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Novel approaches for parenteral protein formulations

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AFFIDAVIT

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At first I want to thank my supervisors Prof. Bernd Nidetzky and Prof. Andreas Zimmer to make this master thesis and all the things I learned on this way possible.

I want to dedicate this master thesis to my sister Johanna, the bravest person I know:

For many years we've shared our lives

One roof we once lived under

Sometimes we laughed, sometimes we cried

Through winter storms and thunder

The younger years have faded fast

We've gone our separate ways

But through all time our friendship lasts

Our bond in life remains

As summer brings the happy times

The autumn winds will whisper

A closer friend I'd never find

Than the one I call my Sister.

And to all the people that believe in me...

ABSTRACT

Finding the optimal protein formulation condition is still dependent on a large amount of experiments. In this study we focused on using the advantage of QbD as statistically tool for in-silico prediction and model to decrease developing time by increasing product understanding.

Accelerated stability studies are employed to test the integrity of IgG1 mAb under harsh conditions for a short period of time with the aim to extrapolate the results to real-time stability prediction. Therefore different kinds of stress – Freeze/Thaw-Cycles, thermal stress and agitation - are employed whereby different optimal formulations may exist for different stressing conditions. Based on the results of the initial screening experiments, MODDE 9.1.1 (Umetrics, Umea, Sweden) – the used DoE software – optimizes the setting for each stressing condition individually, by estimating the most robust area within the design space. The expected output should be a generic formulation optimum for the target protein, by the combination of the several optimized design spaces into a global solution.

Additionally, long time storage experiments were implemented, where pre-chosen formulations were stored under two different conditions – at 25°C with 60% relative humidity and 37°C with 40% relative humidity – for three months.

Using a variety of high- and medium throughput methods – Differential-Scanning-Fluorimetry (DSF), Micro-Flow Imaging (MFI), Nanoparticle Tracking Analysis (NTA), Size-Exclusion-Chromatography (SEC) and CD-Spectroscopy - makes it possible to analyze melting temperature, particle concentration, particle size and conformational changes of pre-stressed formulations.

ZUSAMMENFASSUNG

Um die optimal Bedingung für Proteinformulierungen zu finden bedarf es heutzutage noch einer großen Anzahl an Experimenten. In dieser Arbeit beschäftigten wir uns mit dem Einsatz von QbD als statistisches Werkzeug der in-silico Vorhersage und als Modell der Verringerung der Entwicklungsdauer durch Erhöhung des Produktverständnisses.

Es wurden zeitlich begrenzte Stabilitätsversuche am Modellprotein IgG1 mAb unter ungünstigen Bedingungen durchgeführt um erhaltene Ergebnisse weiterführend für eine Echtzeit-Stabilitäts-Vorhersage heranziehen zu können. Dafür wurden verschiedene Stressmethoden eingesetzt: Einfrier/Auftau-Zyklen, thermaler Stress und Agitation. Dabei kann davon ausgegangen werden, dass für die unterschiedlichen Stressbedingungen unterschiedliche Optima erzielt werden können.

MODDE 9.1.1 (Umetrics, Umea, Sweden), die verwendete DoE Software, optimiert die Parameter jeder Stressbedingung individuell durch die Bestimmung eines sicheren Bereiches innerhalb des Designraumes. Das gewünschte Ergebnis sollte ein generisches Formulierungsoptimum für das Zielprotein sein, erhalten durch die Kombination aller optimierter Designräume in eine globale Lösung.

Anschließend wurden noch Lagerversuche durchgeführt. Dabei wurden aus den Vorversuchen Proben gewählt, die anschließend unter zwei verschiedenen Bedinungen – bei 25°C und 60% relativer Raumfeuchte und 37°C und 40% relativer Raumfeuchte für 3 Monate gelagert wurden.

Durch den Einsatz von Hoch- und Mittel- Durchsatzverfahren wie Differential-Scanning-Fluorimetry (DSF), Micro-Flow Imaging (MFI), Nanoparticle Tracking Analysis (NTA), Size-Exclusion-Chromatography (SEC) und CD-Spectroscopy war es möglich die Schmelztemperatur, Partikelkonzentration, Partikelgröße und Konformationsänderungen der vorgestressten Formulierungen zu analysieren.

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LIST OF ABBREVIATIONS

IgG	Immunoglobulin G
mAb	monoclonal antibody
CP-buffer	citrate-phosphate buffer
T80	Tween 80
T20	Tween 20
CR-EL	Crempohor EL
PTS	Polyoxyethanyl α-tocopheryl sebacate
CCVJ	9-(2-carboxy-2-cyanovinyl)julolidine
F/T	Freeze/Thaw
MT	Melting temperature
DSF	Differential-Scanning-Fluorimetry
MFI	Micro-Flow Imaging
NTA	Nanoparticle Tracking Analysis
SEC	Size-Exclusion-Chromatography
CD	circular dichroism
QbD	Quality by Design
DoE	Design of Experiments

1 INTRODUCTION

The advent of biologics has revolutionized the pharmaceutical market over the last decades. In 1982 Insulin was the first recombinantly produced biological product to gain approval by the FDA. Since then huge progress has been made in the understanding of pharmaceutically active proteins, but there remain a lot of challenges upon bringing protein-based drugs to the market.

