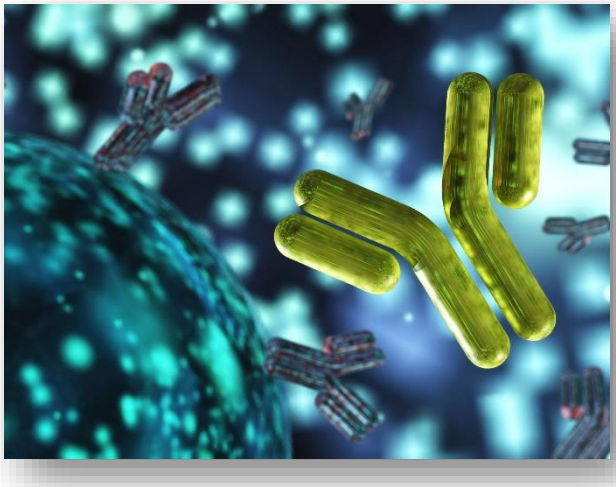
The effect of light in combination with shocks on the physical stability of infusion bags with monoclonal antibodies

Masters research report



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Abstract

Aim: It is known that incorrect storage or mishandling of monoclonal antibodies (mAbs) could lead to aggregation and in the worst case to immunogenic reactions. Therefore, conditions as temperature, light exposure, and exposure to shocks during manufacturing and storage are well monitored and the effect of it is known. Little is known about the effect of these stress factors during administration, for instance when a patient goes on an outside walk. For that reason, the aim of this study was to investigate the effect of light in combination with shocks on the physical stability of infusion bags with monoclonal antibodies.

Materials and methods: Infusion bags were prepared with leftover reconstituted vials of infliximab and pembrolizumab. They were exposed to several types of stress, namely light, shocks and light in combination with shocks. There also was a group of mAb infusion bags that was not stressed (unstressed). Each group also contained 2 NaCl 0.9% infusion bags without anything added to measure background particles. After stressing the infusion bags, they were analysed with dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), size exclusion chromatography (SEC), and micro-flow imaging (MFI). Statistical analysis was done with a one-way ANOVA and Tukey HSD post-hoc test or Brown-Forsythe and Games-Howell post-hoc test depending on the result of Levene's test for equality of variances.

Results and interpretation: In the nanometer range exposure to light seems to negatively influence the physical stability of both infliximab and pembrolizumab based on the results of DLS and SEC. This was only significantly proven for pembrolizumab with SEC ($p = 0.007$). For NTA no reliable results were obtained. In the micrometer range no general effect could be found for infliximab and pembrolizumab. However, MFI detected significantly higher particle concentrations for shocks ($p = 0.002$) and light combined with shocks exposure ($p = 0.002$) compared to unstressed for pembrolizumab. Regarding the requirements of the Pharmacopoeias for parenteral formulations infliximab exposed to any stress factor could still be used, but pembrolizumab exposed to any stress factor could not be used anymore. Despite these requirements further research is needed before administering these products to patients. For example, biological activity should be tested and other analytical techniques that can detect around 1 µm should be used.

Conclusion: This study was not elaborated enough to conclude if infliximab and pembrolizumab can still be administered to patients. However, based on this study, it seems that infliximab and pembrolizumab are both potentially sensitive to light in the nanometer range. To say something about the effect of light in combination with shocks on the physical stability of all mAbs more research is necessary. So, the effect of light in combination with shocks on the physical stability of mAbs remains unknown.

Introduction

Over the past decades therapeutic proteins, such as monoclonal antibodies (mAbs) became indispensable in the treatments of cancer, autoimmune diseases, asthma, viral infections and other diseases. (1,2) Recently mAbs gained even more popularity by their use for the COVID-19 pandemic. (3) Although their successful reputation, mAbs do have some points that require attention. Incorrect storage or mishandling could lead to aggregation. In the worst case aggregates can cause immunogenic reactions. (4–6) This means that the patient neutralizes the therapeutic mAb, which entails loss of efficacy. On top of that, neutralization can lead to cross-reactions with endogenous equivalents, causing immediate hypersensitivity and anaphylaxis. (4,6) Besides these reactions, mAbs often are used as last resort therapeutics. So, loss of efficacy means that the patient cannot be treated anymore. (3,7–10) Therefore prevention of aggregate formation is a must.

Some stress factors that could lead to aggregate formation via various mechanisms are light exposure, shocks and changes in temperature. (4,11) During manufacturing and storage these conditions are well monitored and the effects, such as aggregation formation, are known. (12–14) In contrast little is known about the effect of exposure to these stress factors during administration. Imagine a patient going for a walk during administration and the potential stress factors to which the mAb is exposed during the walk. Therefore, the aim of this study was to investigate what the effect of light in combination with shocks is on the physical stability of infusion bags with monoclonal antibodies.

Background

Aggregates

If a patient goes for a walk during administration, the infusion bag with the therapeutic protein will be exposed to several stress factors, such as light exposure and temperature changes. Additionally, the protein will be exposed to shocks and vibrations, because of the space between the pavement bricks or holes in the way. All these stress factors could lead to aggregation. (4)

First, proteins are sensitive to light. Especially when they contain a lot of aromatic amino acids or cysteine. Monoclonal antibodies contain a lot of these residues and are therefore target for photodegradation. (15–17) Photodegradation can lead to photooxidation, fragmentation, cross-linking, and the formation of radicals. (4) The radicals that are formed by photodegradation are also harmful for proteins and can cause fragmentation, denaturation, oxidation, changes to surface hydrophobicity, conformational changes and even aggregation. (16) Additionally, light exposure can lead to photodegradation of excipients such as polysorbate and histidine. Reactive oxygen species are formed by degradation of polysorbate and can lead to oxidation of proteins. If histidine degrades an important buffer component gets lost which can affect protein stability. (17) To prevent all this, the ICH Q1B guidelines are recommended for testing new active substances and medicinal products. (18) However, the light exposure requirements of these guidelines are very harsh and therefore not comparable with normal light exposure. (4) It is therefore important to learn more about the effect of realistic light exposure in practice on therapeutic proteins.

Second, it is known that mechanical stress as shocks and vibrations can cause aggregation of mAbs. (19) This can for example be caused by cavitation and agitation. In cavitation shocks induce little cavities in the liquid that rapidly collapse and thereby form hot spots. These hot spots are local regions with extreme temperatures and pressure that make aggregation possible. During the collapse hydroxyl and hydrogen radicals are also formed. These can cross-link proteins and can cause formation of aggregation. (4,19–22) For agitation the exact mechanism of causing aggregation is not well established. One of the theories is that the presence of an air/liquid interface causes stress and makes

proteins adsorb to this surface. The proteins partially unfold there, so that the hydrophobic surface adsorbs at the air/liquid interface. This causes monomers at the surface to form aggregates. This process is accelerated by mechanical shear, because of the exchange of new native proteins to the surface and partially aggregated proteins to the bulk. (21,22) Thus, mechanical stress can lead to aggregation via cavitation and agitation.

In short, the physical stress factors mentioned above can denature proteins and form aggregates of different sizes and shapes. (4) Although there is a lot of evidence that the presence of aggregates results in enhanced immunogenicity, the exact mechanism is still unknown. (4,23) A possible theory is that the repeating protein structure, no matter if it is self or non-self, triggers B-cell activation through an evolutionary mechanism that protects the body against viruses and other microbial agents. (24,25) Despite the exact mechanism being unknown, there is consensus that aggregates in the range of 0.1 – 1.0 µm are most immunogenic. (23,25) However, there is no consensus on the maximum allowed number of aggregates in protein therapeutics due to high variance in stability and safety per therapeutic protein. (5) The British and European Pharmacopoeia only specify that no visible particles are allowed. (5,26) Depending on the test, the criteria for parenteral formulations with a volume up to 100 ml are a maximum of 6000 particles per container equal to or greater than 10 µm and a maximum of 600 per container equal to or greater than 25 µm for the light obscuration particle count test. For the microscopic particle count test the requirements are a maximum of 3000 particles per container equal to or greater than 10 µm and a maximum of 300 particles per container equal to or greater than 25 µm. (27) All in all, therapeutic proteins only need to fulfil the requirements of parenteral formulations.

Detecting aggregates

For several reasons it is hard to analyse samples of therapeutic proteins and its aggregates. First, these samples are suspensions in which every particle has its own physical and chemical properties. This makes it challenging to determine the refractive index and do optical measurements. On top of that, there is a lack of appropriate protein calibration standards that have the right properties. Spherical particles of polystyrene or silica are often used, but the refractive index is not comparable with that of protein particles. (28,29) Besides, the formed chemical or physical aggregates cannot easily be distinguished from target proteins. This is because the size range is the same, but the conformation differs. Another problem is that the available analytical techniques are unable to measure over the whole size range in which aggregates can exist (fig. 1). (28) Furthermore, due to the extremely sensitive character of these suspensions, preparing the samples for analysis can already cause aggregation. (28,29) There also is an increased potential to interact with packaging materials due to this highly sensitive character. (28) Besides the overall analysis of the samples, analysis of each individual particle is necessary. Being able to distinguish monomers, aggregates, air bubbles, silicon droplets, and other impurities from each other is crucial. Yet there is no technique that can detect and distinguish all the mentioned above. (28,29) For that reason several analytical techniques were used in this study.

