The effect of pneumatic tube transport on the physical stability of infliximab and pembrolizumab infusion bags

Author: Dalal Ghanim (6330673)
Master student in Pharmacy
Utrecht University

Daily supervisor: drs. Roderick van den Berg
Examiner: prof. dr. Enrico Mastrobattista
Referee: dr. Everaldo Redout

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Abstract

Purpose:

In hospitals, drugs are often transported through a pneumatic tubing transport (PTT). However, so far, therapeutic proteins such as monoclonal antibodies (mAbs) are not allowed to be transported via PTT. Therapeutic proteins have many instability issues which are associated with their protein nature; therefore, mechanical shocks in PTT can lead to protein aggregation and particle formation, which may cause immunogenicity, affecting the efficacy and safety of drug products. Despite many studies on the stability of therapeutic proteins, only a few studies have been conducted on the stability of therapeutic proteins when they are transported by PTT (stressed). This study aims to evaluate the effect of such transportation on therapeutic protein stability. In this study, we examined the impact of PTT on the stability of the models mAb, infliximab, and pembrolizumab, to obtain further insight into the potential stability issues associated with PTT.

Methods: Infliximab and pembrolizumab infusion bags were prepared from the left-over materials. Remsima (infliximab) was provided by Amsterdam Academic Medical Center (AMC), and the left-over Keytruda (pembrolizumab) was provided by Amsterdam VU Medical Center (VUmc). The measurements were carried out in triple for the samples of both therapeutic proteins. However, for 0.9% NaCl samples, the measurements were carried out in diplo. The samples were classified into four groups. Two of these groups (with and without air) were transported by PTT, and the other two groups (with and without air) were not transported by PTT. All the samples were analysed by size-exclusion chromatography (SEC), dynamic light scattering (DLS), microflow imaging (MFI) and nanoparticle tracking analysis (NTA).
Furthermore, mechanical shocks in PTT in Amsterdam AMC and VUmc were measured using MSR shock loggers of 16G and 200G.

Results: From the result obtained, the changes in the size, number of particles, and the ratio of monomer loss can occur post-PPT. However, in this study, not all of the measurements were significant.

Conclusion: The main finding of this study is that the protein products in infusion bags should not be transported in hospital PTT.

Keywords: pneumatic tubing, Monoclonal antibodies, physicochemical stability, microparticles, nanoparticles, particle concentration, infliximab, pembrolizumab,

Background

Therapeutic proteins

A protein is a large molecule composed of a long chain of amino acids folded into a three-dimensional shape. The biological functions of the protein are characterised and determined by specific amino acid sequences and the protein's three-dimensional structure. Therapeutic proteins are highly successful and widely used in clinics (1,2). Abnormal or deficient proteins in specific diseases could be replaced with therapeutic proteins. In addition, genetically modified proteins can resemble the natural proteins they replace.
Therapeutic proteins are generally used in the treatment of cancer, auto-immune diseases, infections, and many other disorders (3,4). On top of that, they can be used to improve the natural protein function by adding specific molecules or groups such as sugar. However, protein modification is a challenge for pharmaceutical industries due to the complicated requirements of protein therapeutics, including post-translational modifications to improve the stability and efficacy of the proteins (5,6). The incorrect modifications of a protein, such as misfolding, glycosylation, proteolysis, and deamidation, can lead to protein aggregation. This may have consequences for patients and could lead to an immune response to therapeutic proteins (7).

Stability and transportation of therapeutic proteins

The mechanism of action of therapeutic proteins relies on their susceptible structure. Hence, in recent years, protein stability has become one of the major challenges throughout the manufacturing process and the development of biopharmaceutical products (5). Currently, there is evidence that demonstrates the impact of protein aggregation and particle formation in protein samples on the stability of protein products. Proteins are in general only stable in their folded form, and their stability is reduced in their unfolded form because it may lead to protein aggregation (2,4). Protein aggregation and particle formation can be caused by a variety of mechanisms. Therapeutic Proteins, for instance, monoclonal antibodies (mAb), can undergo complex degradation processes during various manipulation, manufacturing and development processes (1,8).
These products are very sensitive to many chemical modifications throughout their storage or exposure to mechanical stress as well as different other factors that could influence the quality and stability of these products, such as an accidental freeze-thawing, exposure to light or too high temperature, changing salt concentration or exposure to different stress environments (9–12).
To ensure the safety of these products (medicine) for the patients, different aspects should be taken into consideration during the manufacturing process compared to those small-molecule drugs. To develop a stable therapeutic protein, various analytical techniques and mechanistic approaches should be used to demonstrate and monitor the protein instability as well as to assure the quality of these products and enhance therapeutic protein design. Pharmaceutical industries and manufacturers are responsible to

accomplish these goals and should illustrate the issues with the formation of particles and aggregates within the protein throughout the whole life cycle of the product (1,10,13,14).

On the other hand, the transportation of protein products in containers or in intravenous (IV) bags in hospitals via a pneumatic tube Transportation (PTT) could contribute to mechanical stress that may lead to an unstable protein (10)(42).

Pneumatic Tube Transportation (PTT)

Pneumatic tube transportation PTT is a widely used technique in hospitals to rapidly transport patients' samples and medicines between different delivery points. This system comprises a network of adequately adjusted tubes that propel cylindrical containers between different points by adjusting the air. In the PTT set-up, the IV bags are placed into containers transported at high speeds (15) (figure1). This way of transportation can expose transported products to mechanical stress in their route. Mechanical stress is classified into shock and vibration. Vibrations are composed of a high frequency with low g-force excitation, which partially results in pouring in the filled containers (16,17). This can expose transported items to accelerations of up to 16 g (16). This can form a new air-water interface into the solvent, which will induce the elution of denatured protein from interfacial films. However, the contact between the air-water interfaces and the protein can cause adsorption and denaturation, which induces protein aggregation. Mechanical shock, in contrast, is composed of a low-frequency with a high g-force excitation which can form growth and collapse of cavities in a solution. This can lead to the formation of hotspots, and hydrogen and hydroxyl radicals could be formed. The cavitation bubbles or other air bubbles could also create interface interaction due to violent fluid motion; this may also stimulate the formation of interfaces. Consequently, protein molecules can be damaged when aggregation and particle formation occurs (4).

Figure1: This figure shows a picture of Pneumatic Tube Transportation (PTT) in Amsterdam Academic Medical Centre (Amsterdam AMC).

Both types of mechanical stress can cause various types of aggregation. During transportation and shipping, vibration is the main factor in aggregation formation. Nevertheless, severe stress can be induced during PTT, which may lead to the formation of different types of aggregates. In addition to the many advantages of using PTT for patients' samples, there is hardly any research about the stability and efficacy of therapeutic proteins when transported by PTT. However, most of the studies in this field focus on the stability of laboratory samples. Furthermore, a few recent unpublished studies by Vieillard et al. evaluate the presence of protein aggregates using two specific monoclonal antibodies after their transport via PTT. Moreover, these experimental studies have been carried out only in the presence of headspace air (18,19).

In another study done by john Carpebter.et. al (20), They studied intravenous immunoglobulin (IVIG) and a monoclonal antibody (mAb). For quantifying the particle flow imaging, light obscuration and nanoparticle tracking analysis were used. However, these experimental studies have been carried out only in the presence of headspace air. The mechanical shock in PTT was not measured, but they considered 100 g as a reference since this value was used in an unpublished study discussing the same issue. In this study, they did not mention specifically which type of therapeutic proteins they used, namely intravenous immunoglobulin or monoclonal antibodies (20).

Our study evaluated the effects of PTT on particle generation in protein solutions with and without headspace air in IV bags. For model therapeutic proteins, two monoclonal antibodies, namely pembrolizumab and infliximab, were studied; both are included in lists of products that should not be transported via PTT. The particles formed were characterised using flow imaging microscopy (MFI), nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS). Besides, size exclusion chromatography (SEC) was used to determine soluble aggregate concentration.

