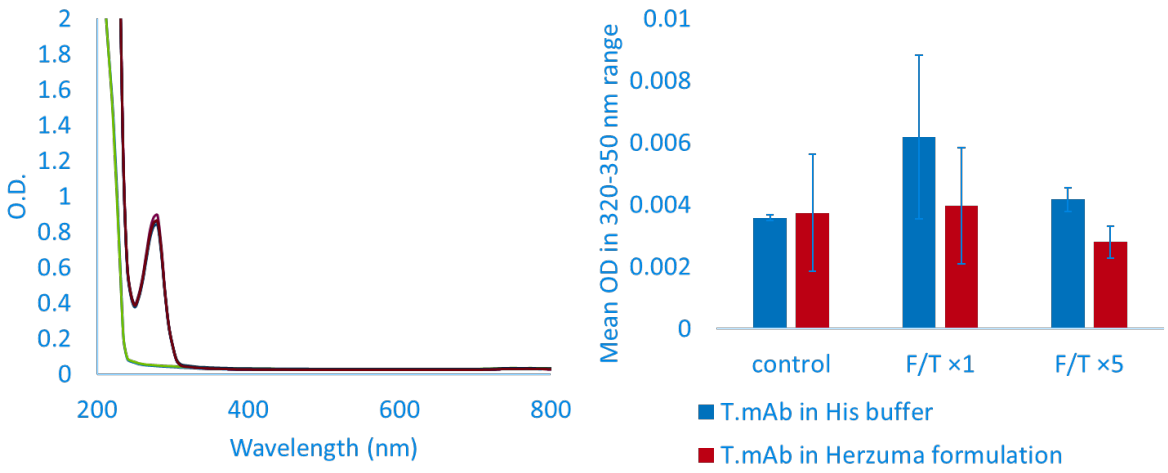



**Figure 4.** (Left panel) UV-Visible spectrum of Tmabs sample, and (Right panel) mean OD of Tmab in His buffer and Herzuma® formulation. The freeze-thaw (F/T) cycling was conducted once (F/T x 1) and five (F/T x 5) times.

**Conclusion**: Aggregation studies indicate no significant change between the control samples and the ones that underwent F/T stress (up to 5 cycles). This was the case for both His buffer and Herzuma® formulation.

## Optmization of the freeze drying cycle without protein

To observe the effect of various excipients on cake quality, we have performed lyophilization of the placebo formulation. In order to set the parameters for our freeze-drying cycle, DSC of the placebo composition was carried out.

*Thermal characterization of the prelyophilization solution.* The DSC scan of excipient composition showed a $T'_g$ at ~ -35°C (Figure 5).