Techniques that can measure over the full-size range were not used in this study because of their absence in the accessible laboratories. Dynamic Light Scattering (DLS) was one of the techniques used. With DLS results in the size range of 1 nm to 1 µm of the overall sample could be obtained. Nanoparticle Tracking Analysis (NTA) is a similar technique that can additionally visualise the particles individually. Therefore, a particle count or concentration can be acquired. Because of the challenging use of NTA, it did not replace DLS but was an adjunct to DLS. Additionally, Size Exclusion Chromatography (SEC) was used, because of its separating properties that provide extra information. SEC can also be used in the nanometer size range. Techniques measuring in the nanometer range are suitable for monomers and small aggregates as a mAb fragment has a diameter of approximately 10 nm. (28) For

measurements in the >1 µm range Micro Flow Imaging (MFI) was used that also visualises each individual particle. All in all, DLS, NTA, SEC, and MFI were used to get a global overview of the realistic particle formation and particle size.

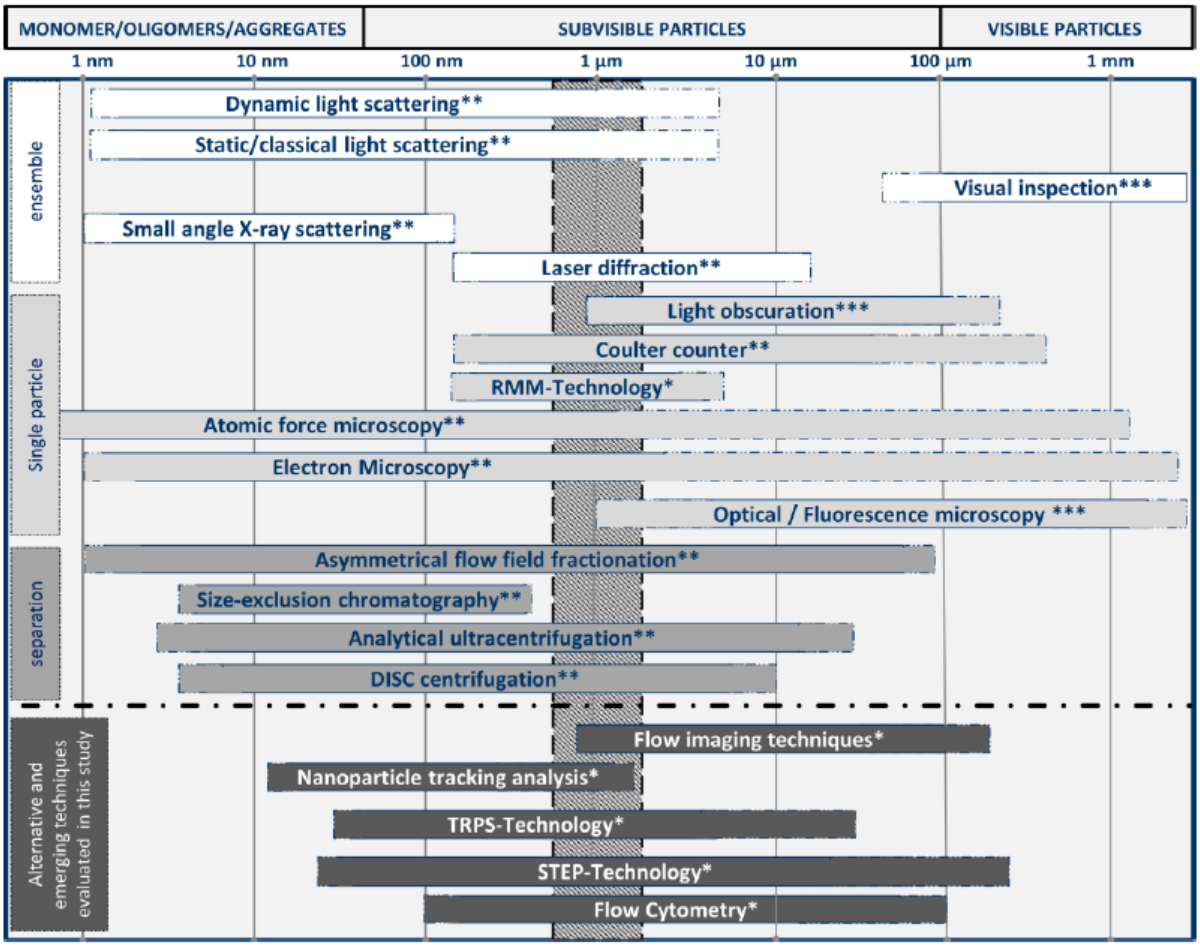

*Figure 1: Overview of particle detecting techniques. Techniques are divided into groups according to their characteristics of measurements. The applicable size range is illustrated on a logarithmic scale. The grey bar shows the detection gap for which most techniques are not suitable. * Emerging technique; ** established and often used techniques for pharmaceutical applications; *** techniques mentioned in US pharmacopeia. RMM = Resonant Mass Measurement, DISC = Disc based centrifugation, TRPS = Tunable Resistive Pulse Sensing, STEP = Space- and Time-resolved Extinction Profile.* (28)

DLS

Dynamic light scattering is a technique in which the Brownian motion of particles is followed over time by detecting fluctuations in scattered light. With this information the diffusion coefficient is obtained. Using the Stokes-Einstein equation the overall hydrodynamic size (z-average) can be calculated. An assumption here is that the particles are spherical and not interacting with each other. With DLS particles in the size range of 1 nm to 10 µm can be measured. (28,29)

A disadvantage of DLS is that average properties of all particles are being calculated. This means that testing highly polydisperse suspensions could lead to incomplete or incorrect rapports of the particle population and particle properties. The polydispersity index (PdI) gives information about the chance of biased results. A high PdI indicates a high variety in particles and thus unreliable results. Another flaw is the bias of the size distribution by intensity at larger sizes. The reason for that is the Rayleigh

approximation in which the scattering intensity depends on the diameter to the power of six. For that reason DLS is mostly suitable for measurements in the nanometer range and less suitable in the micrometer range. (28)

NTA

Nanoparticle tracking analysis (NTA) is a technique that has many similarities with DLS. As for DLS the analysis relies on light scattering and is observed by the Brownian motion of the particles. A charge coupled device (CCD) tracks the particles when the light scattering centres move. In this way the particles are made visible individually which leads to extra insights. The diffusion coefficient, mean and mode hydrodynamic size, particle concentration, and parameters as D10, D50, D90 that describe the PSD in more detail can be obtained with NTA. On top of the extra data that NTA compared to DLS gives, there are more advantages. NTA has most probably better peak resolutions than DLS. Particles with only 1.5-fold differences can be distinguished with NTA. For DLS distinction can take place from 2-3-fold differentiating particles. Besides, the visualisation of the particles provides extra information as the degree of heterogeneity and can exclude particles in the micrometer range, without disturbing the analysis. (28,29) Also, recently Pol et al. found a new application for NTA. Looking at the refractive index of different particle populations may help to differentiate single nanoparticle populations by both scattering intensity and size. The scattering intensity can provide information about shape and composition. (28)

Although this technique might seem to be the better version of DLS, it also has shortcomings. The main drawback is the need of a skilled operator. Unskilled operators could lead to unreliable results. The technique has high user to user variability and is therefore not easy reproducible. Another lack is the detection of particle concentration. To get reliable results the concentration of the sample should be within 10^7 and 10^9 particles/ml. This means that most samples need to be diluted, which can lead to extra stress on the samples and thus changes in sample properties. (28,29) Besides that, particles with a low refractive index as protein particles in water are difficult to detect due to the weak light scatter they have. (28) Because of this low refractive index, most protein particles are detectable from 40-50 nm, which means that most monomers (size about 10 nm) are not detected. (28,29)

SEC

In size exclusion chromatography (SEC) particles are separated on their hydrodynamic volume. The technique is comparable with HPLC but has a few differences. As for HPLC there is a mobile and a stationary phase. The stationary phase is in SEC a column with beads. Smaller particles can pass through small pores in the beads, but bigger ones cannot. For that reason, bigger particles such as small aggregates will have a lower retention time than smaller particles like monomers. Big aggregates and other impurities are removed by the precolumn to prevent blockage of the column. The amount of aggregation is indirectly estimated by the fraction of aggregates and protein particles as a loss in the total peak area. Different kind of detectors, such as light scattering detectors, UV detectors, and fluorescence detectors can be used to estimate the molecular weight of the particles. Although the drawbacks as sample dilution and thus possible changes of protein properties or hydrophobic proteins sticking to the column, SEC is a robust and highly sensitive analytical technique that only uses a little sample volume. (28,29)

MFI

In micro-flow imaging (MFI) the sample fluid passes through a flow cell of 80-400 µm depth. In this flow cell, the sample is illuminated by a bright light and visualized by a CCD camera. Each individual particle is captured in a picture. MFI software automatically generates the data of all particle sizes, shapes, and contrasts. Particles of approximately 1 to 400 µm can be detected, which provides extra

information on the protein therapeutics compared to DLS or NTA. Special software analyses the particle database of the sample. Information as particle count, concentrations (up to about 1,200,000 particles/ml), and characteristic distributions, such as number-weighted PSD can be generated. Because individual pictures are captured, silicone oil droplets, air bubbles, and aggregates in the range above 10 µm can be distinguished. MFI is capable of detecting aggregates and particle formation earlier than SEC. (28,29)