Therapeutic and diagnostic proteins - especially monoclonal antibodies - have become the fastest growing area of biopharmaceutical applications. A total of 21 products are approved in the US and over 200 mAb candidates are currently undergoing clinical studies (state by 2008). Due to the high numbers of global sales, pharmaceutical companies are actively investing in programs dedicated to research and development of therapeutic mAbs [1].

In contrast to traditional small-molecule drugs, proteins are subject to several pathways of destabilization. Protein degradation is generally categorized in physical and chemical processes.

Many chemical reactions like deamidation, oxidation, hydrolysis, rearrangement of disulfide bonds, isomerisation, covalent cross-linking, deglycosylation, etc are responsible for destabilization of protein drugs [2].

The physical degradation mostly cause irreversible aggregate and particle formation, due to physicochemical effects including conformational changes, covalent cross-linking, and non-covalent protein-protein interactions.

Among formulation variables available to minimize protein degradation, optimizing solution pH, ionic strength, and adding pharmaceutical excipients can influence the rate and extent of antibody aggregate formation.

The physical integrity of a protein is defined via its secondary, tertiary and quarternary structure eventually leading to native or non-native protein aggregates. The immunogenic potential of aggregates has been an emerging topic over the last years and therefore needs to be evaluated carefully [3].

Formulation development must ensure that macromolecules remain in their native state both during production, transport and long-term storage. And each production step contains all possibilities of protein damage.

Pharmaceutical companies apply a lot attention to fast and safe formulation development procedures. As a result, there is the need for forced degradation studies to assess potential stress factors affecting product quality and efficacy and the protective effect of different formulations. In drug product development forced degradation studies are commonly used to assess robust formulations in a relatively short amount of time [4].

One important stress factor a protein can encounter - during specific production processes, storage, and shipment e.g. – is elevated temperature.
Thermal stress is therefore a standard procedure for testing of molecule candidates and for formulation screening. The proteins temperature is increased toward the melting temperature (T_m), leading to physical and chemical destabilization of the native protein structure. A suitable temperature for thermal stress testing needs to be established based on structural knowledge of the protein. For a monoclonal antibody – as used in this study – it is important to consider, that Fab and Fc fragments have been shown to denature independent with T_m values above 80°C [4]. For that it is necessary to operate at higher temperatures at with smaller proteins.
The commonly used method for thermostability studies is differential scanning calorimetry (DSC). DSC measures the change of the difference in the heat flow rate.
Differential scanning fluorimetry (DSF), as used in this study, is superior to DSC with respect to throughput. DSF monitors thermal unfolding of proteins in the presence of a fluorescent dye and is typically performed by using a real-time PCR instrument [5].

Another common forced degradation study makes use of Freeze/Thaw-Cycles.
During freezing and thawing, proteins are exposed to a combination of stress factors, including interfacial stresses, temperature fluctuations, cryoconcentration, crystallization of excipients, phase separation and pH shifts. There are no commonly used protocols for F/T-experiments because a large number of formulation and process related variables can influence the results [4]. Therefore, previous test measurements should be realized to find the most suitable setup.
During manufacturing and transport of pharmaceutical products, the formulations are exposed to liquid-air interfaces and shear-stress, which can result in protein instability. To stabilize drug products, excipients - like polysorbate 80 – are added to the formulations to protect the product from aggregation.
Mechanical stress testing is mostly performed as agitation or stirring studies. Especially for this stressing method the setup of the experiment has a large impact on the result [4]. Therefore, the experimental setup should pursue the goal to cause as much force to the protein as possible. Big liquid-air interfaces, as well as long agitation times, are worthwhile.

Another problem causing protein damage are storage time and condition. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidance requires stability against any kind of damage for at least 12 months of storage of protein-based medicines [6] .

For final formulation development it will be necessary to include even more forced degradation studies to assess potential risks for the molecule eventually harming the patients. Those studies include oxidation studies, light stress or incubation with different surfaces or materials.

During screening studies the amount of material is usually limited so experiments have to be carefully designed. It is detrimental to use an experimental setup and analytical tools that use a low amount of material because they are not representative for critical quality attributes. Recent advances in high-throughput screening technology and automation allow for the accumulation of a large amount of data in a short time period. This has the advantage for significant shortening of the time to market, however, data handling and evaluation becomes critical. The use of statistical models – as used by MODDE 9.1.1 - is a major improvement compared to one-factor-at-a-time methods for gaining information.

One big advantage using Design of Experiments (DoE) for experimental screening setup is the possibility of identify not only the influence of single parameters but also interaction effects. Statistical software as MODDE 9.1.1 using appropriate designs (e.g. d-optimal design) so generate robust areas within the design space. However, using different forced degradation studies in DoE runs can lead to different response surfaces depending on the stress methods employed which propose several different optimal formulations.