Techniques to determine protein aggregation

Several analysis techniques can investigate and analyse protein aggregates and proteinaceous particles. These techniques are based on different separation and detection principles, for instance, through various methods, including analytical ultracentrifugation (AUC), microscopic or visual inspection, or mass spectrometry. One of the main challenges in analysing protein aggregates is the unknown character of the formed aggregates and their diameter varieties between some nanometers and some millimetres, reflecting a significant difference in their size ranges. As depicted in figure 2, none of the aforementioned techniques can cover this size range, and each has its advantages and drawbacks; that is why several methods should be combined to cover such a wide size range (21–24). Moreover, the analysis methods available differ in their physical measurement principles and, accordingly, in their obtained information results and outcomes. (21)(23)

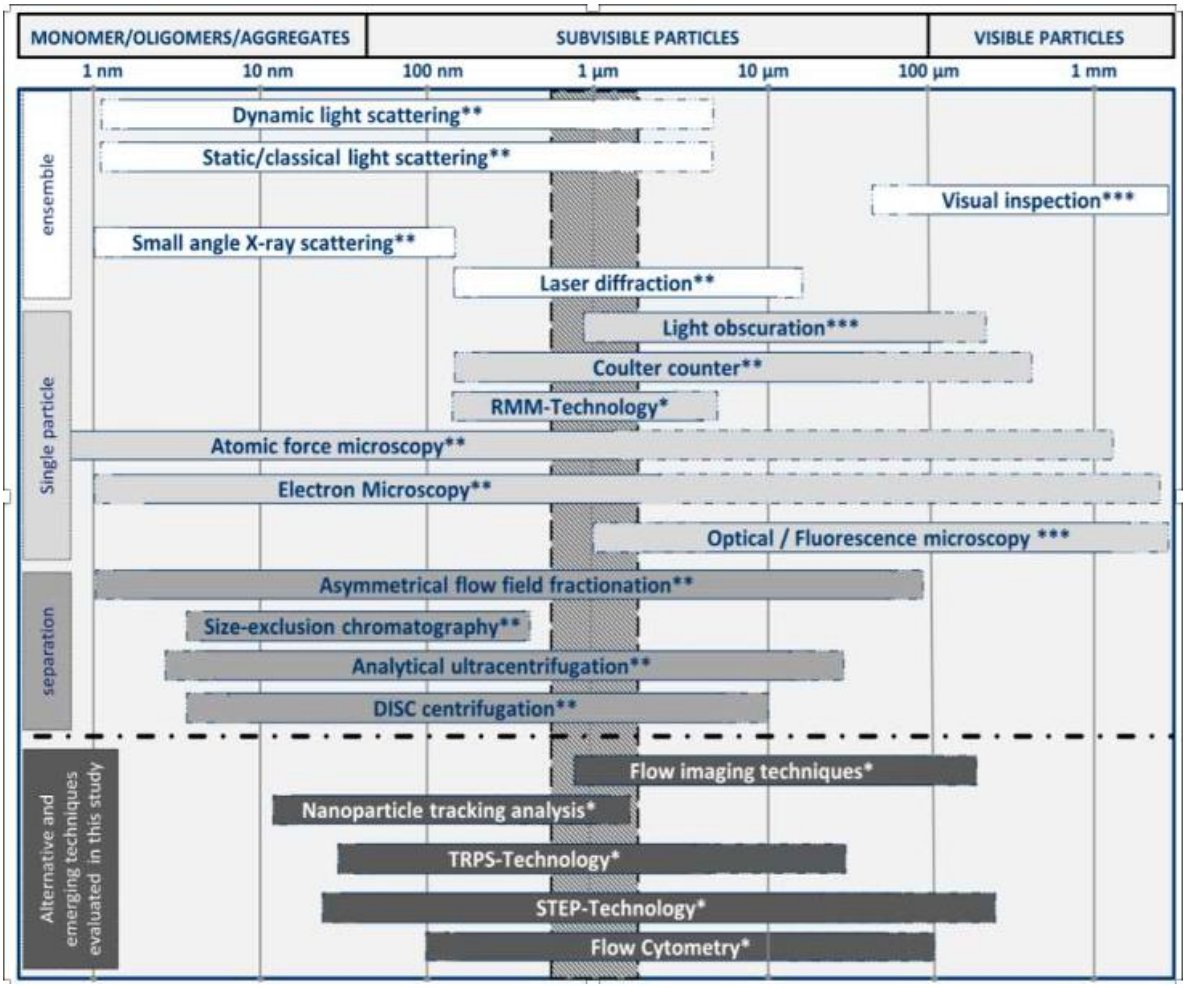

Figure 2: Overview of methods used to detect particle formation and protein aggregation. fixed on the top part and emerging on the lower part methods are oriented based on their size measured range. The logarithmic scale characterises the appropriate size range of these methods at the top. These methods are divided into ensemble, single particle, and separation methods. *Emerging methods. ** validate methods. *** methods stated by European Pharmacopoeia and United States Pharmacopoeia. RMM refers to the resonant Mass Measurement, DISC refers to Disc based centrifugation, TRPS is Tunable Resistive Pulse Sensing, STEP is Space- and Time-resolved Extinction Profile. Adapted from Gross-Rother, et al. (24)

To cover a broad range of particle size, we used in our studies other techniques as well, such as DLS, nanoparticle tracking analysis (NTA) and microflow imaging (MFI) to study the stability of therapeutic proteins when they are transported by PTT, using two types mAb, infliximab and pembrolizumab.

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is a commonly used technique to detect protein aggregation. With this technique, the intensity of the scattered light can be measured and its fluctuation. It measures the size distribution of particles by determining the speed at which the particles move in the solution. The rate of small particles in the solution is higher than the large particles, and consequently, it will lead to a higher frequency of fluctuations in the intensity of the scattered light. Utilising calibration with particles of determining size, the particle size can also be measured, which gives more details about the aggregation process. DLS is useful as it can be performed when only a small amount of product is available because the analysed sample by DLS can be reused for further analysis. The concentration of protein to be measured could be in a range between 0.1–50 mg/ml. Although the susceptibility of this method to detect large particles is not well studied, the quantification of the sample is not possible by DLS. However, with DLS, only a qualitative measurement of the samples can be obtained (24,28).

High- Performance Size Exclusion Chromatography (SEC)

High-performance size exclusion chromatography (SEC) is also one of the widely used techniques for measuring and analysing protein aggregation. In this system, solute molecules can pass through a column which contains beads with pores. Small molecules can easily penetrate the pores. In contrast, the larger molecules or aggregates cannot penetrate. Hence, small molecules will have more retention time in the column and later. However, the ability of the molecules to enter the pores is characterised by molecular size. For the SEC analysis, a relatively small column is required, where small amounts of samples are adequate to obtain results (29).

Different factors could influence the obtained results by SEC, such as low solution ionic strength and hydrophobic interactions, therefore lowering the elution rate and the peak shape. However, the aggregate size detected by SEC is dependent since the larger aggregates can be filtered out from the system by the pre-column. As a result, large protein aggregates could be pulled or vanish in the analysis (30). As a result, large protein aggregates could be removed or vanish in the analysis (30).

Microflow imaging (MFI)

The MFI setup is composed of a system where the fluids can easily pass through to the flow cell, which is elucidated by a pulse of light intensity, as depicted in figure 3. With the aid of a custom magnification system as well as with a broader depth of field, the particle images can be quickly recorded in a short time (31). Before running each sample, a calibration is automatically employed to improve threshold detection. In order to identify the formed

particles an image analysis software is used. The extraction and the saved information will be based on the formed particles' size, contrast, and morphology. MFI is very effective in detecting and visualising very small particles, which can be between 1- 1000 µm(32).

Since this technique can visualise particles by images, it allows for more elaborate aggregation experiments and for determining different types of particles according to their shape and reflected signal intensity. Besides, it is also able to record numerous particles rapidly. But it is difficult to develop a suitable software filter as an inadequate filter may lead to incline results (24,31,32).

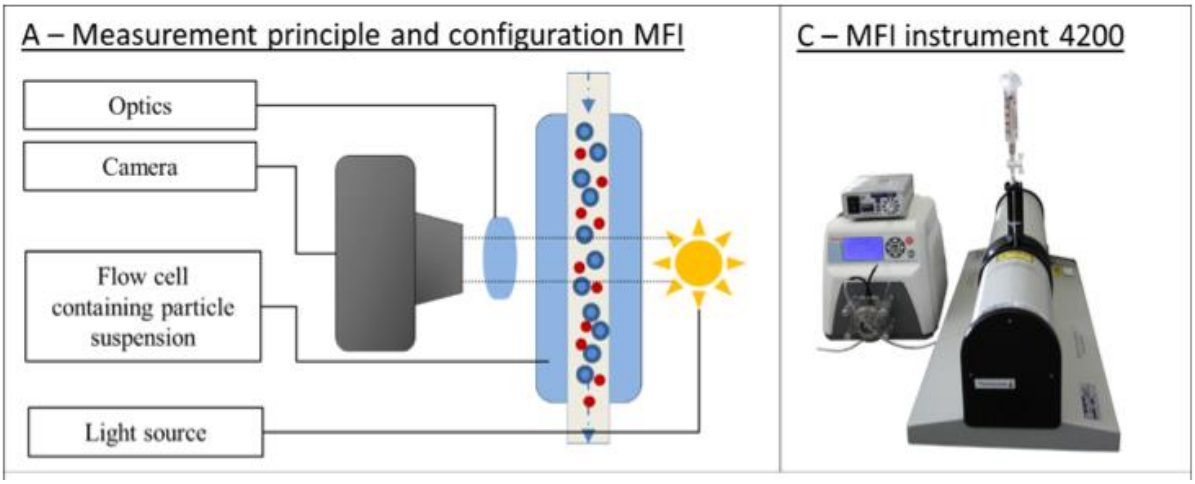

Figure 3: Schematic illustration of Micro-flow imaging (MFI) measurement principle. It indicates when particle suspension moves through the flow cell, the camera reproduces an intense field image like raw data. These raw data will be then analysed by MFI software to determine every single particle. Adapted from Gross-Rother, et al(24).

Nanoparticle tracking analysis (NTA)

NTA is a technique used to record and visualise nanoparticles in a solution by light scattering microscopy connected to a charge-coupled device camera (figure 4). The software of this technique is responsible for the nanoparticles identification on video and correlating their Brownian motion (the speed at which the particles move in solution) with their particle size (33,34).