Although the technique provides valuable information, it also has some drawbacks. First, proteins can stick to the glass of the flow cell. This combined with the fact that only a small portion of the sample is measured, can lead to unrepresentative results. Also, MFI has a detection maximum for particle concentration. Therefore, dilution of some samples might be required, which again could lead to changes in characteristics of the particles. Although the fact that MFI can detect much more particles compared to other techniques, detecting transparent particles is challenging. Nevertheless, MFI still provides extra valuable information compared to the other techniques used. (28,29)

Materials and methods

Monoclonal antibodies

For this experiment infliximab (IgG1) and pembrolizumab (IgG4) were used. (30,31) These mAbs were chosen, because leftover reconstituted flacons of these were available via the Amsterdam University Medical Centre (Amsterdam UMC). Leftover Remsima concentrate (infliximab, 10 mg/ml) was available at the AMC location and leftover Keytruda concentrate (pembrolizumab, 25 mg/ml) was available at the VUmc location of the hospital. At both locations the mAbs were stored at room temperature for several days up to 2 months before picking them up. After picking them up, they were either stored in the refrigerator or stored at room temperature for several days to 2 months. To save space, both mAbs were eventually pooled in syringes of 5 ml or 10 ml (Nipro, luer lock) and stored in the refrigerator.

Sample preparation

To avoid a lack of time for analysis of the samples, the experiments were caried out over 2 days. On the first day, experiments were conducted with infliximab and on the second day experiments with pembrolizumab.

Before adding infliximab or pembrolizumab to infusion bags, the mAb concentrates were filtered with a non-sterile 0.2 µm PES filter (Phenomenex, phenex) to remove large aggregates. A PES filter was chosen because of its relatively low protein binding profile and thus little loss of sample. (32) For infliximab, the filter with a needle attached on it, was directly put on the syringe with pooled infliximab concentrate. When the filter was moistened with the concentrate, 5 ml of infliximab was added to a 50 ml 0.9% NaCl infusion bag (B.Braun Ecobag) to get a low therapeutic concentration of 0.91 mg/ml. (30) These infusion bags were made of polyethylene (PE-LLD). (33) To prevent spillage of concentrate and to save filters, the same moistened filter and connected needle were used for preparing 3 infusion bags. For proper distribution of the protein, the infusion bags were gently mixed afterwards.

Nearly the same actions were performed for pembrolizumab. Instead of 5 ml, only 2 ml of pembrolizumab was added to a 50 ml 0.9% NaCl infusion bag (Baxter Viaflo) to obtain a slightly subtherapeutic concentration of 0.96 mg/ml. (31) The infusion bags used for pembrolizumab were different than the ones used for infliximab due to long delivery times. These bags were made of polyolefin/polyamide plastic. (34) The pooled pembrolizumab was collected in 10 ml syringes (Nipro, luer lock) and therefore not directly usable for injecting pembrolizumab into the infusion bag. This is because 2 ml cannot accurately be added from a 10 ml syringe. The pooled pembrolizumab was

therefore first transferred to a smaller 3 ml syringe (Nipro, luer lock) with a connector. The filter and needle were placed on this smaller syringe and first moistened with pembrolizumab concentrate. The same filter and needle were again used for preparing 3 infusion bags with pembrolizumab.

Stress factor exposure

The infusion bags were divided over 4 experimental groups: unstressed, light, shocks, and light combined with shocks. On the first experimental day each experimental group consisted of 3 infusion bags with infliximab and 2 in fusion bags with 0.9% NaCl for control purposes. On the second experimental day each group consisted of 3 infusion bags with pembrolizumab and 2 infusion bags with 0.9% NaCl.

Infusion bags in the unstressed group were prepared as described above and directly measured afterwards. Infusion bags in the light exposure group were placed in a sunny area for 15 minutes. They were accompanied by a MSR165 data logger (MSR universal data loggers) to measure light intensity and temperature. Infusion bags in the shocks exposure group were wrapped in aluminium foil and hung up on an IV stand that was borrowed from the Tergooi Medical Centre (fig. 2a). Shocks were registered with a MSR165 data logger that was attached on an extra non-sample infusion bag that also hung on the IV stand. The threshold of the logger was set at 1.5 g and the measurement rate at 800 Hz. A 15-minute walk was executed to apply shocks. Infusion bags in the combined stress factor group were stressed in a similar way as in the shocks exposure group except for them not being wrapped in aluminium foil (fig. 2b). Also, a second logger was used and placed on top of the IV stand to measure light exposure and temperature.

Figure 2: a. Samples for the shocks expsore experiments placed on the IV stand. b. Samples for the light + shocks exposure experiments placed on the IV stand.

Measuring samples

The infusion bags were gently mixed before samples were taken from them. An opening was made in the infusion bag by a needle placed on a syringe. The syringe was carefully removed, and the needle remained in the infusion bag. Via the needle tubes were filled with samples. Samples were gently resuspended with a pipet directly before analysis with DLS, NTA, SEC, and MFI.

DLS

A Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) was used for the DLS measurements. 1 ml of each sample was put in a 10-mm path length disposable polystyrene cuvette (Brand, Wertheim, Germany). A laser of 633 nm at an angle of 173° was used. The equilibration time to 25°C was set to 0 seconds. The automatic mode was used for the attenuator settings and the measurement duration.

There were 3 measurements per sample with a varying number of runs per measurement. The Malvern Software version 7.13 (Malvern Instruments, Malvern, UK) obtained the Z-average and polydispersity index (PDI) with which the statistical analysis was executed.

NTA

Measurements were performed with a NanoSight LM20 (Malvern Instruments, Malvern, UK). A laser of 640 nm was used at an angle of 173° to the flow cell. To focus the device a diluted 300 nm (296 +/- 6nm) Nanosphere™ bead standard (Thermo Scientific, Fremont, USA) was used. The samples were automatically injected from a 1 ml syringe (BD Discardit II) to the device. Therefore, an automatic pump was used (Harvard Apparatus, catalog no. 98-4362, Holliston, USA) that was connected to the device by the NTA stage comms software. The camera shutter was set at 1500 and the camera gain at 680. A 30 second video was captured for each sample. Videos were analyzed by Nanosight NTA 2.3 Build 0006 BETA2 software (Malvern Panalytical, Malvern, UK).

SEC

A Waters Alliance e2695 HPLC (Waters Corporation, Manchester, UK) combined with a Waters 2998 Photodiode Array Detector and a Waters 474 Scanning Fluorescence Detector was used for the analysis. The pre-column used was the Phenomenex security guard cartridge GFC-2000 4 x 3.0 mm. The used column was the Phenomenex BioSep-SEC S3000 300 x 7.8 mm 5 micron (p/nl 00H-2146kK0, S/nl H19-172339, B/no 6583-0318). The mobile phase was a solution of 0.9% NaCl dissolved in ultrapure water (obtained from a Milli-Q Advantage A10 Water Purification System, Merck, Darmstadt, Germany). Bovine serum albumin (Sigma-Aldrich, St. Louis, USA) was diluted to a concentration of 1 mg/ml with ultrapure water and used as a standard. Vials for SEC were filled with 1 ml of every sample, but only 10 µl was analysed. During analysis the flow was set at 1 ml/min and the UV absorbance was measured at 280 nm. For sample type, 'inject samples' was chosen. Fluorescence was measured between 280 nm and 340 nm. Data was analysed with Empower pro software (Empower 2 software 2005, Waters Corporation, Manchester, UK). Statistics were run on the ratio of the area of loss of monomer divided by the area of remaining monomer.

MFI

An MFI 5200 device (ProteinSimple, Santa Clara, CA, USA) was used to analyse the samples. The results were made visible with MFI View System Software version 5.1.1.104 (ProteinSimple, San Jose, USA). Before using the device, it was cleaned with 4 ml of a 2% hellmanex II solution (Hellma, Müllheim, Germany) and after that with 4-6 ml ultrapure water (obtained from a Milli-Q Advantage A10 Water Purification System, Merck, Darmstadt, Germany). To make sure that the 100 µm silane coated flow cell of the device was cleaned well, a test with ultrapure water as sample was performed. The particle count needed to be <400 particles/ml for this test. Before every sample measurement, the system was flushed with 3 ml and primed with 1 ml of the sample and the 'optimize illumination' procedure of the device was performed. In total 500 µl of each sample was analysed. Results were analysed with MFI Image Analysis version 1.1.0.24 (ProteinSimple, San Jose, USA). The obtained particle concentrations were compared between the 4 experimental groups. Additionally, some filters were applied, and the data was analysed again. Edge, stuck, and slowly moving particles were removed. To make sure that air bubbles and possible silicone oil droplets were removed, particles with a circularity and aspect ratio of ≥ 0.9 were excluded. The ECD was set at ≥ 5 µm, because of low accuracy of the filter below 5 µm, hence the data ≥ 5 µm with and without filter were compared to each other. (35,36)

Statistics

To statistically analyse the data a one-way ANOVA or Brown-Forsythe test was performed in SPSS, depending on the result of the Levene's test for equality of variances. Tukey HSD post-hoc test was

used if the ANOVA gave a significant result. For a significant result for the Brown-Forsythe test, the Games-Howell post-hoc test was used.