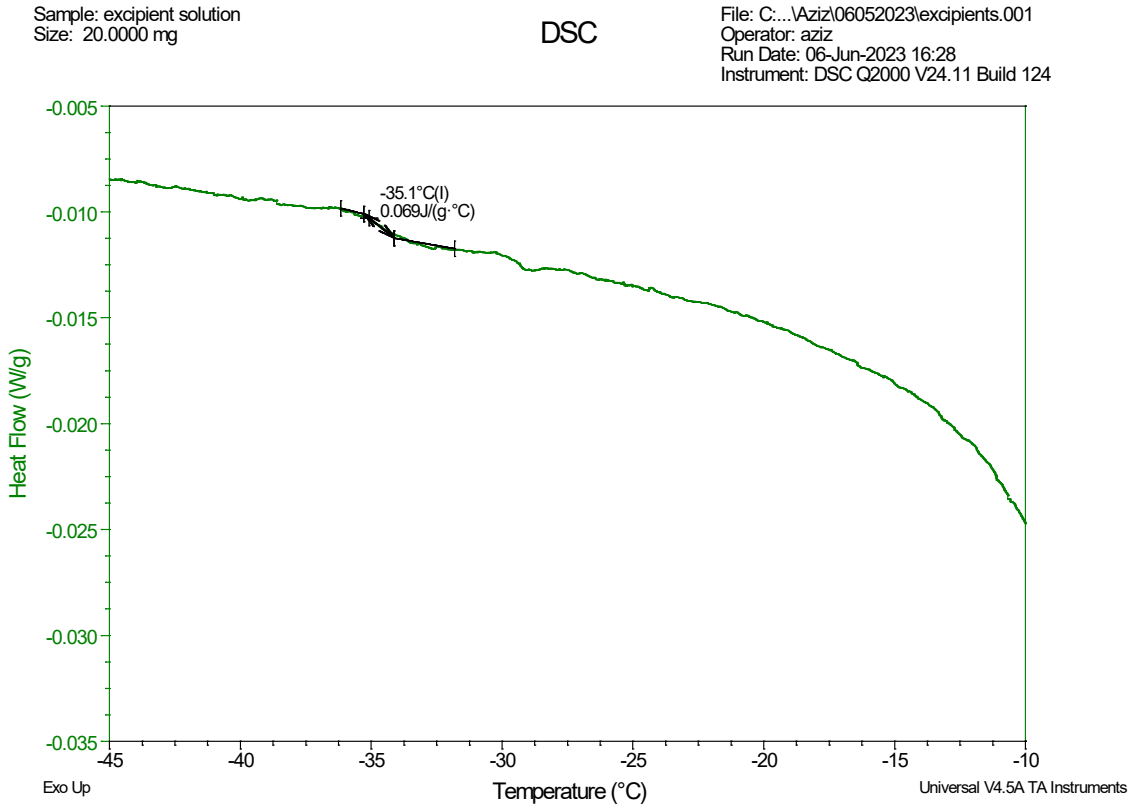



**Figure 5.** DSC heating curve of placebo solution. The solution was initially cooled from RT to -60 °C, held for 15 min and then heated back to RT. Only the heating curve is shown. A thermal event, attributable to $T'_g$ of trehalose freeze-concentrate, was observed at ~ -35 °C.

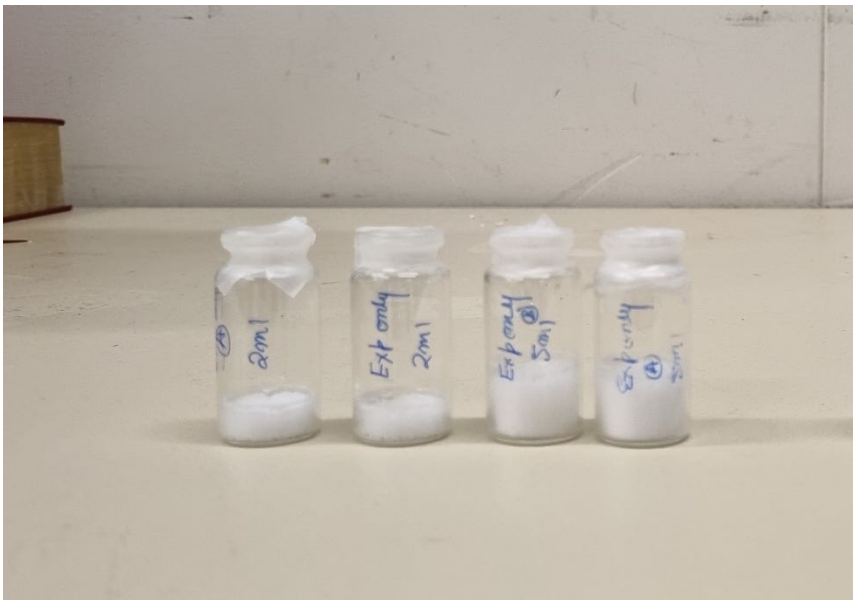


**Figure 6.** Lyophilized cake obtained after freeze-drying the placebo formulation.

The cycle yielded an elegant cake, with very little shrinkage (Figure 6).

# Optimizing the freeze-drying cycle with a model protein (BSA)

BSA was used as the model protein.