NTA can be utilised to measure particle size and size distribution in a range between 30 - 1000 nm. Since NTA is counting single particles, it is capable of detecting an expanded range of population ratios in one sample and determining the accurate size of them using a high peak resolution, which is not the case with DLS (33,35). Moreover, NTA has a small concentration range between (10*7 to 10*9 particles/ml), in which a sample dilution may be needed; therefore, there is a possibility of protein damaging or aggregates forming. With this technique, a small sample volume can be analysed and measured. Lastly, NTA is longer time intensive than the DLS instrument, where software settings are corrected to enhance video capturing and particle detection is required. This technique was not used in this study because of technical problems in the apparatus (24,35).

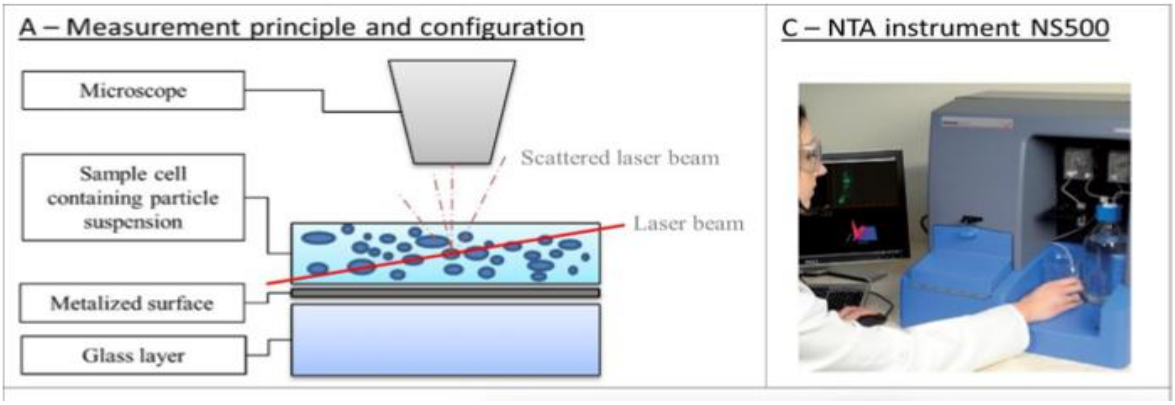

Figure 4: Schematic illustration of the measurement's principle and configuration of NTA instruments. It shows when particle suspension moves through the sample cell, where the laser beam exposes the particle inducing light scattering. The scattered light for each particle is then recorded and analysed using a microscope and NTA Software to find out the Brownian motion for the particle and afterwards its size. Adapted from Gross-Rother, et al (24).

Materials and methods

For this experiment, the leftover materials are used to prepare infusion bag samples. For the preparation of infliximab samples, leftover martial of reconstituted remsima (Celltrion Healthcare®, infliximab, anti-TNF-α monoclonal antibody) solution of 10 mg/ml was used, which was provided by Amsterdam Academic Medical Centre (Amsterdam AMC). Further, to prepare the samples of pembrolizumab, the leftovers of Keytruda (Celltrion Healthcare®, Pembrolizumab, humanised monoclonal anti-programmed cell death-1 (PD-1) antibody) solution of 25 mg/ml which was provided by VU University Medical Centre (VUmc) was used. The left-over reconstituted vials were first stored at room temperature at the hospitals for a few days to a few weeks. Then these vials were congregated and stored at a temperature of 2-8°C, where they could be stored for a few days to a few months. Before the experiment days, the leftovers of the protein solutions were pooled in the Laminar flow cabinet (laf-cabinet). Afterwards, the samples were stored at 2-8 °C for several weeks. This process was aseptically done.

The vial of infliximab (remsima) of 10 ml composed of 100 mg infliximab, 500 mg sucrose, 0.5 mg polysorbate 80 (PS80), (2.2 mg) sodium dihydrogen phosphate, and (6.1 mg) disodium hydrogen phosphate dihydrate (36). The vial of pembrolizumab (Keytruda) of 4 ml composed of 100 mg pembrolizumab, 6.2 mg L-histidine, 0.8 mg polysorbate 80 (PS80) and 280 mg sucrose (39).

The infusion bag for NaCl 0.9% (VIAFLO®) of 50 ml is made of a flexible plastic container fabricated from a multilayer sheeting (PL-2442) composed of Polypropylene (PP), Polyamide (PA) and Polyethylene (PE). The infusion bag of 0.9% Sodium Chloride was composed of USP (NaCl) with an osmolarity of 308 mOsmol/L (calc) and 9 g/L Sodium Chloride (NaCl). Which includes 17.1 mmol of Na and 17.1 mmol Cl. (38)

The prepared sample and the exposed stress

The measurements were carried out in triple for the samples of both therapeutic proteins. However, for 0.9% NaCl samples, the measurements were performed in diplo. The samples were classified into four groups. Two of these groups (with or without air) were transported by PTT, and the other two groups (with or without air) were not transported by PTT, as illustrated in table 1. In this study, it was considered that the infusion bags that were not transported via PTT to be unstressed groups and the infusion bags which were indeed transported via PTT to be stressed groups.
The experiments with the two therapeutic proteins were performed on two separate days. However, in both experiments, 0.9 %NaCl samples were analysed (table 1)

Table 1: Study design of sample content and condition regarding stressed or unstressed and with or without air.

Sample number	Sample condition
1,2 and 3	**Infliximab/ pembrolizumab in 0,9% NaCl infusion bags with air unstressed**
4,5 and 6	**infliximab/ pembrolizumab in 0,9% NaCl infusion bags with air stressed**
7,8 and 9	**Infliximab/ pembrolizumab 0,9% NaCl infusion bags without air unstressed**
10,11 and 12	**infliximab / pembrolizumab 0,9% NaCl infusion bags with air stressed**
13 and 14	**0,9% NaCl infusion bags with air unstressed**
15 and 16	**0,9% NaCl infusion bags with air stressed**
17 and 18	**0,9% NaCl infusion bags without air unstressed**
19 and 20	**0,9% NaCl infusion bags without air stressed**

Infusion bag preparation

To prepare infliximab samples, infliximab solution was pooled from several leftover remsima vials in 10 ml syringes and gently mixed. To prepare the samples, the pool leftover materials were filtered over a 0.2 µm PES filter as described by Tian et al. and added to the 0,9% NaCl infusion bags. The PES filter was first attached to the syringe containing the pooled sample, and then a needle was tied up to the filter. Firstly, 1 ml was poured in to make the filter wet. Then, 5 ml of the leftover pooled infliximab was injected into the infusion bag, which contained 50 ml 0,9% NaCl; then, it was mixed three times gently. This resulted in a 0.91 mg/ml infliximab solution in 0,9% NaCl, a common concentration used in clinical practice (36).

For this experiment, 12 infliximab samples were made. The headspace air of six of these samples was removed from the infusion bags using a 50 ml syringe until no air remained. The headspace air was removed before adding the infliximab to the infusion bags.

To prepare pembrolizumab samples, pembrolizumab solution was pooled from several leftover vials of pembrolizumab in a 50 ml syringe and gently mixed. Then for each sample, approximately 3,5 ml of the pooled material was transferred to a 5 ml syringe by a connector in order to get a precise amount of solution. The PES filter was first attached to the 5 ml syringe, which contained the pooled sample, and then a needle was tied up to the filter. Firstly 1 ml was poured in to make the filter wet. Then 2 ml of the leftover pooled pembrolizumab was injected into the infusion bags which contained 50 ml 0,9% NaCl; then it was mixed three times gently. This resulted in a 0,96 mg/ml pembrolizumab solution in 0,9% NaCl, which is a concentration similar to clinical practice (37).

For this experiment, 12 pembrolizumab samples were made. The headspace air of six of these samples was removed from the infusion bags using a 50 ml syringe until no air remained. The headspace air was removed before adding pembrolizumab to the infusion bags.

Pneumatic tube transport (PTT)

Infusion bags were transported using the PTT system available at the Amsterdam AMC. The route between the clinical pharmacy and the poli-oncology was used in each experiment. No visible particles or foam were observed in samples before or after PTT.
Afterwards, the samples were stabilised in a box and transported by car to Leiden Academic Centre for Drug Research (LACDR), where they were analysed. During the trip to Leiden, an MSR logger was used to determine the shock and vibration forces in the samples transported by car.

Stability of aggregates post-PTT

To assess the stability of particles formed by PTT, infliximab infusion bags, pembrolizumab and 0.9% NaCl infusion bags were analysed by DLS, MFI, SEC, and NTA stressed and unstressed, with and without the presence of headspace air.

Dynamic Light Scattering (DLS)

Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), equipped with a 633-nm He-Ne laser fixed at an angle of 173°, was used for DLS measurements. Single-use DTS0012 polystyrene cuvettes were used. For each measurement, 1 ml of the sample was taken. A SOP was then used with the following settings: Material sample: polystyrene latex with IR of 1.590 and absorption of 0.010. Dispersant: water, General options: used dispersant viscosity as sample viscosity. The temperature was set at 25 °C and equilibrated for 0 second. Cell: DTS0012. Measurement duration was in automatic mode and 3 runs for each sample. Data processing was in general purpose mode. The polydispersity index (PdI) and Z-average diameter are calculated using the correlation function.