In the present study we show a formulation approach fully compliant with Quality by Design principles. To get a thorough understanding of product and process modelling tools can assist to predict the behaviour of proteins under certain conditions. This approach is very valuable as it allows scaling down the experimental work since several conditions can be ruled out beforehand as promising formulation regions. For a fast and resource-effective approach we were combining DoE studies with high- and medium throughput techniques for IgG1 mAb as model system applying different forced degradation studies.

2 SCREENING EXPERIMENTS OF IGG1 MAB

2.1 MATERIALS AND METHODS

2.1.1 Materials

Materials:

Pipettes 1 mL, 200 µL, 20 µL, 10 µL
Pipette tips 1 mL, 200 µL, 20 µL, 10 µL
Falcon Tubes 15 mL
Falcon Tubes 50 mL
Eppendorf Tubes 1.5 mL
UV cuvettes
CD 0.1 cm cuvette
Vivaspin 20 centrifugal concentrators (Sartorius AG, Germany)
Minisart high flow Syringe Filters (Sartorius AG, Germany)
Syringe
Syringe canula
unskirted 96-well PCR plates (Bio-Rad)
optical foil (Bio-Rad)
HPLC vials

Substances:

IgG1 mAb (Sandoz GmbH, Kundl, Austria)
CCVJ (Sigma-Aldrich ,St. Louis, MO, USA)
Ethanol absolute, Cat.No. 107017 (Merck, Millipore, Canada)
Liquid nitrogen
D-Sorbitol > 98%, Cat.No. 6213.1 (Carl Roth GmbH + Co. KG, Germany)
Sodium chloride ≥99,5 %, p.a., ACS, ISO, Cat.No. 3957.1 (Carl Roth GmbH + Co. KG, Germany)
di-sodiumhydrogenphosphate ≥99 %, p.a., ACS, water free, Cat.No. P030.2 (Carl Roth GmbH + Co. KG, Germany)
sodiumdihydrogenphosphate – monohydrate ≥98 %, p.a., ACS, Cat.No. K300.2 (Carl Roth GmbH + Co. KG, Germany)
citric acid ≥99,5 %, p.a., ACS, water free, Cat.No. X863.2 (Carl Roth GmbH + Co. KG, Germany)
Calibration solution (Bio-Rad)

ultra-pure water (Barnstead MicroPure, Thermo Scientific)
Tween 20 viscous liquid, Cat.No. P1379 (Sigma-Aldrich, Austria)
Tween 80 viscous liquid, Cat.No. P1754 (Sigma-Aldrich, Austria)
PTS 15 wt. % in H_2O, Cat.No. 698717 (Sigma-Aldrich, Austria)
Kolliphor EL (BASF Chem Trade GmbH, Germany)
Kolliphor P188 (BASF Chem Trade GmbH, Germany)

Instruments:

Thermomixer (Eppendorf)
Table centrifuge (Eppendorf)
Inolab 720 pH meter (WTW)
DU 800 UV/VIS - Spectrophotometer (Beckman Coulter)
iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA)
HPLC System D-7000 (Merck-Hitachi)
Micro Flow Imaging (ProteinSimple, Santa Clara, CA, USA)
NanoSight nanoparticle analysis instrument (Malvern Instruments Ltd, GB)
Jasco J715 spectropolarimeter (Jasco, Groos-Umstadt, Germany)
Tosoh TSKgel G3000SWxl column (Tosoh Bioscience LLC, PA)

Software:
MODDE 9.1.1 (Umetrics, Umea, Sweden)
NTA 2.3 Analytical Software (Malvern Instruments Ltd, GB).

2.1.2 Buffer and protein sample preparation

The used IgG1 mAb – provided by Sandoz - was prepared in 25 mM citrate buffer at pH 6.5. The concentration of the protein stock was 30 mg/mL. IgG1 mAb was stored frozen at -20°C until further use. Buffer exchange for the assigned buffer (citrate-phosphate buffer at a concentration of 10 mM) was implemented by ultrafiltration. Therefore, the frozen protein samples were centrifuged in spin columns (20 mL) at 4000 g at 4°C for 30 min, including two time buffer exchange. The concentrated protein samples were then re-diluted in 10 mL CP-buffer. Afterwards, the protein concentration was measured by absorbance at 280 nm. Used concentrations of protein (1 mg/mL) and CCVJ (10 µM) based on former studies [5].

Stock solutions of tested surfactants (T80, T20, CR-EL, Pluronic F68, PTS, sorbitol and sodium chloride) were prepared in CP-buffer at used pH values. Used surfactants and buffer concentrations were selected based on previous studies (concentration of final formulations see Table 1, Table 2, Table 3, Table 4) [6]- [10]. Therefore, the constraints of these variable factors were defined by using the pharmaceutical area of application. MODDE 9.1.1 then calculate the suitable number of experiments necessary to build a statistical valuable design space. For optimization, constraints were defined manually based on screening results of each stressing method. Afterwards, MODDE 9.1.1 again calculate the suitable number of experiments within the predefined constraints. As seen in Table 2 – Table 4, only two non-ionic surfactants showed remarkable influence in the screening experiments. Optimization experiments were therefore not only set up to improve stability by one surfactant. Possible interactions that can lead to higher stability were also tested using both surfactants in one formulation.