Results

Exposure to stress

On the first experimental day, experiments with infliximab were conducted. The light exposure group had an estimated mean exposure of 20,800 lux for 15 minutes, see supplementary material (Fig. S1A). For the shocks exposure group the sum of the intensity over threshold (IoT) of the 10 most important shock events was 18,957.97. The estimated mean of light exposure was 32,000 lux for 15 minutes for the light and shocks exposure group (Fig. S1B). For this group the sum of the IoT of the 10 most important shock events was 7,905.42.

On the second experimental day, experiments with pembrolizumab were conducted. The light exposure group had an estimated mean exposure of 38,500 lux for 15 minutes (Fig S1C). For the shocks exposure group the sum of the IoT of the 10 most important shock events was 59,860.62. The estimated mean of light exposure was 50,000 lux for 15 minutes for the light and shocks exposure group (Fig S1D). For this group the sum of the IoT of the 10 most important shock events was 41,762.91.

For all experiments the maximum temperature measured did not exceed 36 °C.

DLS

The tests of homogeneity of variances were for both Z-average and PdI and for both infliximab and pembrolizumab significant, so the Brown-Forsythe test was conducted for the experiments. There were no significant differences between groups for the Z-average of infliximab ($F(3, 3.944) = 1.914$, $p = 0.27$), the PdI of infliximab ($F(3, 4.268) = 0.763$, $p = 0.568$), the Z-average of pembrolizumab ($F(3, 2.652) = 2.440$, $p = 0.259$, and the PdI of pembrolizumab ($F(3, 2.930) = 5.961$, $p = 0.091$) (Fig. 3 & 4 and S2 of the supplementary material).

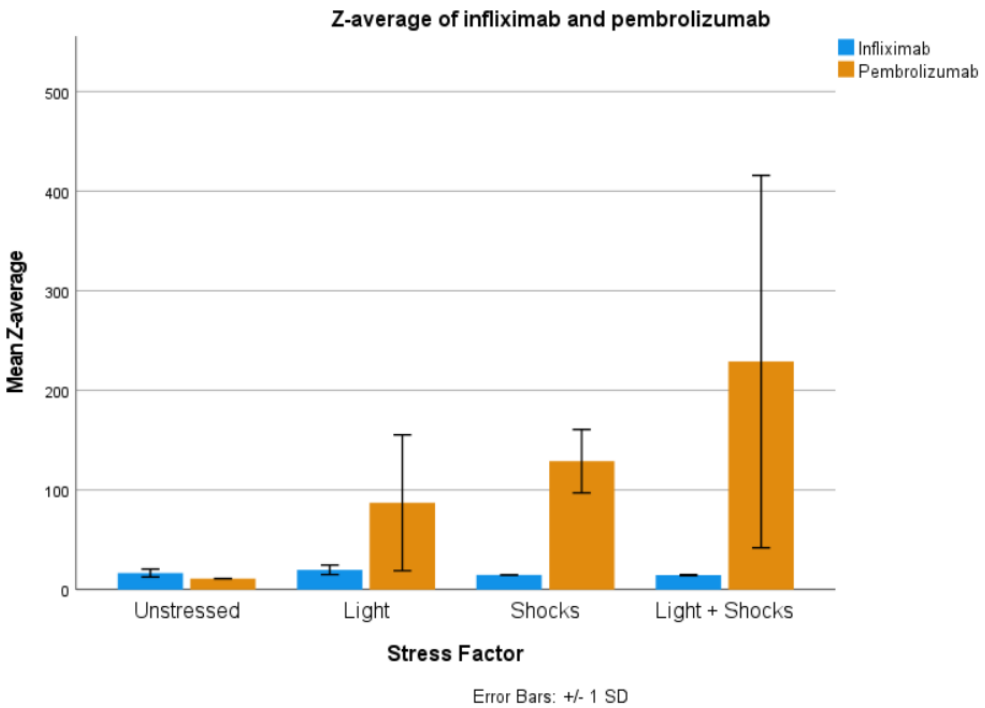

Figure 3: Mean Z-average of infliximab and pembrolizumab exposed to different stress factors. No significant effect was found.

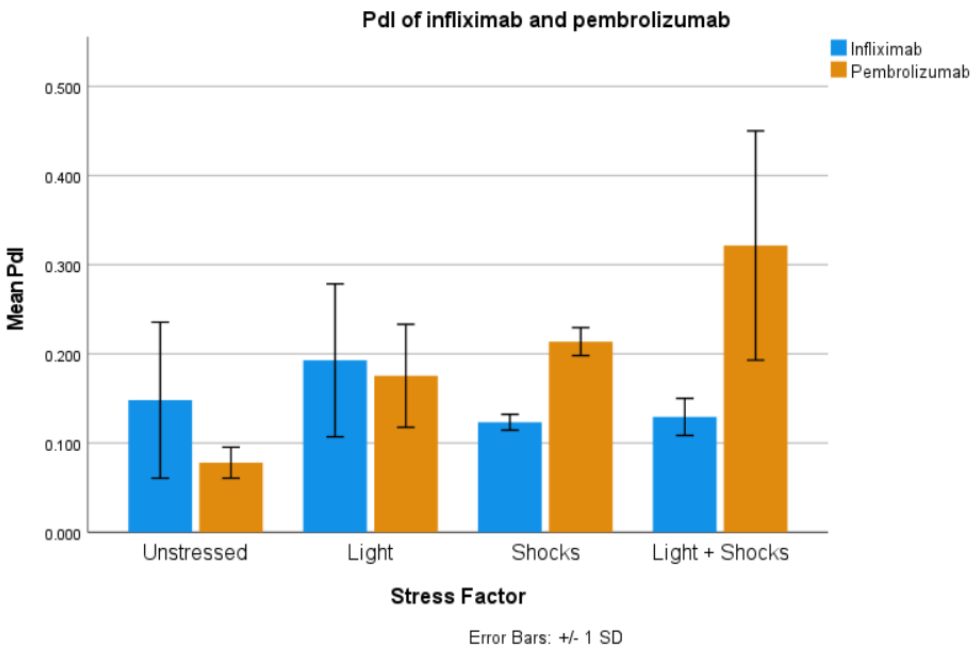

Figure 4: Mean PdI of infliximab and pembrolizumab exposed to different stress factors. No significant effect was found.

NTA

No reliable results were obtained from NTA, because the operator was not skilled enough.

SEC

For infliximab 3 peaks were detected with SEC. The peak at approximately 8.8 minutes was the monomer peak. The peak at 7.4 minutes was seen as loss of monomer (or potential aggregates). The last peak at about 13.4 minutes was also seen at all NaCl samples (data not shown) and was therefore not included in the loss of monomer/monomer ratio (Fig. S3).

For pembrolizumab a couple of peaks were detected with SEC at around 9.1, 10.4, 10.9 and 12.2 minutes. The monomer peak was at 12.2 minutes. The other peaks were considered as loss of monomer (or potential aggregates) (Fig. S4).

For both infliximab and pembrolizumab the test of homogeneity of variances was not significant. For infliximab the ANOVA detected no significant differences between any of the groups ($F(3, 8) = 3.326$, $p = 0.077$) (Fig. 5).The ANOVA for pembrolizumab found significant differences between some of the groups ($F(3, 8) = 16.205$, $p < 0.001$). In the Tukey HSD post-hoc analysis the following significant differences between groups were found: the unstressed and light ($p = 0.007$), unstressed and light + shocks group ($p = 0.013$), the light and shocks group ($p = 0.002$), and the shocks and light + shocks group ($p = 0.004$) (Fig. 6).

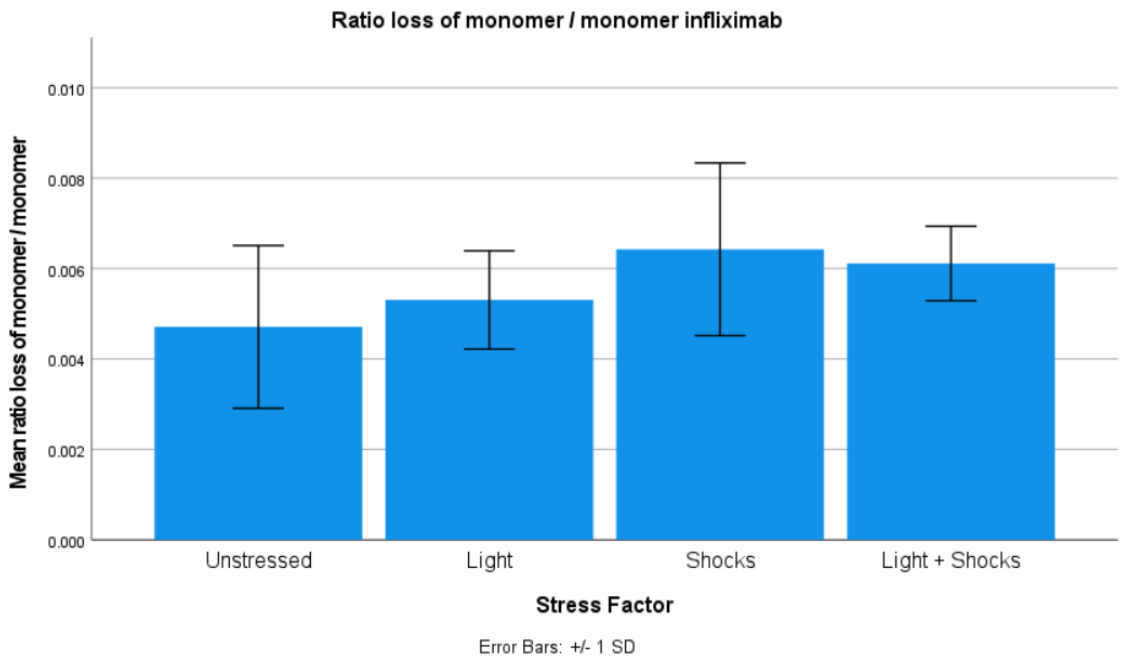

Figure 5: Mean ratio of loss of monomer area divided by monomer area of infliximab obtained by SEC. No significant results were found.