Microflow imaging (MFI)

The particle size range between 1-1000 µm was characterised and counted with MFI 5200 device (Protein Simple, Santa Clara, CA, USA) with View System Software (MVSS) Version 2 at LACDR. For each run, 1 ml of the sample was used. To detect the particle, 0.5ml was used in the MFI device. Before each run, a 100 µm silane-coated flow cell was flushed with 3 ml of the sample at the maximum speed. Further, flow cell cleanliness was checked on each measurement day. To optimise the illumination, 0.5 mL was used before each measurement. Analysis was carried out with (MVAS) version 1.2 and MFI Image Analysis version 1.1.0.24. For this study, all the sizes of the particles were included, and no filter was used.

Size Exclusion Chromatography (SEC)

SEC measurements were obtained using a Waters Alliance e2695 HPLC (Waters Corporation, Manchester, UK) combined with a Waters 2998 Photodiode Array Detector and a Waters 474 Scanning Fluorescence Detector. Phenomenex BioSep-SEC S3000 300 x 7.8 mm 5 microns (p/nl 00H-2146kK0, S/nl H19-172339, B/no 6583-0318) column was used with a pre-column Phenomenex security guard cartridge GFC-2000 4 x 3.0 mm. For the mobile phase, 0.9% NaCl was dissolved in ultrapure water, and 1 mg/ml of serum albumin was used as a standard. The flow was set on 1 ml/min during the analysis. The UV was measured at 280 nm, and the fluorescence was measured between 280 nm and 340 nm. To analyse our data, empower pro software (Empower 2 software 2005, Waters Corporation, Manchester, UK) was used. The loss of monomers was calculated by summing up all the volumes from the non-monomers. The ratio of monomer loss area divided by the remaining monomer area was calculated and used for statistics.

Nanoparticle tracking analysis (NTA)

For this study, NTA was not used because of a technical problem in the instrument belonging to the LACDR.

Statistical analysis

For the statistical analyses, SPSS version 28.0 (IBM Corp., Armonk, New York) for Windows. The mean, standard deviation, minimum and maximum for different values were determined using the frequency function of SPSS. Furthermore, continuous variables were compared between the different groups/experiments using an independent samples t-test. The values of the four groups were compared by using the ANOVA test in SPSS. A two-tailed P-value < 0.05 was determined as statistically significant. The graphs are made using Excel for Mac (version 16.44), running on macOS Mojave 10.14.6.

MSR loggers' measurements

MSR Logger 455877 16 g and MSR Logger 456127 200 g are used to measure the forces and vibration in PTT. These loggers have a Lithium polymer battery of 100 mAh and is rechargeable via USB connection. The size of this devices is 39x23x72 and weight is ca 69 g. (43)

The forces in PTT in both Amsterdam AMC and Amsterdam VUmc are determined by MSR Loggers. Further, during the transportation of Pembrolizumab and Infliximab samples in both experiments, the loggers were used to measure the applied force. Unfortunately, Due to the time limitation of this study and technical problems, only the results of VUmc with one logger (200 g) are analysed.

Preparation of logger in PTT experiment

Both loggers were attached to the infusion bag by using duct tape and then transferred through PTT. (Figure 5)

Figure 5: This figure shows 0.9% NaCl with 200 g logger.

All measurements are done in triple. The measured groups are 1- only one 50 ml 0.9% NaCl infusion bags in the tube 2- five of 50 ml 0.9% NaCl infusion bags in the tube. 3- only one 50ml 0.9% NaCl infusion bags in the tube wrapped in foam (figure 6) 4- only one 250 ml 0.9% NaCl infusion bags in the tube. (Figure 7)

Figure 6: This figure shows the infusion bag wrapped in a foam in PTT tube.

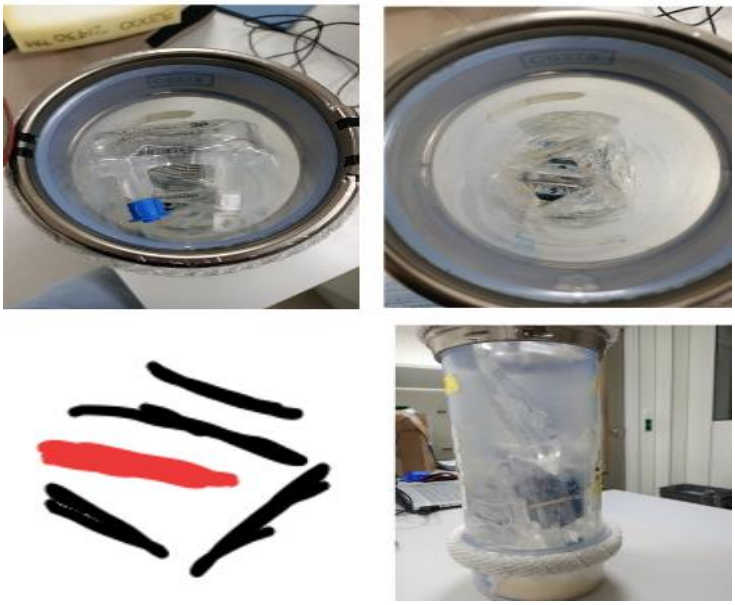

Figure 7: This figure shows five of 0.9% NaCl infusion bags of 50 mL in the PTT.

MSR data analysis

To determine the mechanical shock and vibration are the mean intensity over Threshold (IoT) is and the mean maximum acceleration max.
The analyses are performed using MSR pc software (standard) (2022-04-25, v6.07.04, MD5: 37823d82a39e71d569389e5aaa7df13a). System requirements: Windows 7 or higher. And MSR165 ShockViewer software MSR165 ShockViewer (2021-06-04, v1.02.24, MD5: 98f1eb12aadcfe5474e26b25e56516a7). Further, the figures are made by using Microsoft excel. (44)

Results

The results of the different techniques used in this experiment.

Results of DLS

1-Mean Z-average

The change in the mean Z-average of both proteins was determined in several conditions using DLS. The blue bars depicted in (figure 8) represent the results of pembrolizumab, and the orange bars represent the results of infliximab.

The mean z-average of infusion bags containing the therapeutic proteins with air unstressed was compared to the mean z-average of infusion bags containing proteins with air stressed. As it is illustrated in (figure 8), the mean Z-average of pembrolizumab stressed with air (21.11 nm, std 19.75) was higher than the mean Z-average of the corresponding group unstressed with air (15.44 nm, std 7.36). These results were significant with a p-value of 0.045. However, this was not indicated in the group pembrolizumab without air, where the mean Z-average was lower mean z-average without air stressed (11.33 nm, std 0.57) in comparison with the mean Z- average of the infusion bags without air unstressed (12.56 nm std 2.21). These results are significant, with a p-value of 0.001.

Comparing the results of pembrolizumab without air unstressed (12.56 nm std 2.21) with the results of pembrolizumab with air unstressed (15.44 nm, std 7.36). The mean z-average is lower in the group without air. These results are significant, with a p-value of 0.007

The mean Z-average of infliximab infusion bags with air stressed is slightly higher (18.65 nm std 0.083) than the mean Z-average of infliximab infusion bags with air unstressed (17.82 nm std 2.28). However, the error bars of the mean Z-average of these two groups are overlapping, and the p-value is 0.148, which indicates that these results are not significant. The infusion bags of infliximab without air stressed had a mean Z-average of (14.84 nm std 0.01), which is less than the mean Z-average of the unstressed infliximab infusion bag without air (16.99 nm std 0.087). The error bars of both mean Z-averages are relatively small and do not overlap with each other, and the p-value is 0.001; thus, this means that these results are significant.

Comparing the results of infliximab without air unstressed (16.99 nm, std 0.087) with the results of infliximab with air unstressed (17.82 nm std 2.28). The mean z-average is lower in the group without air. These results are not significant, with a p-value of 0.095

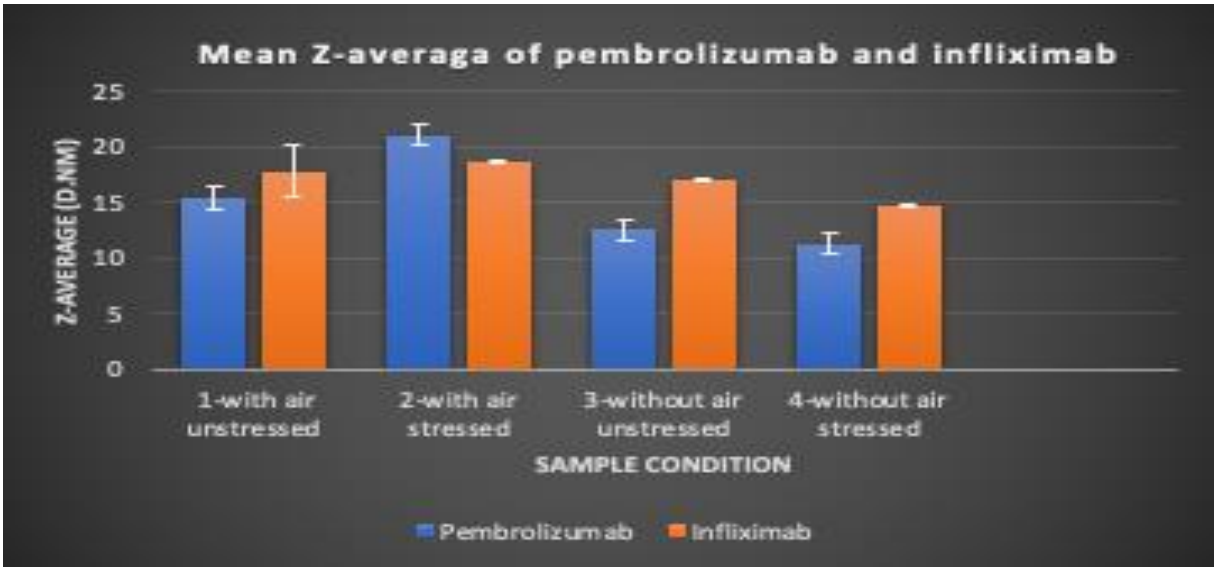

Figure 8: This figure illustrates the outcomes of the mean Z-average in nm of infliximab 0.91mg/ml and pembrolizumab 0.96mg/ml using DLS. These results were measured under different conditions: 1-with air unstressed, 2-with air stressed 3-without air unstressed 4-without air stressed. The orange bars represent infliximab, and the blue bars represent pembrolizumab. The grey lines represent the error bars. The grey lines represent the error bars.