Table 1: protein formulation conditions for used additives for the screening study

Sample No.	Surfactant	Surfactant concentration [%]	Sorbitol concentration [%]	Ion strenght [mM]	pH
N1	T80	0	0	150	4
N2	T80	0	12	0	4
N3	T80	0	0	0	8
N4	T80	0	12	150	8
N5	T80	0.4	0	0	4
N6	T80	0.4	12	150	4
N7	T80	0.4	0	150	8
N8	T80	0.4	12	0	8
N9	T80	0.2	6	75	6
N10	CR-EL	0	0	0	4
N11	CR-EL	0	12	150	4
N12	CR-EL	0	0	150	8
N13	CR-EL	0	12	0	8
N14	CR-EL	0.4	0	150	4
N15	CR-EL	0.4	12	0	4
N16	CR-EL	0.4	0	0	8
N17	CR-EL	0.4	12	150	8
N18	T20	0	0	0	4
N19	T20	0	12	150	4
N20	T20	0	0	150	8
N21	T20	0	12	0	8
N22	T20	0.4	0	150	4
N23	T20	0.4	12	0	4
N24	T20	0.4	0	0	8
N25	T20	0.4	12	150	8
N26	T20	0.2	6	75	6
N27	PluronicF68	10	0	0	6
N28	PluronicF68	5	12	0	4
N29	PluronicF68	0	6	0	8
N30	PluronicF68	5	0	150	8
N31	PluronicF68	0	12	150	6
N32	PluronicF68	10	6	150	4
N33	PluronicF68	0	0	75	4
N34	PluronicF68	10	12	75	8
N35	PTS	0.2	0	0	8
N36	PTS	0.4	12	0	6
N37	PTS	0	6	0	4
N38	PTS	0	0	150	6
N39	PTS	0.4	12	150	4
N40	PTS	0.4	6	150	8
N41	PTS	0.4	0	75	4
N42	PTS	0	12	75	8
N43	PTS	0.2	6	75	6
N44	PTS	0.2	6	75	6
N45	PTS	0.2	6	75	6
N46	PTS	0.2	6	75	6
N47	PTS	0.2	6	75	6

Table 2: protein formulation condition of used additives for thermal stress optimization study

Sample No.	T80 [%]	CR-EL [%]	Sorbitol concentration [%]	Ion strenght [mM]	pH
N1	0	0	5	0	6
N2	0	0	10	80	8
N3	0	0	5	80	8
N4	0	0	10	0	6
N5	0	0.2	10	0	8
N6	0	0.2	5	0	8
N7	0	0.2	10	80	6
N8	0.2	0	5	80	8
N9	0.2	0	10	0	8
N10	0.2	0	5	0	6
N11	0.2	0	10	80	8
N12	0.2	0.2	5	0	8
N13	0.134	0.2	10	0	6
N14	0.2	0.067	10	0	6
N15	0.2	0.2	8.34	0	6
N16	0.067	0.2	5	80	7.34
N17	0.2	0.2	5	80	6
N18	0.134	0.2	10	80	6
N19	0.2	0.134	10	80	6.67
N20	0.2	0.2	10	80	6
N21	0	0.2	6.67	80	6
N22	0	0.2	5	53.34	6
N23	0.2	0.2	5	53.34	6
N24	0.2	0.2	10	26.67	6
N25	0.1	0.1	5	40	8
N26	0.1	0.1	7.5	40	7
N27	0.1	0	7.5	40	7
N28	0.1	0.1	7.5	40	7
N29	0.1	0.1	7.5	40	7
N30	0.1	0.1	7.5	40	7
N31	0.1	0.1	7.5	40	7
N32	0.1	0.1	7.5	40	7

Table 3: protein formulation condition of used additives for F/T-stress optimization study

Sample No.	T80 [%]	CR-EL [%]	Sorbitol concentration [%]	Ion strenght [mM]	pH
N1	0	0	10	80	4
N2	0	0.2	5	80	4
N3	0	0.2	10	0	4
N4	0	0.2	5	0	6
N5	0	0.2	10	80	6
N6	0.2	0	5	80	4
N7	0.2	0	5	0	6
N8	0.2	0	10	80	6
N9	0.2	0.2	5	0	4
N10	0.2	0.2	10	80	4
N11	0.2	0.2	5	80	6
N12	0.2	0.2	10	0	6
N13	0.067	0	5	0	4
N14	0	0	5	0	5.34
N15	0.2	0.134	10	0	4
N16	0.134	0	10	0	6
N17	0	0.067	10	0	6
N18	0	0	10	0	4.67
N19	0.2	0	8.34	0	4
N20	0.067	0	5	80	6
N21	0	0	6.67	80	6
N22	0	0	5	53.34	4
N23	0.2	0	10	26.67	4
N24	0	0	10	26.67	6
N25	0.1	0.1	5	40	5
N26	0.1	0.1	7.5	40	4
N27	0.1	0.2	7.5	40	5
N28	0.1	0.1	7.5	40	5
N29	0.1	0.1	7.5	40	5
N30	0.1	0.1	7.5	40	5
N31	0.1	0.1	7.5	40	5
N32	0.1	0.1	7.5	40	5