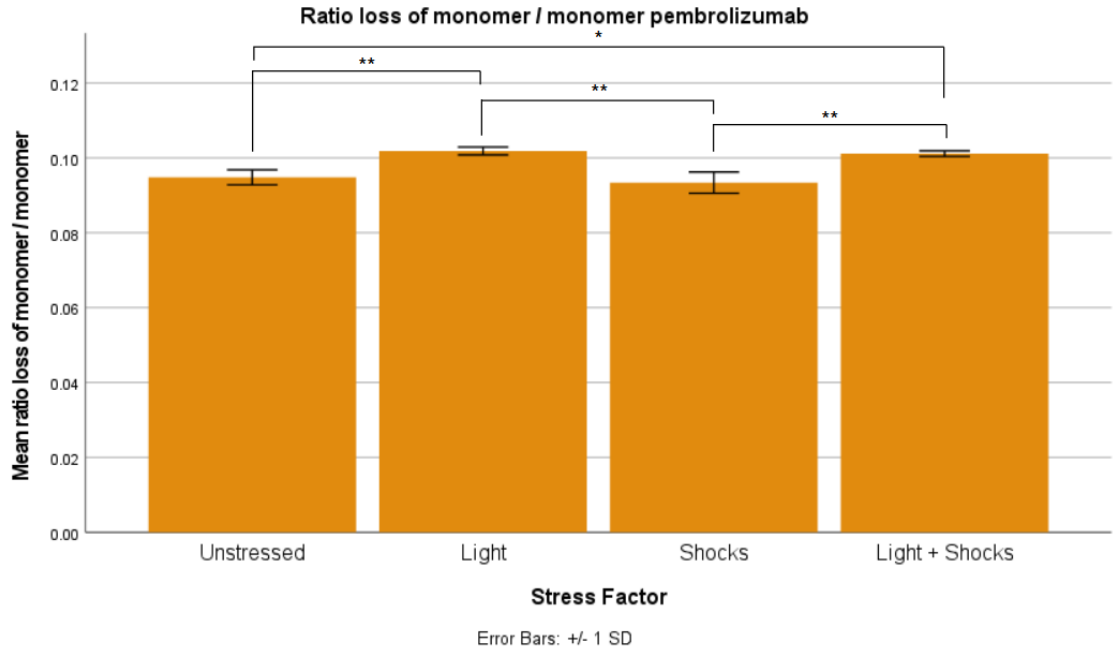

*Figure 6: Mean ratio of loss of monomer area divided by monomer area of pembrolizumab obtained by SEC. *: $p < 0.05$, **: $p < 0.01$*

MFI

Total particle concentration was compared between the 4 experimental groups for infliximab. The test of homogeneity of variances was significant ($p = 0.007$). The result of the Brown-Forsythe test comparing the experimental results for infliximab was non-significant ($F(3, 2.356) = 5.509$, $p = 0.131$) (Fig. 7).

For pembrolizumab total particle concentration was also compared between the experimental groups. The test of homogeneity of variances was not significant (p = 0.098). There was at least one significant difference between the experimental groups according to the ANOVA (F(3. 8) = 13.928, p = 0.002). Tukey HSD post-hoc test found significant differences between the unstressed and shocks group (p = 0.002), and the unstressed and light + shocks group (p = 0.002) (Fig. 8).

A software filter was applied on data ≥ 5 µm for infliximab (fig. 9), pembrolizumab (fig. 10), and their infusion bag background. It reduced particle concentration in each group. For more detailed information about pembrolizumab see figure S5 and S6 of the supplementary material.

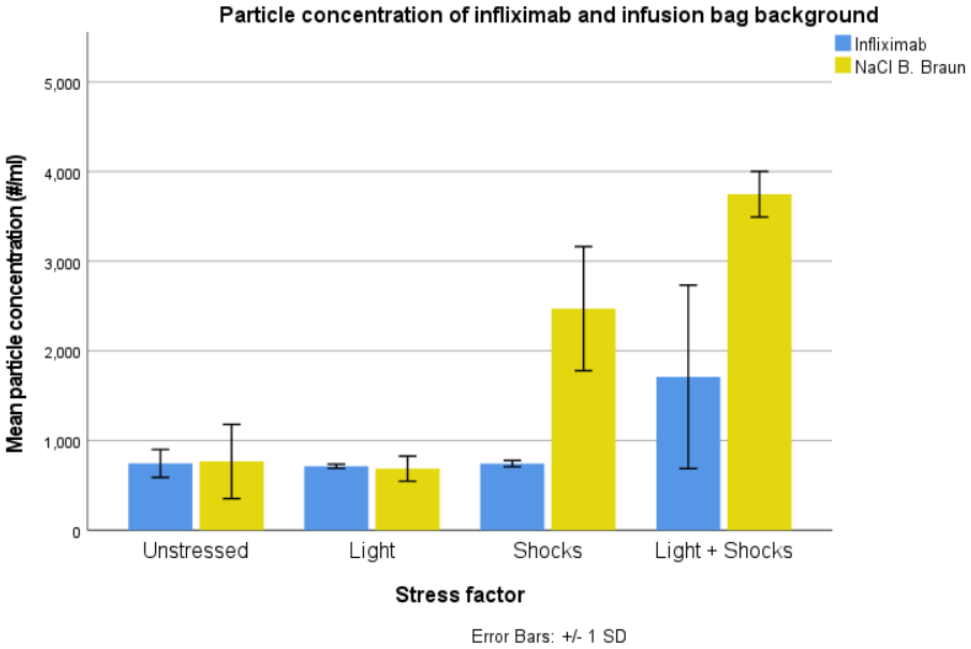

Figure 7: Comparison of total particle concentration for particles > 1 µm obtained from MFI for each experimental group for infliximab and its infusion bag background (NaCl B. Braun). No significant results were found between the infliximab groups.

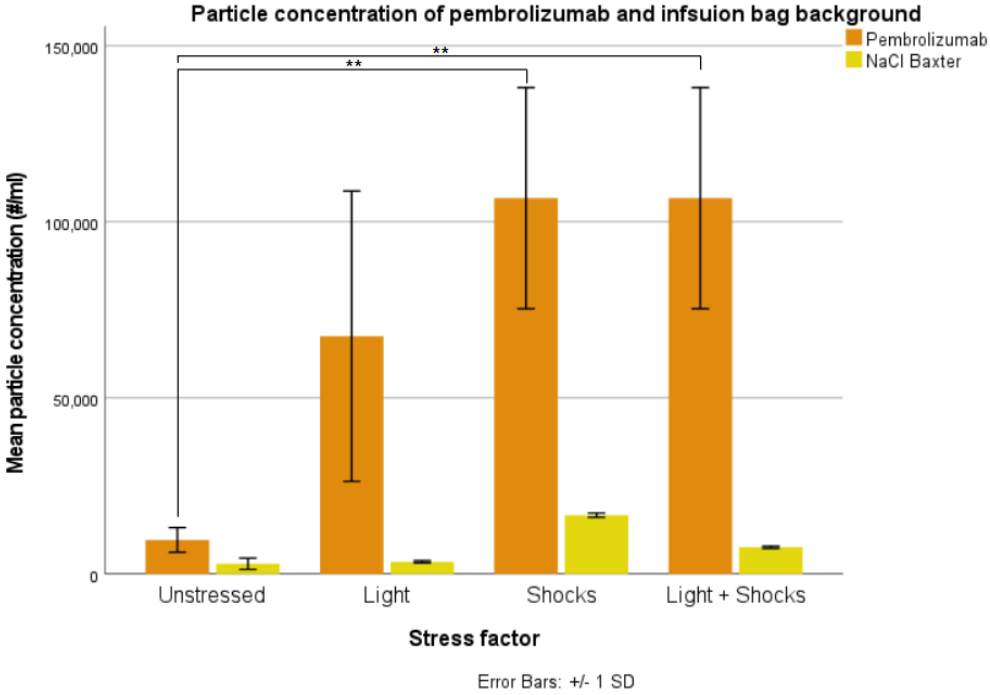

*Figure 8: Comparison of total particle concentration for particles > 1 µm obtained from MFI for each experimental group for pembrolizumab and its infusion bag background (NaCl Baxter). **: p < 0.01*