2-Average PDI:

Pembrolizumab

In figure 9, the mean average PDI of pembrolizumab is shown, which is also measured with the aid of DLS. It can be seen that the mean average of PDI of pembrolizumab when stressed with air is relatively higher (0.18 std 0.038) than the mean average of PDI of pembrolizumab with air unstressed (0.11std 0.076). The error bars of these two outcomes overlap, and the p-value is 0.076, which means that these outcomes are not significant. Looking at the bars, which represent the mean average PDI of pembrolizumab without air and stressed, it can be seen that the mean average PDI (0.09 std 0.062) is lower than the mean average PDI (0.12 std 0.065) of pembrolizumab unstressed and without air. The error bars of these two outcomes overlap, and the p-value is 0.791, which means that these outcomes are not significant.

Comparing the results of pembrolizumab without air unstressed (0.12 std 0.065) with pembrolizumab with air unstressed (0.11std 0.076). It can be seen that the mean PDI is higher in the group without air. These results are not significant, with a p-value of 0.932. The error bars are overlapping.

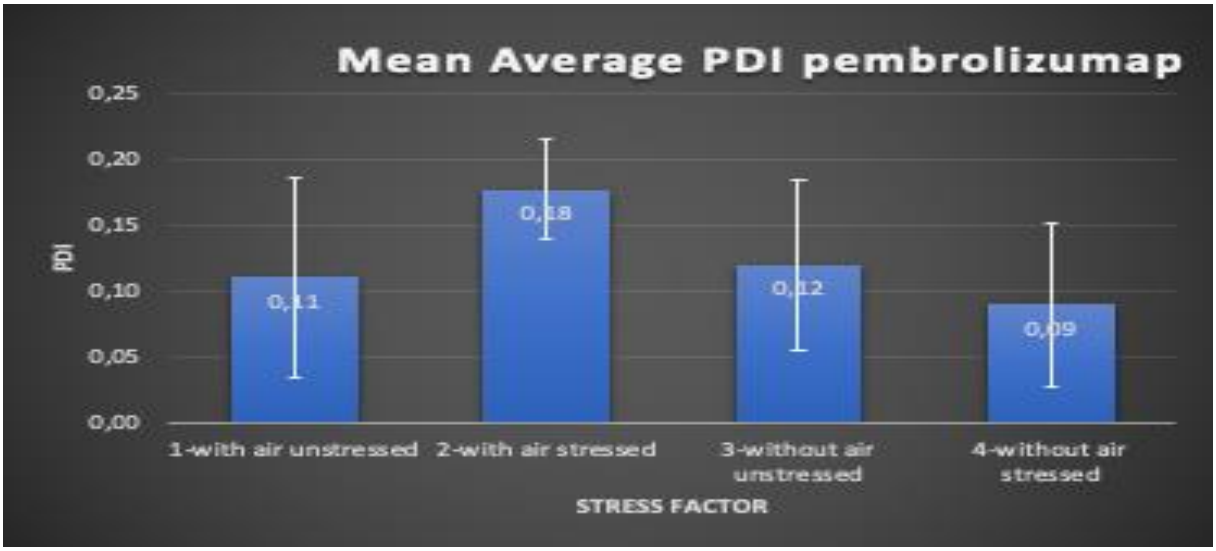

Figure 9: This figure shows the outcomes of the mean average PDI of pembrolizumab 0.96mg/mL using DLS. These results were measured under different conditions: 1-with air unstressed, 2-with air stressed, 3-without air unstressed 4-without air stressed. The grey lines represent the error bars. The grey lines represent the error bars.

Infliximab

The bar graph (figure 10) illustrates the mean average PDI of infliximab. It can be seen that the mean average of PDI with air when stressed (0.243, std 5.67) is slightly higher than the mean average of PDI of infliximab with air unstressed (0.223, std 0.074). Furthermore, the error bars of one of these measurements is notably high and overlap with the error bars of other groups. Further, these two groups have a p-value of 0.479, which indicates that these results are not significant. Looking at the two bars, which represent the mean average of PDI of infliximab without air, it can be seen that the level of mean average PDI stressed is slightly lower (0.11, std 2.17) than the mean average of PDI unstressed (0.17, std 7.29).
Furthermore, the error bars of both of these measurements are also notably high and do overlap, which indicates that these results are not significant.
Comparing the results of infliximab without air unstressed (0.17, std 7.29) with the results of pembrolizumab with air unstressed (0.223, std 0.074). It can be seen that the mean PDI is lower in the group without air. However, these results are not significant, with a p-value of 0.352. Further, the error bars are overlapping.

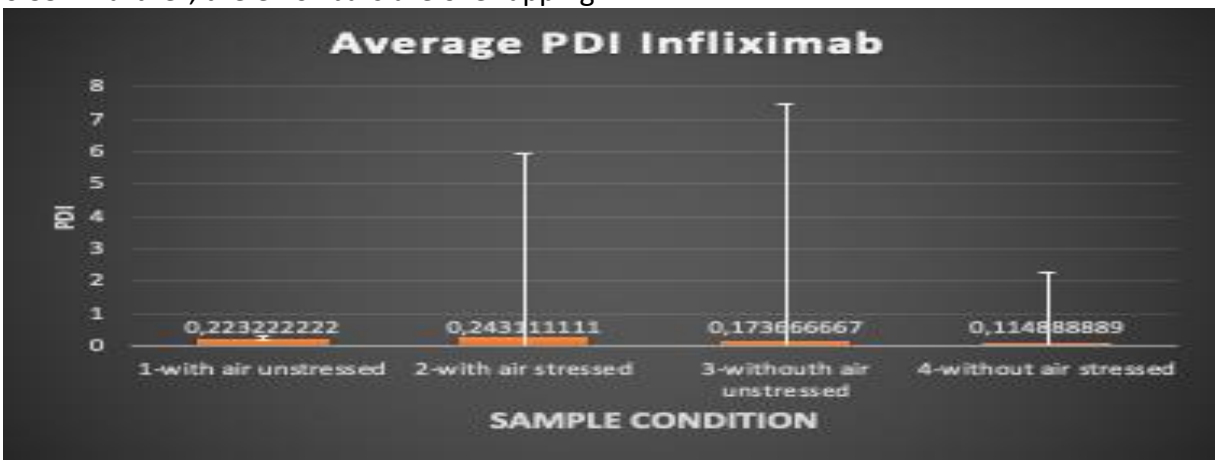

Figure 10: This figure shows the outcomes of the mean PDI of infliximab 0.91mg/mL using DLS. These results were measured under different conditions: 1-with air unstressed, 2-with air stressed, 3-without air unstressed 4-without air stressed. The grey lines represent the error bars.

Results of MFI

Pembrolizumab

The results of MFI were given in the number of particles per concentration unit. Figure 11 shows the number of particles of pembrolizumab and the corresponding 0.9% NaCl measured on the same day under the same conditions. From this graph, it can be seen that the number of particles of pembrolizumab in the group with air stressed (101278.51 particles, std 4554.49) is much higher than the group with air unstressed (43015.61, std 4074.89). The error bars are relatively small in both cases; they do not overlap, and the p-value is < 0.001, which means that these outcomes are significant.

From the graph below, an increased particle formation in 0.9% NaCl with air stressed was observed (10389.65 particles, std 15.10) compared with the samples of 0.9% NaCl with air unstressed (2240.23 particles, std 459.80) with a p-value < 0.001, which means that these results are significant.

The last two bars in the graph below show that the number of particle formation in the samples without air are extremely low. The number of particles formed in the samples containing pembrolizumab without air stressed (5164.96 particles, std 116109) is slightly higher than in the group pembrolizumab without air unstressed (4229.16 particles, std 80515). The p-value is 0.15, which means that it is not significant.
Also, the number of the particles in group 0.9% NaCl without air stressed (2174.12 particles, std 69,82) is slightly higher than in the group 0.9% NaCl without air unstressed (1856.49 particles, std 69.81). The P -value is 0,28, which is not significant.

However, the results of the pembrolizumab group without air unstressed are different from the results of the group with air unstressed. It can be seen that the number of particles in the group without air unstressed was lower than the number of particles in the unstressed group with air with a p-value < 0.001, which means it's significant.