Table 4: protein formulation condition of used additives for agitation-stress optimization study

Sample No.	T80 [%]	CR-EL [%]	Sorbitol concentration [%]	Ion strenght [mM]	pH
N1	0	0	5	60	6
N2	0	0	5	0	8
N3	0	0	10	60	8
N4	0	0.2	5	0	6
N5	0	0.2	5	60	8
N6	0	0.2	10	0	8
N7	0.2	0	5	0	6
N8	0.2	0	5	60	8
N9	0.2	0	10	0	8
N10	0.2	0.2	10	0	6
N11	0.2	0.2	5	0	8
N12	0.2	0.2	10	60	8
N13	0	0.134	10	0	6
N14	0	0.2	8.34	0	6
N15	0.134	0	5	60	6
N16	0.067	0.2	5	60	7.34
N17	0	0	10	60	6
N18	0.2	0.067	10	60	6
N19	0.2	0	10	60	6.67
N20	0	0	8.34	60	6
N21	0.2	0.2	6.67	60	6
N22	0	0	5	40	6
N23	0.2	0.2	10	20	6
N24	0.2	0	10	40	6
N25	0.1	0.1	7.5	30	8
N26	0.1	0.2	7.5	30	7
N27	0.2	0.1	7.5	30	7
N28	0.1	0.1	7.5	30	7
N29	0.1	0.1	7.5	30	7
N30	0.1	0.1	7.5	30	7
N31	0.1	0.1	7.5	30	7
N32	0.1	0.1	7.5	30	7

Table 5: protein formulation condition of used additives for the storage experiment

SampleNo.	Surfactant	Surfactant concentration [%]	Sorbit concentration [%]	Ion strenght [mM]	pH
N10	CR-EL	0.1	0	0	6
N20	T20	0.1	0	150	8
N30	PluronicF68	5	0	150	4
N3	T80	0.1	0	0	8
N14	CR-EL	0.4	0	150	6
N42	PTS	0.1	12	75	8
N15	CR-EL	0.4	12	0	4
N2	T80	0.1	12	0	4
N29	PluronicF68	1	6	0	8
N7	T80	0.4	0	150	8
N25	T20	0.4	12	150	8

2.1.3 Design of experiment and statistical analysis

The specific experimental design software, MODDE 9.1.1 (Umetrics, Umea, Sweden) was created for design of experiment and multivariate data analysis. For a preliminary screening, a d-optimal design was used. D-optimal design is useful to constrain the region. It makes it possible to generate a manageable experimental design including depiction of desired effects and interactions.
The model includes five variable factors: four quantitative factors (ion strength, surfactant concentration, sorbitol concentration, and pH) and one qualitative factor (type of surfactant). The response variable was T_m, in the case of the DSF measurements, and the particle concentration and particle size distribution, in the case of the MFI measurements. All measurements were performed in duplicated and averaged.

For visualization of factor effects, contour and predictions plots were generated. MODDE 9.1.1 also illustrate if the experimental data fit the model by calculating the model validity, R^2 (which represents how well the date fit the model), Q^2 (which represents how well the model predicts new data) and the reproducibility.

2.1.4 Analytical methods

2.1.4.1 Thermostability screening by Differential Scanning Fluorimetry (DSF)

Differential Scanning Fluorimetry (DSF) makes it possible to analyze the increasing fluorescence signal based on protein degradation, measuring with high-throughput.

DSF measurement was carried out following the general protocol outlined by Niesen et al. (2007) and previous thermostability studies [5], [9]. For the screening experiments, NaCl, sorbitol and surfactant concentrations were varied. Furthermore, various pH values and types of surfactant were used. The final concentration of IgG1 mAb was 1 mg/mL and the final concentration of CCVJ was 10 µM. 30µl of prepared samples – 24 µl formulation plus 6 µl CCVJ (50 µM) - were added to white, unskirted 96-well PCR plates (Bio-Rad) and sealed with optical foil (Bio-Rad). Each formulation was measured in duplicates.
The thermostability studies were realized on an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The samples were exposed to a temperature ramp from 25°C to 95°C at a heating rate of 1°C/min and at 0.5°C increments. For measurements, a specific filter for CCVJ (Ex/Em: 435/500 nm) was installed.

T_m is defined as the temperature corresponding to the maximum value of the first derivative of the first fluorescence transition and was analyzed by the iCycler program. The reported T_m values are an average of a repeat determination [9].

2.1.4.2 Particle formation and thermostability screening of Freeze/Thaw-stressed protein formulations by Micro-Flow Imaging (MFI) and Differential-Scanning-Fluorimetry (DSF)

Micro-Flow Imaging is an imaging technology that detects and measures subvisible and visible particles in protein-containing solutions.