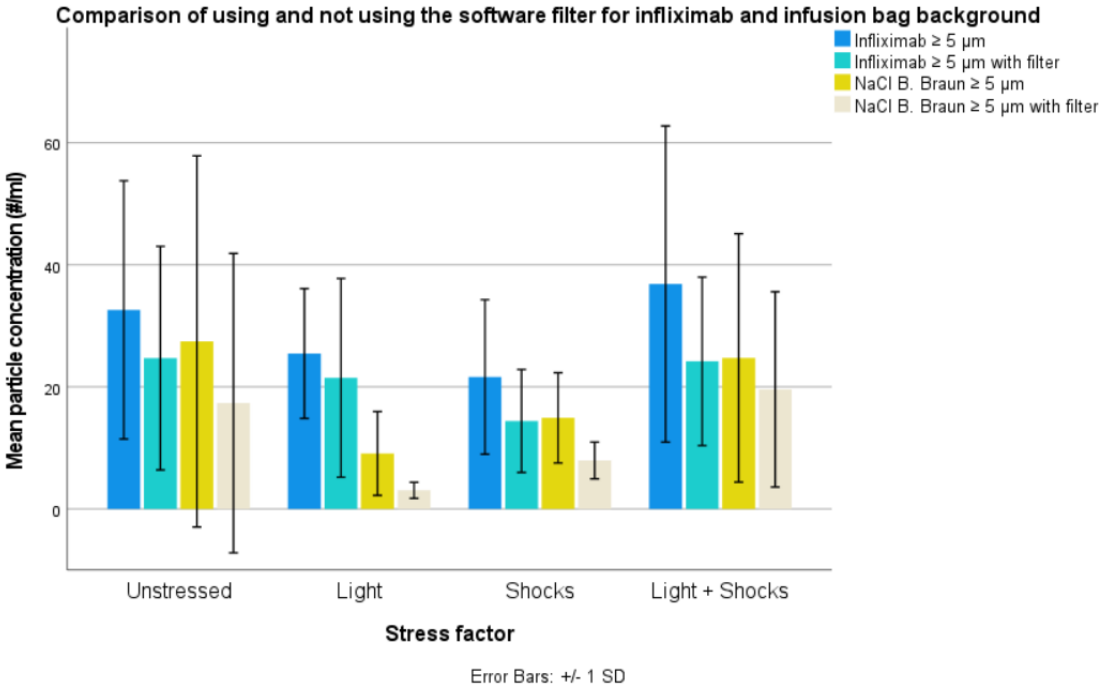

Figure 9: Comparison of applying and not applying the software filter on infliximab and infusion bag background (NaCl B. Braun) data ≥ 5 µm.

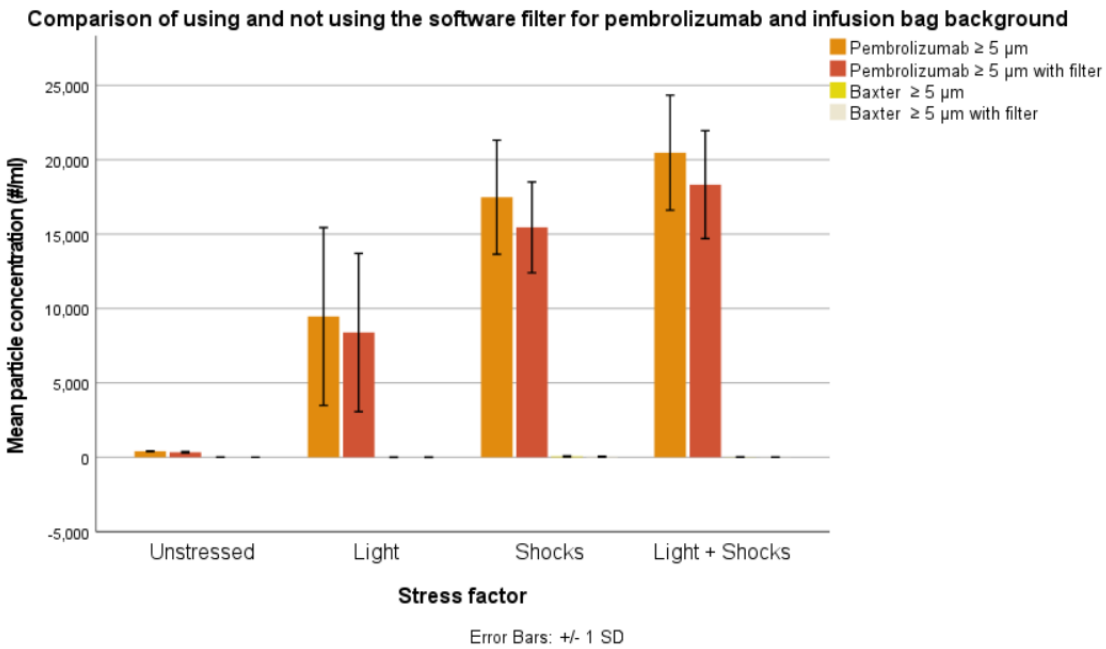

Figure 10: Comparison of applying and not applying the software filter on pembrolizumab and infusion bag background (NaCl Baxter) data ≥ 5 µm. For more detailed information see supplementary material figure S5 and S6.

Discussion

General effect of stress factors

First, it should be noticed that a change in Z-average, PdI, loss of monomer/monomer ratio and particle concentration not necessarily implies aggregation of therapeutic proteins. Changes in these parameters may also be caused by particles released from the infusion bag or air bubbles. It can also mean that dimerization or trimerization took place. However, a change in any of these parameters is considered here as a potential change in the physical stability of the proteins.

For infliximab the results of DLS, SEC, and MFI agree that there are no significant differences between the experimental groups. DLS only showed a slightly higher Z-average and PdI for the light exposure group compared to the unstressed group (fig. 3 & 4). SEC showed a little higher ratio for all stressed groups compared to the unstressed group, but it is debatable if this can be called a trend, since the largest difference in ratio was only 0.002 (fig. 5). So, the results for SEC contradict the results for DLS for the shocks and light + shocks group. MFI only showed a higher particle concentration for the light + shocks exposure group, but the NaCl background gave a higher particle concentration in this group (fig 7). So, it is likely that the effect seen here for infliximab only is because of particle release from the infusion bag or air bubble formation. Also, whenever the software filter was applied on data ≥ 5 µm the difference between the unstressed and light + shocks exposure group vanished. On top of that, particle concentration of NaCl background is lower than for infliximab when only data ≥ 5 µm is displayed (fig. 7). Thus, particles < 5 µm must be responsible for the differences seen in figure 5. Concluding, both DLS and SEC showed a not significant possible effect of light exposure on infliximab in the nanometer range and no effect was seen in the micrometer range with MFI.

For pembrolizumab the situation was different. No significant results were found for DLS, but both Z-average and PdI were raised in all experimental groups compared to the unstressed group (fig. 3 & 4). Comparing ratios for SEC, the light and light + shocks ratios differed significantly from the unstressed exposure group. The ratio for the shocks exposure group was nearly equal to the unstressed group (fig.

6). For MFI total particle concentration differed significantly for the shocks and light + shocks exposure groups compared to the unstressed group. For the shocks exposure group this contradicted the results of SEC. NaCl background was also higher for these groups than the unstressed group but did not influence the results for pembrolizumab much. Particle concentration of the light exposure group was also higher, but not significantly, than the unstressed group and background seemed to be equal to the unstressed group (fig. 8). Particle concentration decreased for both pembrolizumab and background with at least factor 5 when only data ≥ 5 µm was analysed compared to total particle concentration (fig. 8, 10 & S5). Background particle concentration was negligible compared to pembrolizumab. So, again most particles could be found in the size range < 5 µm. After applying the software filter, all exposure groups still had a higher particle concentration compared to the unstressed group (fig. 10). However, no statistics were run on these data. All in all, in the nanometer range pembrolizumab is sensitive to light and light + shocks and in the micrometer range to shocks and light + shocks and possibly also to light. Nonetheless, the possible effects found for light exposure could be an overestimation. One infusion bag in this group namely fell from a table and was therefore also exposed to a shock and another infusion bag was pierced by a needle, which could have caused extra release of particles from the infusion bag. The refined conclusion is that in the nanometer range pembrolizumab is likely sensitive to light and surely sensitive to light combined with shocks and in the micrometer range pembrolizumab is likely sensitive to light and surely sensitive to shocks and light combined with shocks.

Comparing the two therapeutic protein products used in the experiments, no general significantly proven pattern can be found in the effects of applying different stress factors. Although, both infliximab and pembrolizumab products seem to be sensitive to light in the nanometer range. In the micrometer range no similarities were found.

Clinical relevance

As mentioned before, the British and European Pharmacopoeia only specify rules for the maximum number of subvisible particles in parenteral formulations. These rules are based on the light obscuration test or microscopic particle count test. In this study, MFI was the only technique used from which a particle concentration could be obtained. It is known that MFI is more sensitive for detecting proteinaceous particles than light obscuration. (37,38) Based on figure 9 a 50 ml infusion bag with infliximab exposed to any stress factor does meet these criteria. Based on figure 10 pembrolizumab exposed to any stress factor possibly does not meet these criteria. However, the Pharmacopoeias specify rules for particles ≥ 10 µm. Based on figure S6 pembrolizumab exposed to any stress factor still does not meet the criteria. Pembrolizumab unstressed (average of 54.9 particles/ml ≥ 10 ml) did meet the criteria in a 50 ml infusion bag. Even if the sensitivity for MFI is twice as high as for light obscuration, the criteria would not be met for pembrolizumab exposed to any stress factor. So, regarding the requirements for subvisible particles for parental formulations of the Pharmacopoeias, infliximab in a 50 ml infusion bag could still be administered to patients and pembrolizumab in a 50 ml infusion bag not.