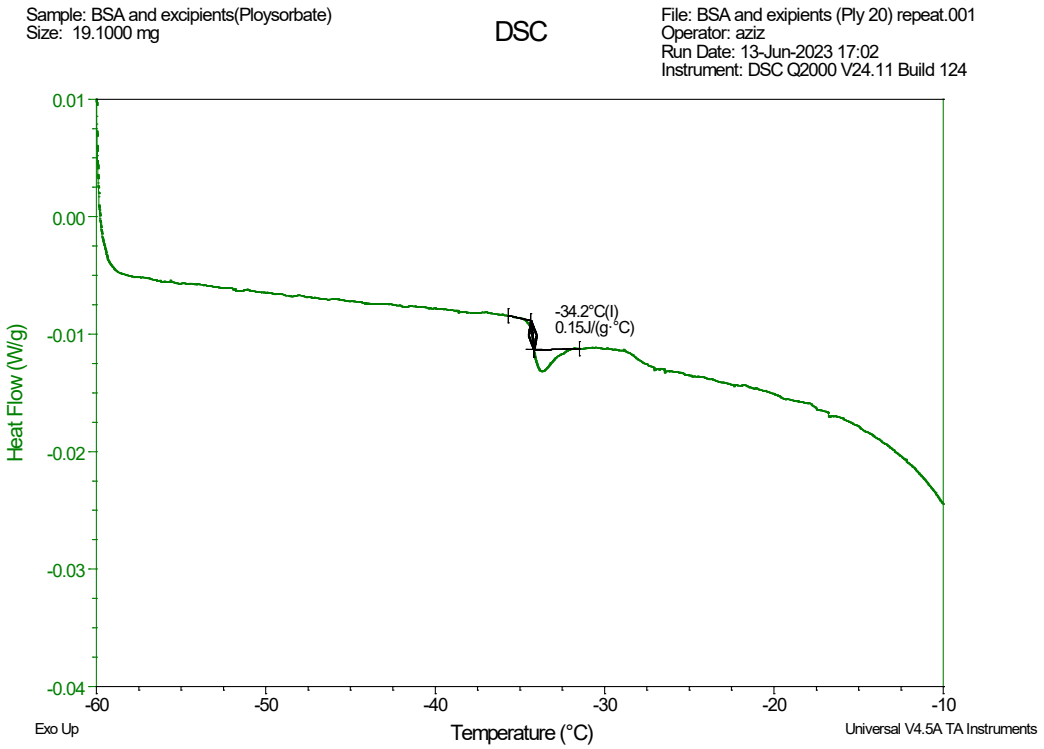



**Figure 7.** DSC heating curve of BSA prelyophilization solution. The solution was initially cooled from RT to -60 °C, held for 15 min and then heated back to RT. Only the heating curve is shown. The expanded profile (inset) reveals $T'_g$ accompanied by enthalpic recovery, at ~ -35 °C.

*Thermal characterization of the prelyophilization solution.* As is evident from Figures 5 and 7, the $T'_g$ of the prelyophilization solution, in the presence and absence of BSA were virtually identical (~ -35 °C) (Figure 5 and 7). Therefore, freeze-drying cycle parameters used for the excipient and the BSA solution were identical. The cake quality of BSA formulation was also good, though we observed some shrinkage (Figure 8).