The suspended particles pass through a sensing zone and are automatically analyzed to provide a digital archive of particle parameters. MFI offers the advantages to reach high sensitivity and to measure a broad size range using only a small amount of sample for analysis [11].

MFI analysis was carried out using the screening setup seen in Table 1. The used MFI 5200 was provided by ProteinSimple (Santa Clara, CA, USA), measuring in a size range from 1 – 70 µm.

The different protein formulations were pre-stressed by Freeze/Thaw-Cycles. Freeze/Thaw-stressing combines a number stressing factors leading to protein denaturation and/or aggregation, which will lead to the increase of particle concentration, measured by MFI [12]. Furthermore, the thermostability of pre-stressed samples was measured using DSF technology (see 2.1.4.1).

Samples were frozen for five minutes in liquid nitrogen and de-frozen for five minutes at 25°C, ten times repeat. Afterwards, the pre-stressed samples were analysed by MFI and DSF.

For MFI analysis, the particle concentration and particle size distribution was measured using 500 µl of pre-stressed protein formulations.

2.1.4.3 Particle formation screening of agitation-stressed protein formulations by Micro-Flow Imaging (MFI)

Former studies demonstrated that non-ionic surfactants, like castor oils or poloxamers, are commonly used to inhibit surface-induced protein aggregation [13]. In difference to ionic surfactants, they can be used in pharmaceutical formulations because they are non toxic.
Therefore, the effect of agitation on different protein formulations was demonstrated using the already mentioned screening protocol for pre-stressed formulations (see Table 1). To get a large air-water interface, a rotation wheel was used. Falcon tubes (15 mL) were filled with 1 mL of final protein formulation and rotated with a rotation time of 20 rotations per minute for five days at room temperature. Afterwards, the particle concentration was analysed by MFI (see 2.1.4.2).

2.1.4.4 Storage experiments

For the storage experiments, 11 protein formulations were chosen based on previous studies. To get a good overview of protein behaviour under different storage conditions, five formulations with low (N15, N2, N29, N7, N25), two formulations with medium (N14, N42), and four formulations with high (N10, N20, N30, N3) aggregation behaviour were chosen. 30 mL of these formulations were prepared and stored under 25°C with 60% humidity and 37°C with 40% humidity for three months (see Table 5). Before storage, 1 week, 2 weeks, 2 months and 3 months later, samples were taken and particle size and distribution was analysed using MFI, SEC and NTA.

2.1.4.4.1 Particle formation of stored protein formulations using Micro-Flow Imaging (MFI)

MFI was implemented to show the formations of particles of stored formulations in the micrometer range.
MFI analysis was carried out using the screening setup seen in Table 5. The used MFI 5200 was provided by ProteinSimple (Santa Clara, CA, USA), measuring in a size range from 1 – 70 µm.
For the MFI measurement, 500 µL of stored formulations was analysed. Each formulation was measured in duplicates and particle concentrations were averaged (see 2.1.4.2).

2.1.4.4.2 Particle formation of stored protein formulations using Nano-Tracking-Analysis (NTA)

The NTA was done by the NanoSight nanoparticle analysis instrument using NTA 2.3 Analytical Software (Malvern Instruments Ltd, GB).
For the NTA, five (one bad, one medium and three good) formulations were chosen. For each analysis, 1 mL of sample – with a concentration of 1 mg/mL - is injected into the glass chamber.
The proprietary NTA software records a video file of 90 seconds duration. Each formulation was measured five times and the outcome was averaged by the program. Afterwards, a cumulative plot was created, highlighting D50, D70 and D90. The D value stands for the particle size distribution. For example, if D50 = 50 nm, then 50% of the particles in the sample are larger than 50 nm and 50% are smaller.

2.1.4.4.3 Formulation behaviour under log-time storage using CD-Spectroscopy

Circular Dichroism (CD) measurements were performed on a Jasco J715 spectropolarimeter (Jasco, Groos-Umstadt, Germany) equipped with an external thermostat. Spectra were measured from 260 to 190 nm – recorded in triplicate - in a 0.1 cm cuvette with a protein concentration of 1 mg/mL.

2.1.4.4.4 Particle formation of stored protein formulations using Size-Exclusion-Chromatography (SEC)

SEC was done using Tosoh TSKgel G3000SWxl column (Tosoh Bioscience LLC, PA). TSK gel G3000SWxl column is well suitable for measuring monoclonal antibodies. With their mass of 150 kDa they lie perfectly in-between the mass range of the column of 10 – 500 kDa.
The samples – with a concentration of 1 mg/mL - were centrifuged at 4000 g for 10 min to get rid of lager particles that may plug the column and 500 µl of the supernatant was filled in injection vials for measurement. For each measurement, 100 µl of samples was injected into the column. Each formulation was measured twice.
For calibration all used excipients and the IgG1 mAb were dissolved in CP buffer and measured separated to find out their retention time.
The mobile phase consisted of 10 mM sodium phosphate and 0.3 M NaCl at pH 7.5 with a flow rate of 1 mL/min. The components of the mobile phase remained constant throughout the separation (isocratic elution). The run time was 30 min. Elution of protein from the column was detected by UV spectrophotometry at 215 nm.