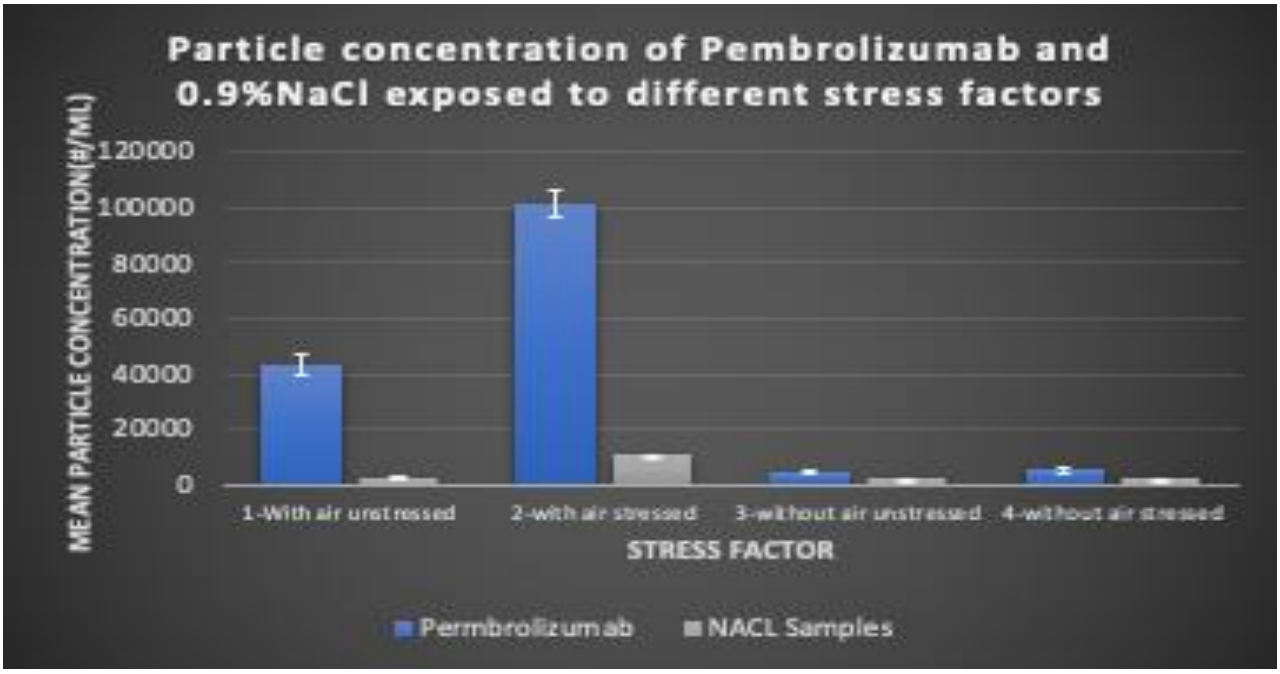

Figure 11: This figure shows the results of particle concentration of pembrolizumab 0.96mg/mL and 0.9% NaCl using MFI. These results were measured under different conditions: 1-with air unstressed, 2-with air stressed, 3-without air unstressed 4-without air stressed. The grey bars represent 0.9% NaCl samples, and the blue bars represent the pembrolizumab samples. The grey lines represent the error bars.

Infliximab

From the graph below (figure 12), it can be seen that in the group of infliximab with air stressed (4178.69 particles, std 338.17), the number of particles after the transport is somehow higher compared to infliximab with air unstressed (2558.92 particles, std 8004.52). The p-value is 0.016, and the error bars do not overlap, which means that the results are significant.

Figure 12 also shows that the number of particles in the group of 0.9% NaCl with air stressed (9259.59 particles, std 311.78) is obviously higher than the number of the particles in the group 0.9% NaCl with air unstressed (2635.56 particles, std 443.94). Both these outcomes have very small error bars and do not overlap. The p-value is < 0.001, which indicates that these results are significant. Looking at the bars representing the groups without air, it can be seen that the number of particles in the group infliximab without air stressed (4501.18 particles, std 1010.10) is higher than the number of the particles in the group infliximab without air unstressed (1322.95 particles, std 319.26). Both these outcomes have relatively small error bars and do not overlap. with a p-value < 0.001, which indicates that the results are significant.

Furthermore, it can be also seen from Figure 12 that the number of the particles in the group 0.9% NaCl without air stressed (1251.10 particles, std 104.46) is slightly higher than the number of particles in the group 0.9% NaCl without air unstressed (604.41 particles, std

101.55). Both these outcomes have very small error bars and do not overlap. The p-value is 0.01, which indicates that the results are significant.
The number of particles in group infliximab without air unstressed is higher than the number of the particles in the group infliximab with air unstressed. The errors do not overlap, and The p-value is 0.03, which means that the results are significant.

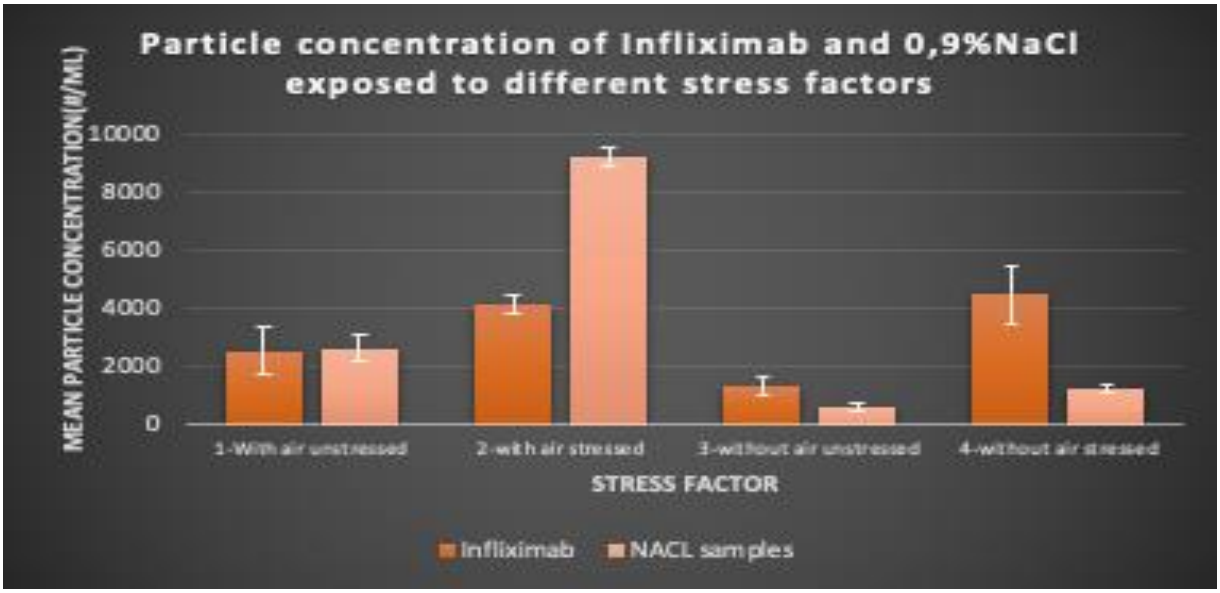

Figure 12: This figure shows the results of particle concentration of infliximab and 0.9% NaCl using MFI. These results were measured under different conditions: 1-with air unstressed, 2-with air stressed, 3-without air unstressed 4-without air stressed. The light orange bars represent 0.9% NaCl samples, and the dark orange bars represent the pembrolizumab samples. The grey lines represent the error bars.

Results of SEC

Pembrolizumab

In the bar graph below (figure13), the results of pembrolizumab are shown after analysing the particles with Size-exclusion chromatography (SEC). The ratio of monomers/ monomers loss in the group with air stressed (0.093, std 0.0016) is slightly lower than the ratio of monomers/ monomers loss in the group with air unstressed (0.096, std 0.0015). However, the error bars of these outcomes overlap, with a p-value of 0.95 which means that the results are not significant.
Furthermore, it can also be seen that the ratio of monomers/ monomers loss in the group without air stressed (0.097, std 0.0012) is slightly higher than the ratio of monomers/ monomers loss in the group without air unstressed (0.096, std 0.0026). However, the error bars of these outcomes overlap, with a p-value of 0.147, which means that the results are not significant.