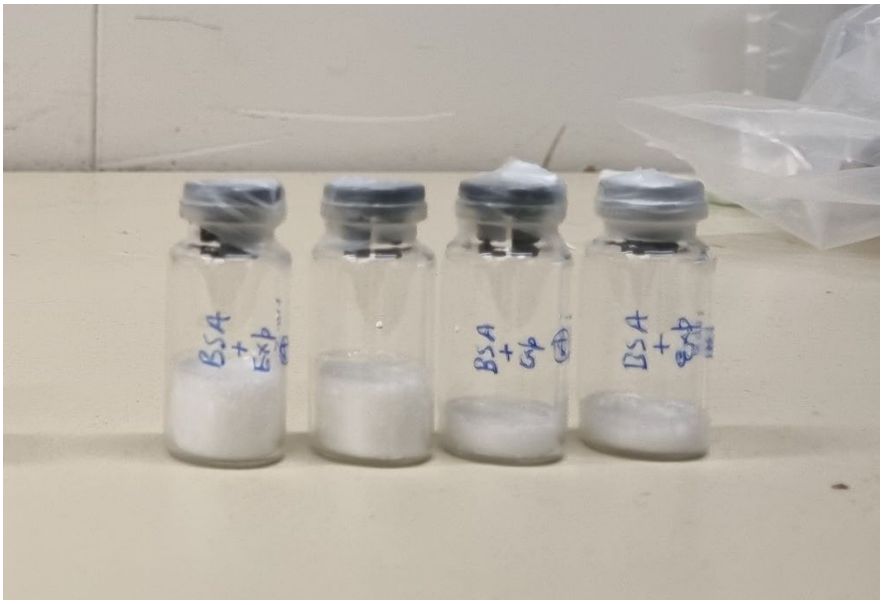


**Figure 8.** Lyophilized cake obtained after freeze-drying the BSA formulation**.**

## First batch of freeze drying cycle of Tmab (composition of Herzuma)

Since the excipient composition of Tmab formulation was similar to that of BSA formulation (Table 4), the process parameters for this lyophilization cycle were the same as those for the BSA formulation. In contrast to BSA formulation, both the excipient and Tmab concentrations were half of what was desired (Table 4). Despite this difference, while the Tmab cake had a good appearance, the shrinkage was more pronounced than in the BSA formulation.

## Characterization of Tmab lyophile formulated at UMN

We have characterized the Tmab lyophile, specifically the water content, crystallinity and $T_g$, using several analytical techniques. The water content, determined by Karl-Fischer titrimetry, was 5%, in agreement with TGA analysis. TGA revealed a weight loss of ~ 5%, between 30 and 116° C. This is attributed to the loss of sorbed water (Figure 9). The weight loss at higher temperatures is attributed to lyophile degradation (Figure 9).

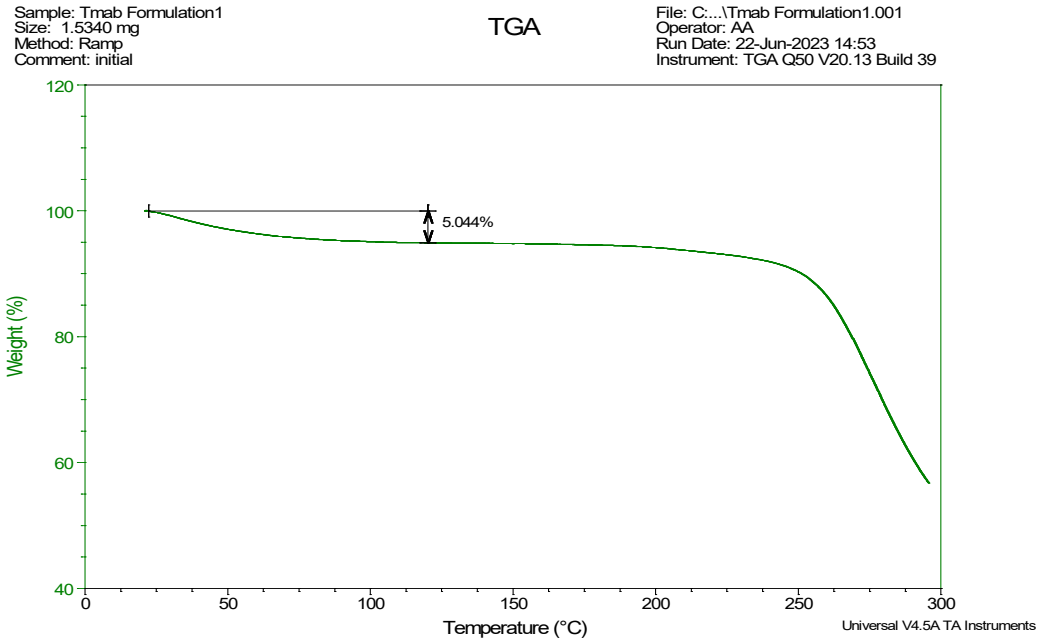



**Figure 9.** TGA of reformulated Tmab (same composition as in Herzuma).

DSC heating curve of Tmab lyophile also revealed a broad endotherm between 40 and 120 °C, again attributable to loss of water (Figure 10).