3 RESULTS & DISCUSSION

3.1 SCREENING EXPERIMENTS

3.1.1 Thermostability screening by Differential Scanning Fluorimetry (DSF)

A really important factor that causes protein degradation is temperature. The development of a stable formulation, which can resist variation of temperature, is a big goal in formulation development.

DSF is a rapid and inexpensive high-throughput screening method that monitors unfolding of proteins in presence of fluorophores. This method uses thermal stress to measure protein stability in different formulation conditions. Therefore, a Real-Time PCR is used, which offers the advantage to measure 96 samples at the same time with different conditions and a small amount of protein [5].

A fluorescence molecular rotor (CCVJ), which exhibits high fluorescence when bound to exposed hydrophobic core residues of the protein due to thermal unfolding, was used as fluorescent dye based on previous studies [5],[10]. Previous studies show, that CCVJ is well-suited to detect aggregation of thermal stressed, polysorbate-containing protein formulations with the advantage of less background fluorescence [7],[10].

To calculate the T_m values, the temperature of hydrophobic exposure, the used iCycler program determines the maximum of the first derivative [5], [9], [10]. A high T_m value indicates high protein stability [8].

The experimental setup was generated using MODDE 9.1.1 d-optimal design (see Table 1).

The iCycler program generates a curve diagram by plotting the temperature [°C] against the fluorescence signal [RFU]. The received peak illustrates the melting temperature. For calculate the final melting temperature of the samples, the start fluorescence signal [RFU] of each sample was subtracted. All samples were measured in duplicates and averaged (see 2.1.4.1).

The obtained melting temperatures were used as response factors in the experimental screening setup and illustrated using contour and prediction plots (see Figure 1, Figure 2).

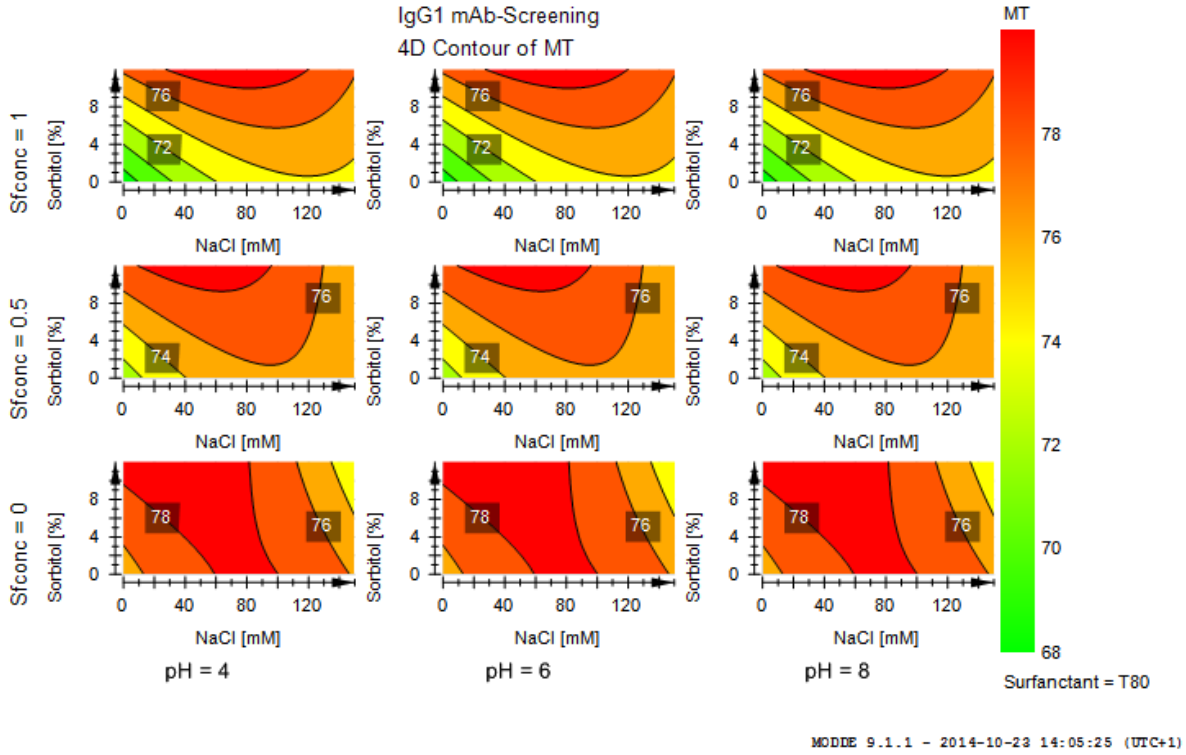


Figure 1: The obtained melting temperatures of the thermal stress screening were illustrated using 4D Contour-plot. The colour bar shows the increasing melting temperature. The highest melting temperatures were reached with a high sorbitol concentration and low ion strength. The surfactant type, surfactant concentration and pH value have less positive impact on the melting temperature of the formulations.