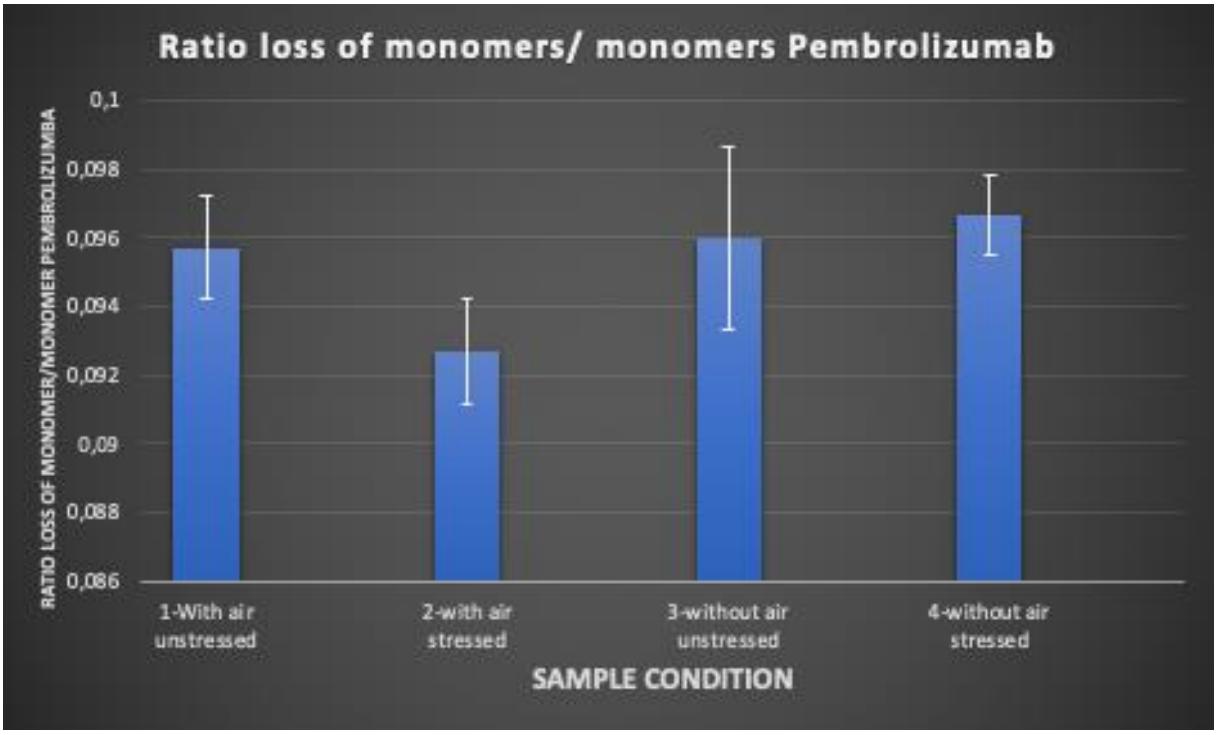

Figure 13: This figure illustrates the results of the ratio loss of monomers of pembrolizumab 0.96mg/mL using SEC. These results were measured under different conditions: 1-with air unstressed, 2-with air stressed, 3-without air unstressed 4-without air stressed. The grey lines represent the error bars.

Infliximab

The graph below (figure 14) shows the results of infliximab measured by SEC. It is clear that, in general, the ratio of monomers/ monomers loss is very low. However, in the group with air stressed (0.0077, std 0.0015), this ratio is relatively lower than the ratio in the group with air unstressed (0.013, std 0.0049). However, the error bars of these outcomes overlap, with a p-value of 0.88, which means that the results are not significant. Furthermore, it can be seen that the ratio in the group without air stressed (0.0075, std 0.00035) is slightly lower than the ratio in the group without air unstressed (0.010, std 0.0042). However, the error bars of these outcomes also overlap, and the p- values is 0.062, which means that the results are not significant.

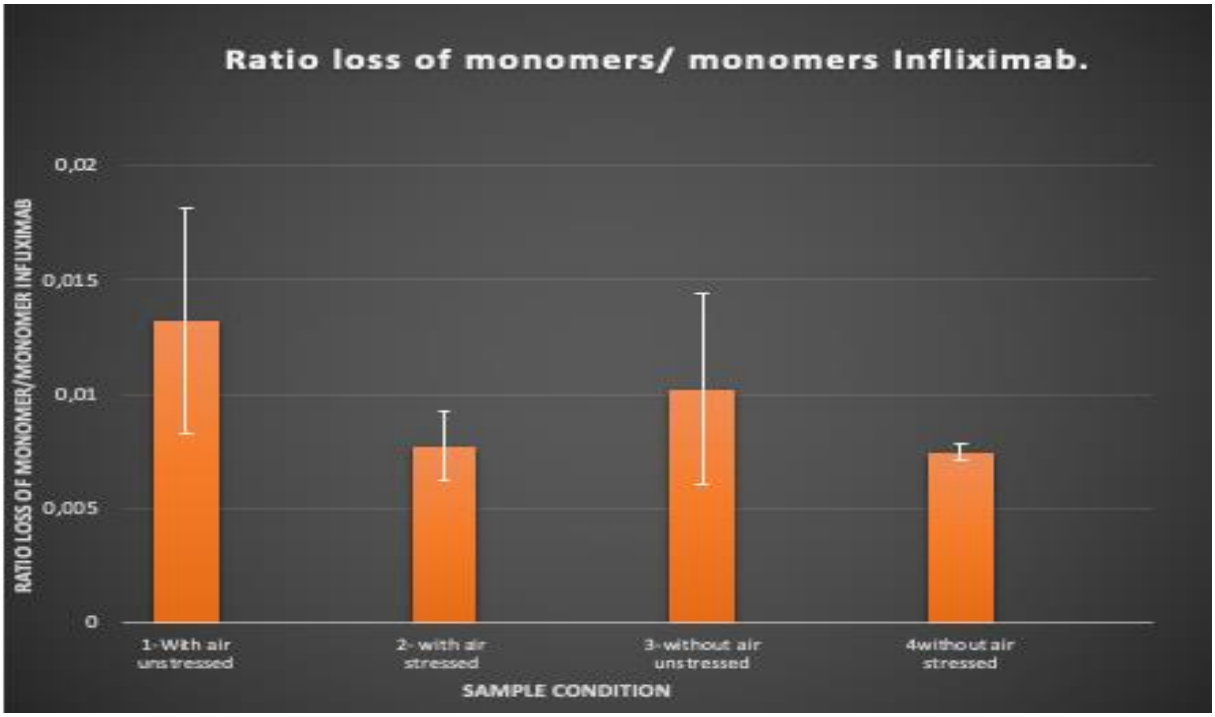

Figure 14: This figure represents the results of the ratio loss of monomers of infliximab 0.91mg/mL using SEC. These results were measured under different conditions: 1-with air unstressed, 2-with air stressed, 3-without air unstressed 4-without air stressed. The grey lines represent the error bars.

Results of Loggers' data

In PTT different forces and vibration occurred (figure15). The software showed the 10 major events each time.

From figure 16 it can be seen that in group 1 the mean of maximum acceleration max was (131,78g std is 15,19) which is higher than group 4 (77,3 g std, 11,51). Further, in group 4 it was higher than in group 3 which was (26,15 g std, 7.18). However, group 2 was the lowest (20,89g, std 4,21) among all other groups.

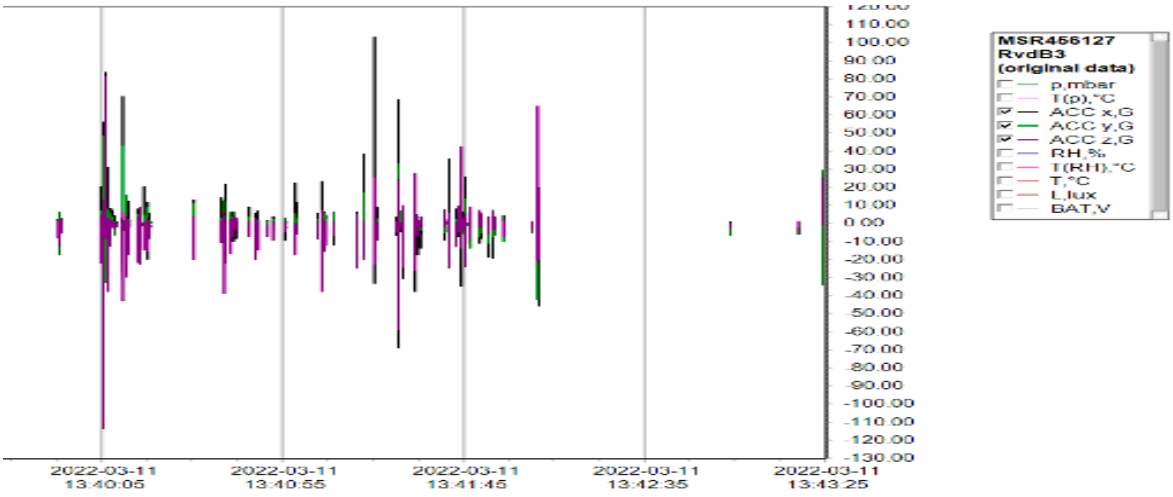

Figure 15: This figure shows an example of the forces in g in one route when a 50 mL of 0.9% NaCl infusion bags are transported via PTT in VUmc.