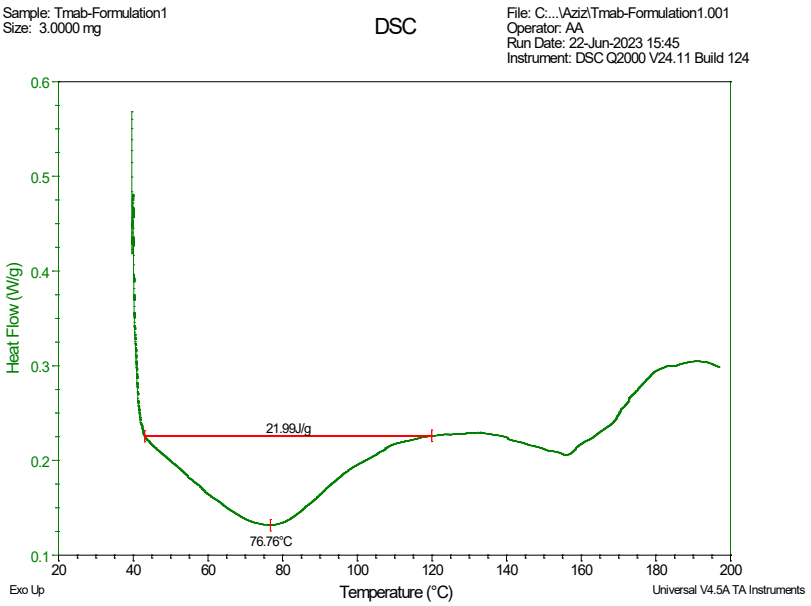



**Figure 10.** DSC heating curve of reformulated Tmab (same composition as in Herzuma).

Further, the XRD patteren of Tmab lyophile revealed  no sharp peaks, which indicates its amorphous nature (Figure 11).

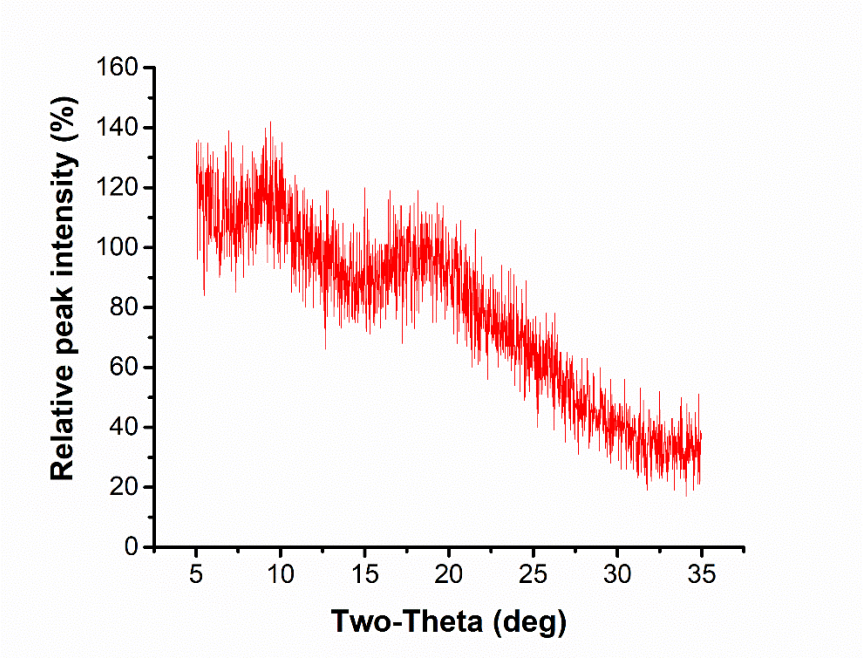



**Figure 11.** XRD patteren of reformulated Tmab (same composition as in Herzuma).

# Characterization of reformulated Tmabs as Herzuma (lyophilized)

## HP-SEC

Reconstituted Tmab formulations with approximate concentration of 1 mg/ml were analyzed by SEC using phosphate buffer as a mobile phase. The monomer and dimer peaks were observed, evident from Figures 12 and 13. High molecular weight percentage (maninly dimers) were calculated for the three vials, Tmab-a, Tmab-c and Tmab-d, and were 1.23%, 1.58%, and 1.53% respectivley. These results provide the %HMW for the T0 samples in this formulation. It is noteworthy that a detectable dimer peak was observed in these samples and the total HMW% is over 1% whereas this peak was not present in the Tmab formulations that were tested prior to lyophilization. We would like to mention that the differences in monomer content were not taken into account in this particular study because the volume of the injected samples may have been slightly different. Representative chromatograms are presented as a reference.