Even though infliximab in a 50 ml infusion bag fulfils the criteria of the Pharmacopoeias, much more should be investigated before administering it to patients. For example, the biological activity of the protein products was not investigated after exposure to stress. Also, most particles were found in the <5 µm range for both protein products. These particles are not considered when only the requirements of the Pharmacopoeias are met and the immunological effect of them is still unknown. Additionally, in this experiment the proteins were put in 50 ml infusion bags. In practice, it is possible that proteins are solved in infusion bags of larger volumes which may cause a raise in particle concentration.

Requirements of the Pharmacopoeias could in that case not be met. So, further investigation is necessary.

Strengths and limitations

A strength of this study was the realistic study design. No laboratory set-up could have simulated reality better. However, this strength also was a limitation. For example, the experiments were conducted on different days, which resulted in different exposure to stress factors. The exposure to light for pembrolizumab was almost twice as high as for infliximab. Shock exposure also differed much according to the sum of the IoT of the 10 most important shock events, despite the same route was walked. However, it is debatable if the sum of the 10 most important shock events is a good way to express the total exposure to shocks. One walk could have less intense, but more shock events than the other. This can result in a high sum of the IoT for the last one, while the first one had more exposure.

Besides the variations due to the study design, there were more variations between the experiments. For example, the infusion bags for infliximab and pembrolizumab differed in brand and material due to slow delivery. Also, starting conditions of the therapeutic proteins differed. Infliximab was stored in the reconstituted vials at a concentration of 10 mg/ml while pembrolizumab was stored at 25 mg/ml. It could be possible that long term storage at a higher concentration automatically leads to more physical instability. This would also explain the fact that pembrolizumab showed more peaks than infliximab for SEC (fig. S3 & S4). So, it may be possible that pembrolizumab was more contaminated at the beginning than infliximab. This possible limitation is caused by another limitation of this study, namely that leftover reconstituted mAbs were used. The physical stability could therefore already be affected in advance and differ between batches. Also, the leftover material was not completely pooled before adding it to the infusion bags. This could have led to an unequal distribution of the particles present before applying stress on it. If new flacons with mAbs were used, this would not have been an issue.

Future directions

To be able to say something about the effect of light in combination with shocks on the physical stability of monoclonal antibodies more than 2 monoclonal antibodies should be investigated. The mAbs used in this experiment differed from each other in for example IgG type and iso-electrical point, which could have led to different results. Therefore, more mAbs should be investigated before a general conclusion can be drawn for all mAbs.

As mentioned before the most immunogenic particles are in the size range of 0.1 – 1.0 µm. (23,25) The techniques used in this experiment do have a detection gap at around this critical 1 µm. In future experiments techniques that can measure around 1 µm must be included. A possible technique for this could be flow cytometry. Also, techniques used in this study were not fully able to distinguish between proteinaceous particles and other materials. It would be of great value if techniques could do this in the future.

Conclusion

Regardless of whether the criteria of the Pharmacopoeia are met, this study was not elaborated enough to conclude if infliximab and pembrolizumab can still be administered to patients. However, based on this study, it seems that infliximab and pembrolizumab are both potentially sensitive to light in the nanometer range. To say something about the effect of light in combination with shocks on the physical stability of all mAbs more research is necessary. So, the effect of light in combination with shocks on the physical stability of mAbs remains unknown.

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Supplementary material

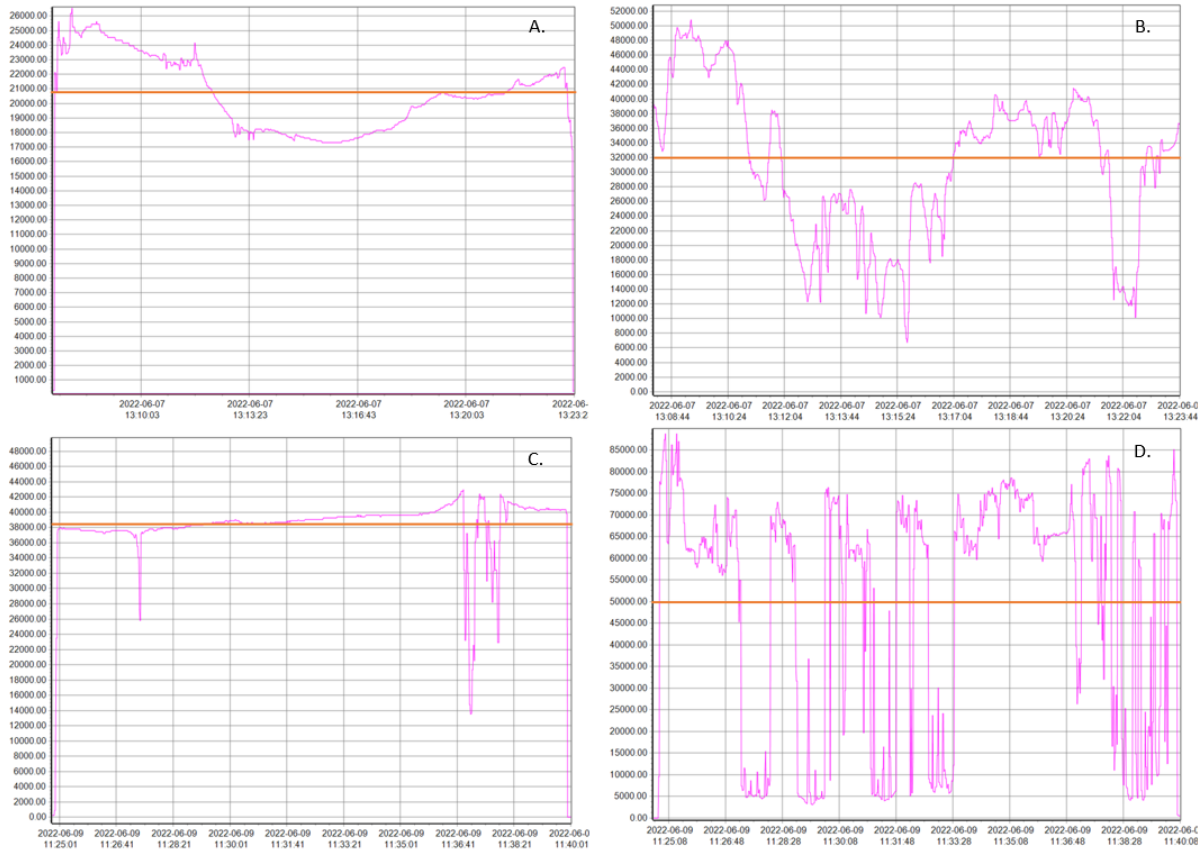

Figure S1: Estimated light intensity in lux (orange line) measured during the experiments. A: Infliximab light only exposure group. B: Infliximab light + shocks exposure group. C: Pembrolizumab light only exposure group. D: Pembrolizumab light + shocks exposure group.

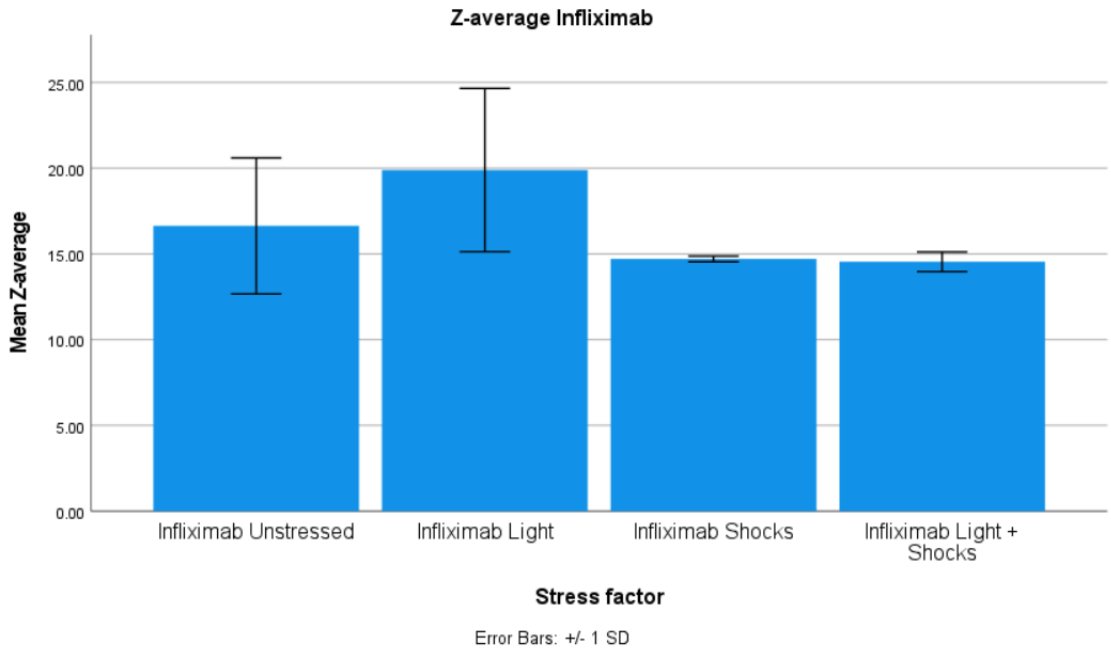

Figure 2S: Mean Z-average of infliximab exposed to different stress factors.