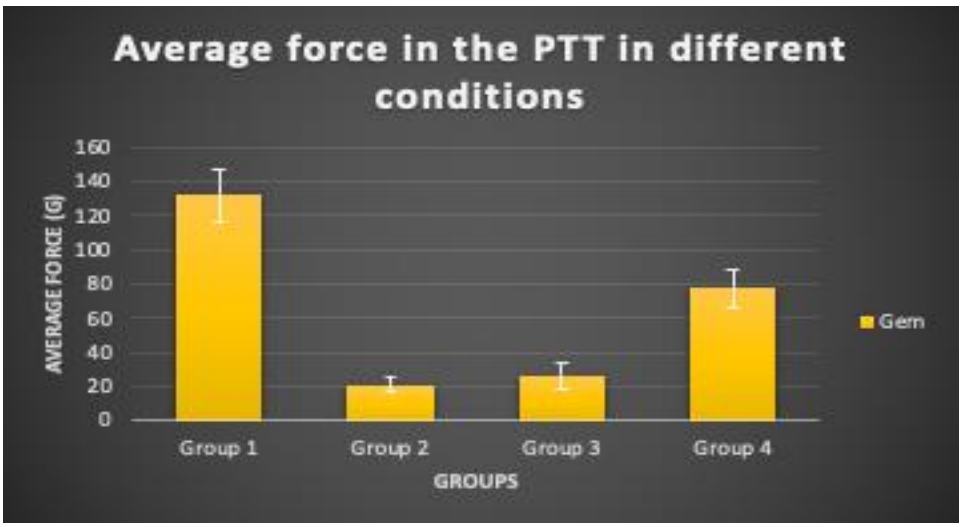

Figure 16: This shows the average of maximum acceleration max. The x axis indicates the following groups 1- only one 50 ml 0.9% NaCl infusion bags in the tube 2- five of 50 ml 0.9% NaCl infusion bags in the tube. 3- only one 50ml 0.9% NaCl infusion bags in the tube wrapped in foam 4- only one 250 ml 0.9% NaCl infusion bags in the tube. The y-axis indicates the average force in g. The grey lines represent the error bars.

From figure 17 It can be seen that in group 1 the mean of IoT was (1920.48 std is 103.26) which is higher than group 4 (1806.59 std, 164.49). Further, in group 4 it was higher than in group 2 which was (137.24 std,118.95). However, group 3 was the lowest which was(612.74, std58.05) among all other groups.

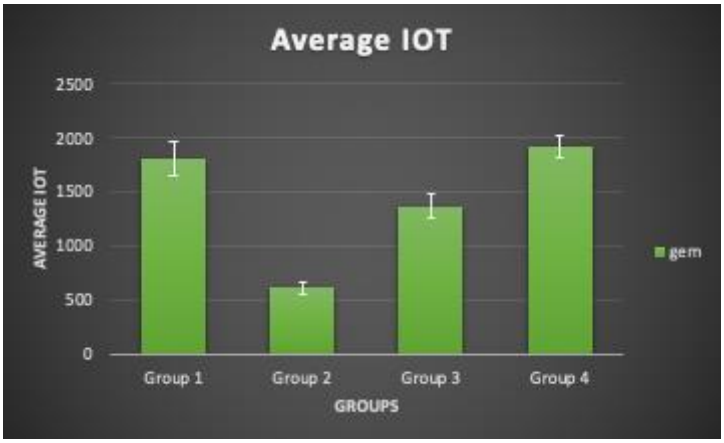

figure 17: This figure indicates the average of IoT. The x axis indicates the following groups 1- only one 50 ml 0.9% NaCl infusion bags in the tube 2- five of 50 ml 0.9% NaCl infusion bags in the tube. 3- only one 50ml 0.9% NaCl infusion bags in the tube wrapped in foam 4- only one 250 ml 0.9% NaCl infusion bags in the tube. The Y axis indicates the average of IoT. The grey lines represent the error bars.

Discussion:

After the analysis with DLS it was expected that an increase in the mean Z-average of infliximab and pembrolizumab stressed samples will be seen compared with unstressed samples. Since that Z-average could indicate changes in the particle and aggregation formation. In addition, an increase in the mean z-average was seen in the stressed samples with the air of both proteins compared with unstressed samples (figure 8). This can be explained by the fact that mechanical stress applied to the samples leads to more aggregation. This is in line with previous observations by Vieillard et al. (18,19), where the increase in particle formation was observed when mAbs were transported by PTT. However, this increase was not significant in infliximab samples; in contrast to the rise in pembrolizumab samples which was significant.

In the airless groups of both proteins, it was observed that the mean Z-average was lowered after the stress, which was not expected. However, these results were not significant for without air samples of pembrolizumab because of the overlapping of the error bars. Further, it was Significant for the sample infliximab (figure 8). This is opposed to the observations of Vieillard, et al. (18,19) and john Carpenter, et,al (20)

For both proteins the mean of the z-average without air unstressed is lower than the mean of the Z-average with air unstressed. That was not expected because the same material, the same solution and the same infusion bags were used, and logically no difference would be seen because no stress was applied, except for the ride from Amsterdam AMC to LACDR, which had very few shocks/ vibrations with mechanical stress (max 10g).

After the MFI analysis, it was expected that the particle concentration of both proteins would be higher after transport than before transport and that the concentration of particles with and without air would be the same in the unstressed condition (16-8). In addition, it is also expected that NaCl groups have a lower concentration of particles than the corresponding infliximab or pembrolizumab groups. Looking at the (figure 11,12) with air samples, the concentration of particles in the stressed groups is higher than in unstressed groups. In the group without air, the concentration of particles in both groups, both pembrolizumab and NaCl, both unstressed and stressed, is very low. However, when the unstressed conditions are compared, namely the unstressed pembrolizumab with air and unstressed pembrolizumab without air, the number of particles in the group without air was much lower than in the unstressed pembrolizumab with air. This may be due to the fact that it contains more air bubbles and, therefore, larger numbers of particles. Strikingly, many more particles in the NaCl group with air stressed were present in comparison with the group of in the infliximab group with air stressed. This was not expected, given that the infusion fluid was NaCl. This may be related to the fact that infliximab contains a surfactant which could have a beneficial effect on the release of particles from the infusion material or less formation of air bubbles. (40) These are not filtered out. In the airless infliximab group, fewer particles were measured than with air unstressed in both cases. This can be explained by the fact that there are fewer air bubbles in the group of no air; these were not filtered.

In the results of SEC, it was expected that the mean ratio after the transport of both investigated proteins would be higher than the mean ratio for the transport with and without air (21-24). In figure 10, where the results of pembrolizumab were depicted and

figure 10, where the results of infliximab were depicted, the ratio with air after the stressed is lower than with air unstressed. However, this is not what was seen in the results of DLS n MFI. An increase in the average ratio was seen in groups without air. However, this result is not reliable and not significant because error bars overlap.

It has been observed that not the same pattern is seen for the same sample in different analysis methods or within the methods themselves. As an example, infliximab without air stressed has a lower z-average than without air unstressed; however, the PDI of these groups is not significant. From SEC measurements it can be seen that the ratio loss/monomer infliximab without air stressed is lower than without air unstressed.

Increased particle formation does not straightforwardly mean that therapeutic protein aggregates were formed. This can be clarified in different ways, such as nanobubbles formation or particles leaching from used infusion (41)

Future perspective:

Both Gross-Rother, et al. and Vieillard, et al. used in their studies size exclusion chromatography (SEC) and dynamic light scattering (DLS) to quantify protein aggregation after using PTT. However, using these techniques will not cover a large range of particle sizes. To cover a broad range of particle sizes, we used in our studies other techniques in addition to the aforementioned ones, such as DLS and microflow imaging (MFI), to study the stability of therapeutic proteins when they are transported by PTT, using two types of mAb, infliximab and pembrolizumab (18,19,24).

In our study, one type of 0.9% NaCl (VIAFLO®) infusion bag was used, while in the study by John Carpenter, et a (27). They have concluded that the aggregation of monoclonal antibodies during PTT depends on the type of the infusion bag. For future research, more types of infusion bags need to be tested. Further, Carpenter et al. looked into different solvents, namely 5% glucose and 0.9 % NaCl. They have indicated that they could influence the stability of the therapeutic protein. However, further experiments are subsequently required to establish that.

For future studies and research, new vials should be used and thus not the leftover materials, Since the used vials may contain previously aggregation, which may affect the obtained results. (10) In this study, the effect of infliximab with pembrolizumab as a therapeutic protein has been established. However, to give a general view of the stability of biological, different other proteins should be first tested and investigated.

Furthermore, with the available and the used techniques, it cannot be said whether the particles are formed or not. Yet, various other methods should be used to demonstrate that, for example, using NTA and flow cytometry to determine a broad range of sizes. (24)

The number of particles measured after transportation by PTT in the group infliximab and pembrolizumab, both with air, does not meet the requirements of European Pharmacopoeia (EP) and the United States Pharmacopoeia (USP). In which they stated that the number of particles should be < 6000 particles < 10 µm and 600 particles/container >25 µm in 100 ml. However, for the outcomes of MFI, which measured the number of particles, no filter was used to filter out the air bubbles. That is why it is not sure if they meet the requirements or

not. It should also be considered that when it does meet the requirements of (EP) and (USP), this doesn't mean that it is always safe to give it to the patient. (27).

However, Wunder C, Länger G et.al (16) indicated that the measured max force in PTT was 16 g while in our study the measured G force was very high and it was in some cases higher than 130 g.

Conclusion

From the obtained results, it can be seen that the changes in the size, number of particles and the ratio of monomer loss can occur post-PPT. However, in this study, not all of the measurements were significant. Further, there was no common pattern within different techniques for the same sample. Further research, using several techniques, is required to investigate whether aggregate formation occurs in different types of mAbs. The main conclusion of this study is that protein products in infusion bags should not be transported in hospital PTT.

From loggers' data the g forces In PTT obtained could rise up to 130 g and this may have an effect on the stability of therapeutic protein when it is transported by PTT.

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