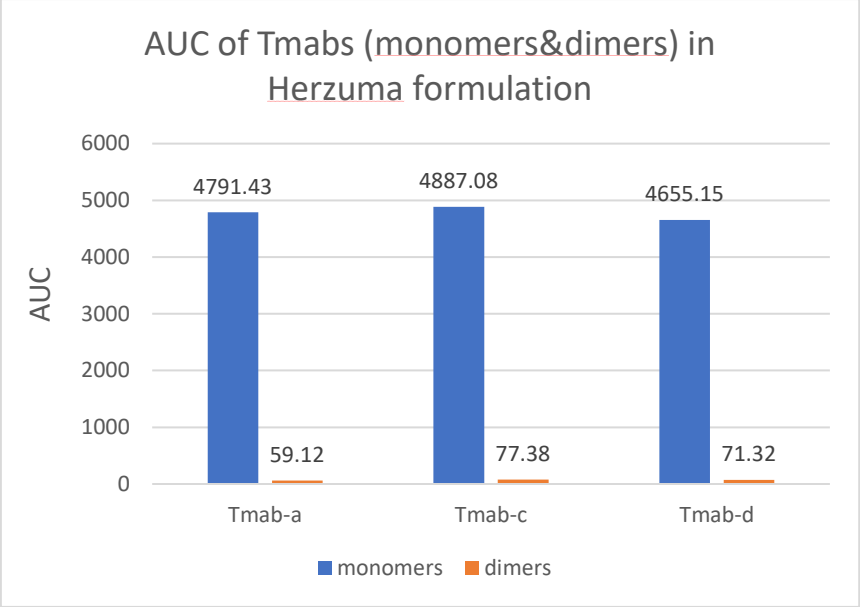



**Figure 12.** AUC of monomers & dimers in reformulated Tmab (same composition as in Herzuma).

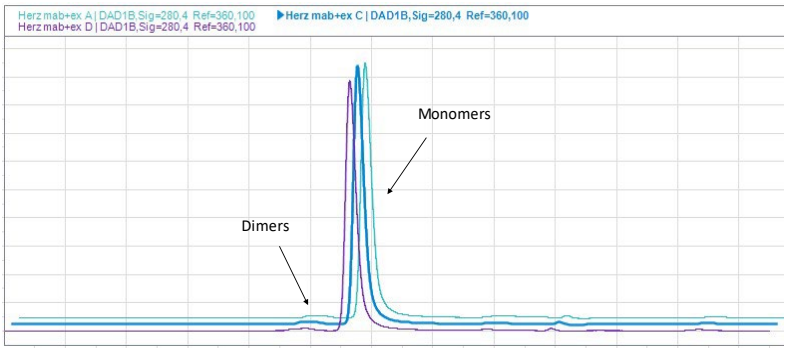



**Figure 13.** SEC chromatogram of reformulated Tmab (same composition as in Herzuma).

## DLS

Reconstituted Tmab formulations (1mg/ml) were also analyzed using DLS. Diameter and PDI were measured. Tmab samples show similar particle diameters around 10.3 nm and PDIs are below 0.4. Here too, it is noteworthy that the average particle diameter is higher than that of the formulations prior to lyophilization. We also perfomed DLS on reconstituted excipients-only formulations and obtained the normalized intensity for these samples and compared it with those of the reconstituted Tmab formulation. As expected the Normalized intensity was higher for Tmab formulations.