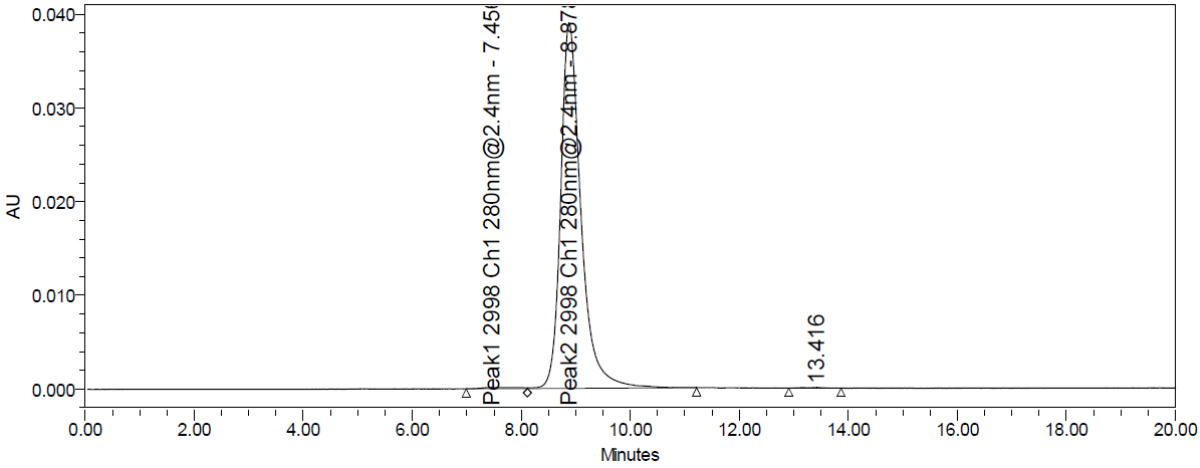

Figure S3: An example of a chromatogram of infliximab. All samples consisted of 3 peaks around 7.4, 8.8, and 13.4 minutes.

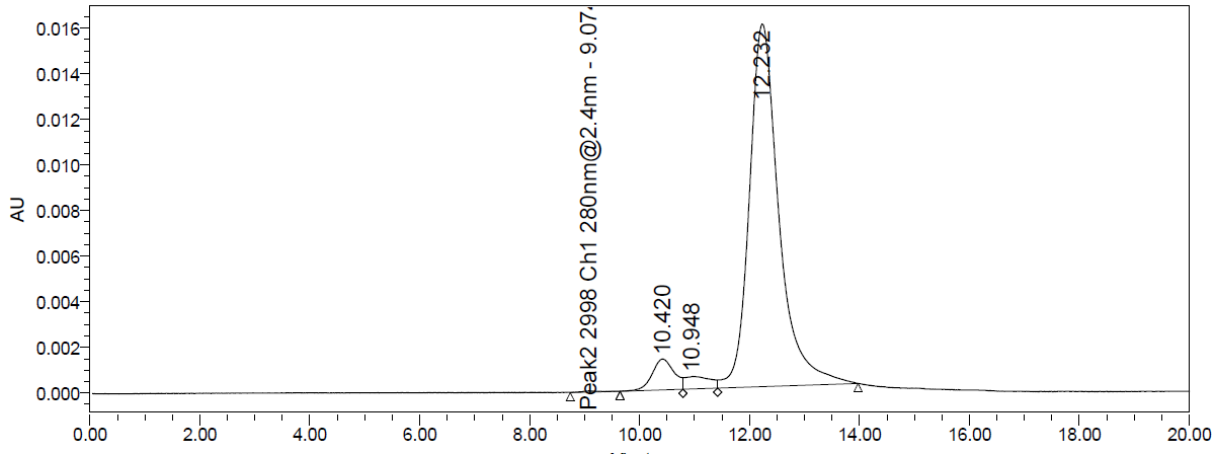

Figure S4: An example of a chromatogram of pembrolizumab. There were a couple of different peaks at around 9.1, 10.4, 10.9, and 12.2 minutes.

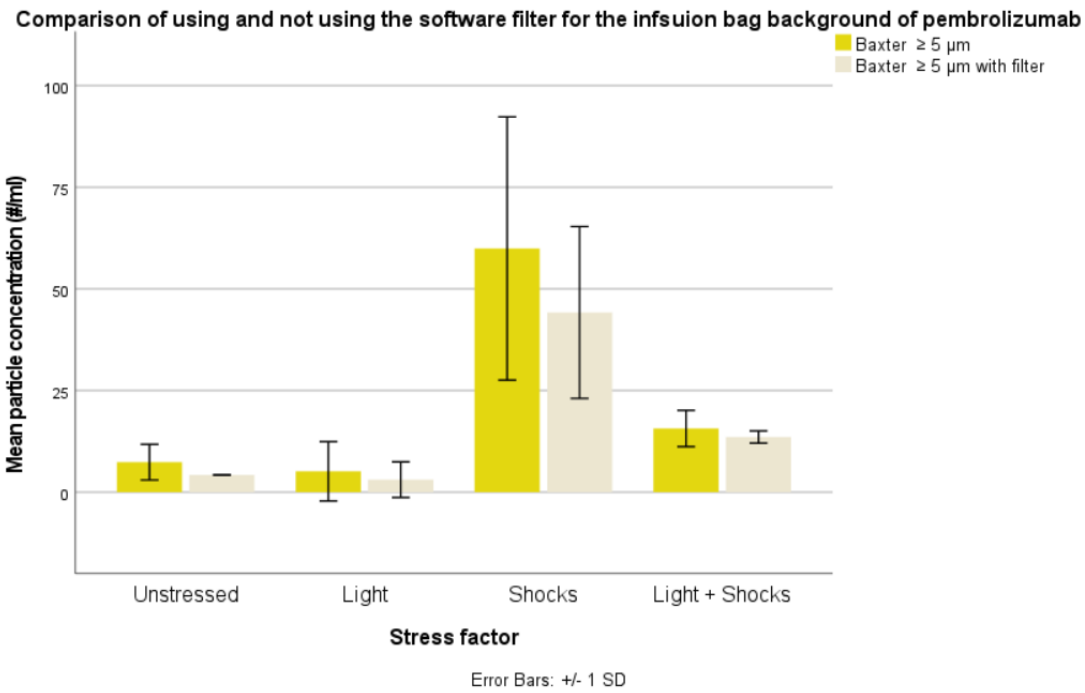

Figure S5: Comparison of applying and not applying the software filter on pembrolizumab background (NaCl B. Braun) data ≥ 5 µm.

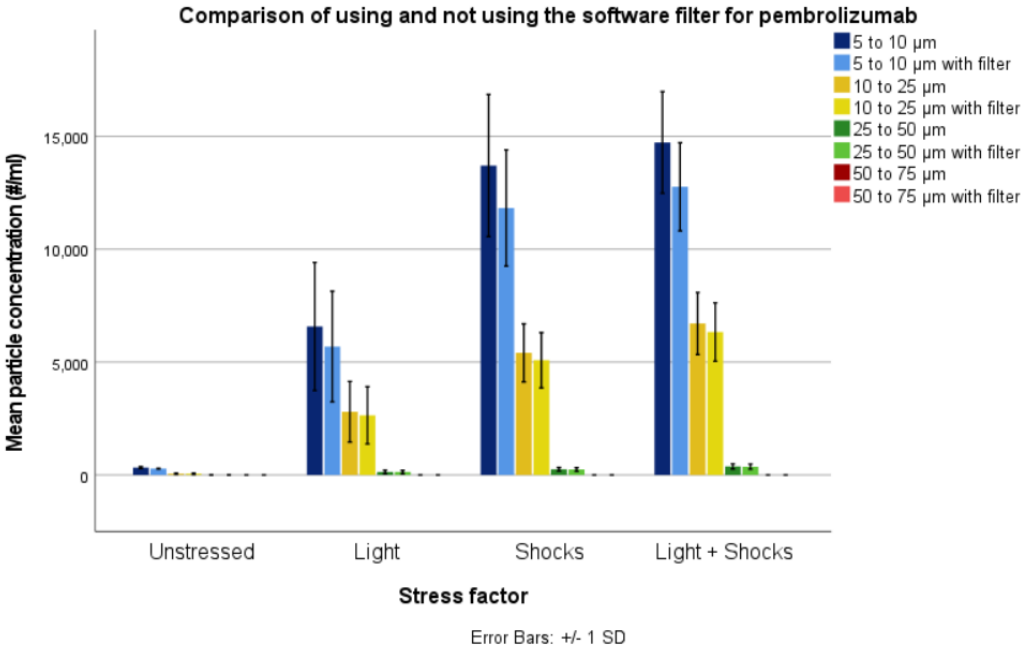

Figure S6: Comparison of applying and not applying the software filter for pembrolizumab data ≥ 5 µm.