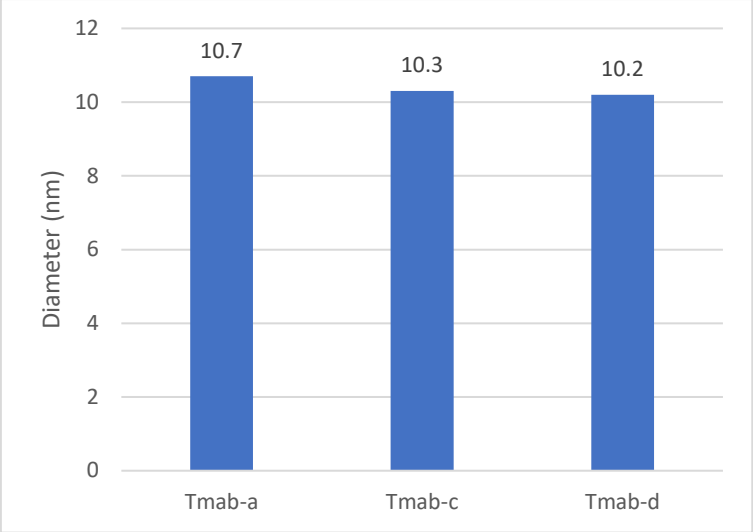



**Figure 14.** Size measurements of the reformulated Tmab (same composition as in Herzuma).

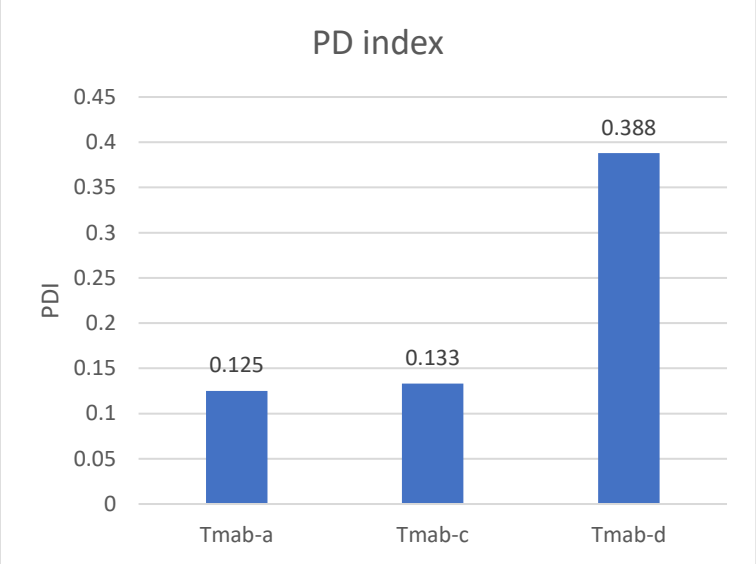



**Figure 15.** PD Index of reformulated Tmab (same composition as in Herzuma).

**Table 5.** Normalized intensity of Tmabs DLS measurements

| | Tmab-a | Tmab-c | Tmab-d | Excipients-a | Excipients-c | Excipients-d | buffer |
|---|---|---|---|---|---|---|---|
| Normalized intensity | 4255095 | 7332366 | 7260113 | 501445 | 508485 | 463333 | 670375 |

## UV-Vis spectroscopy

Reconstituted Tmab and placebo formulations (1 mg/ml) were scanned from 200 nm to 500 nm using a UV-VIS spectrophotometer to assess the extent of potential aggregation. The average of OD from 340 nm to 360 was calculated and the values are below 0.005 which indicates that samples do not contain high concentration of larger particles (aggregates). As expected, the formulations contining Tmab have a higher OD350 values than the placebo formulations but both are essentially clear.

**Table 6.** Averaged optical density of Tmabs.

| Tmab-a | Tmab-c | Tmab-d | Excipients-a | Excipients-c | Excipients-d |
|---|---|---|---|---|---|
| 0.004 | 0.001 | 0.002 | 0.0003 | 0.00092 | 0.00045 |

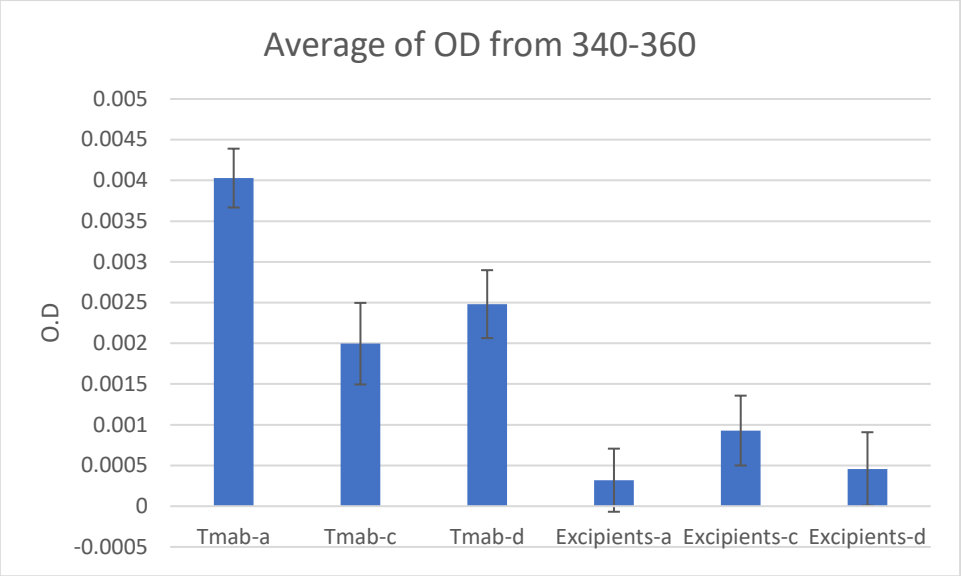



**Figure 16.** OD measurements of reformulated Tmab (same composition as in Herzuma) between 340-360 nm.

# Information Across the Entire Project

## Regulatory impact of the findings

Establishing analytical comparability is an obligatory step for filing for regulatory approval, involving both analytical and functional characterization of the biosimilar. The interference caused by presence of the excipients in the formulation is a major hurdle when evaluating comparability of the drug products with biosimilar manufacturers resorting to a variety of methods for isolation of the biotherapeutic protein from the drug product formulation. This is why in this study we examined the impact of the destabilization caused by continuous excipient addition/removal to the API molecule. In this study we have focused on the impact with respect to charge variant profiles and non-reversible degradation of secondary and tertiary structures in innovator and biosimilar products. We demonstrate that repeated extraction does have significant impact on product quality and is likely to impact product stability as well. Hence, we recommend that perhaps the best practice would be for biosimilar manufacturers to first make the drug product for their biosimilar and then extract the protein from the drug product like they do for innovator product for an equitable comparison.

## Public communications

No presentations, abstracts, poster have been done yet.

## Conclusions

- No visible aggregation was witnessed up to 8 cycles of excipient extraction and addition to API through SEC and DLS analysis
- There is a change in charge heterogeneity with increase in basic impurities as excipient extraction proceeds between formulation and CEX elution buffer as compared to control samples
- Both secondary and tertiary structures are affected as excipient extraction proceeds between formulation and CEX elution buffer as compared to control
- Protein loss through buffer exchange is much more in CEX elution buffer than in formulation buffer
- Both innovator and biosimilar behave in the same way in both buffer systems, but the innovator molecule appears to be more stable than the biosimilar.

## Future Plans

- Peptide mapping of Eleftha and Herclon samples to understand site/sequence modifications for up to 8 cycles of excipient extraction.
- Complete the excipient extraction process for Herclon product as control, similar to Eleftha control samples already done.
- Check repeatability and verify results obtained with a third biosimilar product, possibly non-Indian biosimilar.
- Testing lot-to-lot variability using different lots of innovator and biosimilar products.
- Test different buffer systems and compare results to understand the effect of buffers in excipient extraction.
- Test each excipient individually to understand it’s effect on API stability during excipient extraction.

## Budgetary Information

All budgeted manpower is in place and project activities have started. Efforts towards purchase of instruments that were sanctioned for Year 1 have been initiated and we expect to place the orders in the coming months. We will shortly be invoicing NIPTE for transfer of funds that have been spent thus far.