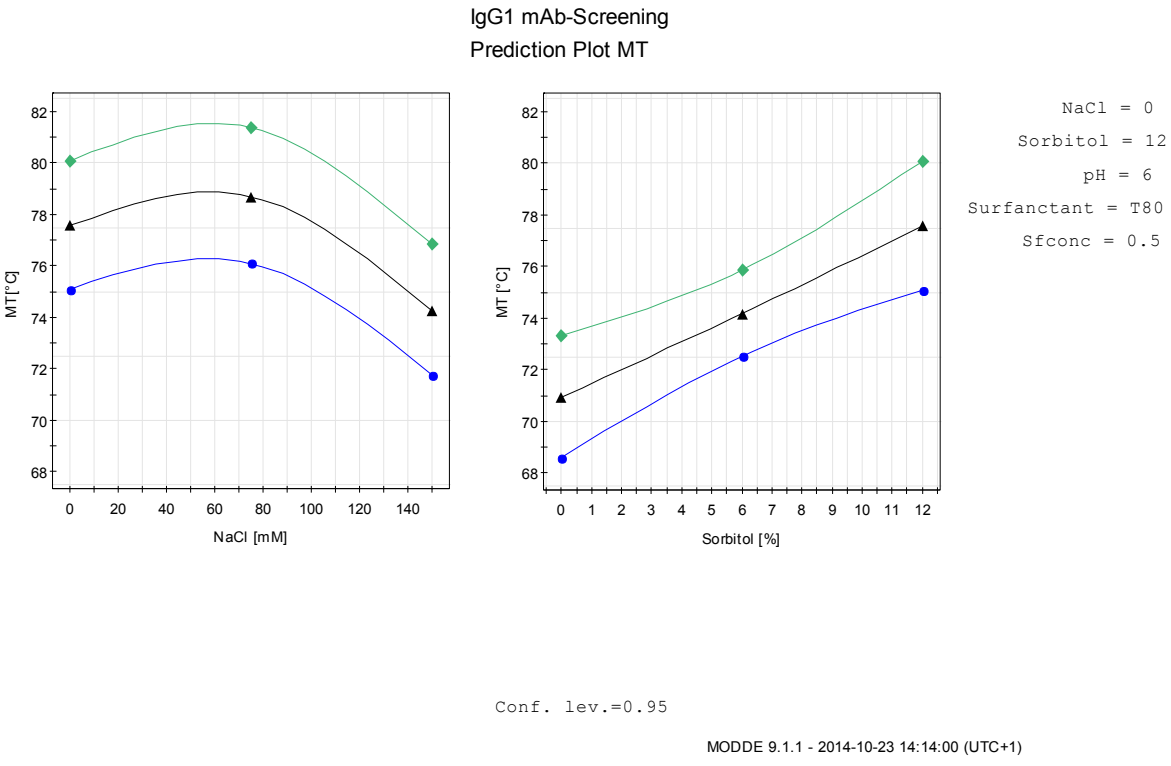


Figure 2: The obtained melting temperatures of the thermal stress screening were illustrated using a prediction plot. The melting temperature is plotted against the ion strength and the sorbitol concentration. The black line shows the melting temperature. The green and the blue lines show the upper and lower confidence levels. A high sodium chloride concentration as well as a low sorbitol concentration leads to a lower melting temperature.

3.1.1.1 Discussion of thermostability screening by DSF

The 4D Counter Plot created by MODDE 9.1.1 shows, that the most influencing factors concerning thermostability are the sodium chloride and the sorbitol concentration of the formulation. Surfactants as well as the pH value show no remarkable stabilizing support against thermal dependent denaturation of IgG1 mAb. A high or really low salt concentration leads to a decrease of melting temperature, whereas increasing concentrations of sorbitol have a positive influence on the stabilization of the native conformation of IgG1 mAb.

The positive effect of sugar alcohols – equal to sugars – can be explained by the preferential exclusion mechanism, first described by Timasheff et al. in 1993. This means that sugars are excluded from the immediate domain of protein molecules, leading to a water rich environment around the protein. This behaviour misleads the protein to a denser packing to avoid exposure of hydrophobic protein parts at the surface [22].
Furthermore, reduced protein surface due to preferential exclusion reduces the chemical potential and is thus less prone to oxidative processes [23].

Salts are very important to stabilize the pH of the protein formulation. However, salt can increase the surface tension of water, leading to interfacial aggregation of protein.
He et al. (2010) already demonstrated salt induced aggregation at high temperature or agitation of IgG1 mAb. Although, they could specially observe the negative impact of salt at a pH value lower six. In this work we could not observe any influence of the pH value at thermostability of IgG1 mAb.
The convex prediction plot for NaCl can have two causes. In most cases, at low and high salt concentrations either salting-in or salting-out occurs, respectively. Salting-in is observed when electrostatic interactions between the salt ions and charged residues of the protein are favourable. Salting-out occurs when the salt ions are excluded from the protein, which is mainly caused by unfavourable interactions between the salts and hydrophobic regions of the protein. There are examples, however, in which proteins are soluble at high salt concentrations.
The ionic strength can also mediate protein-protein interactions [13], [24].

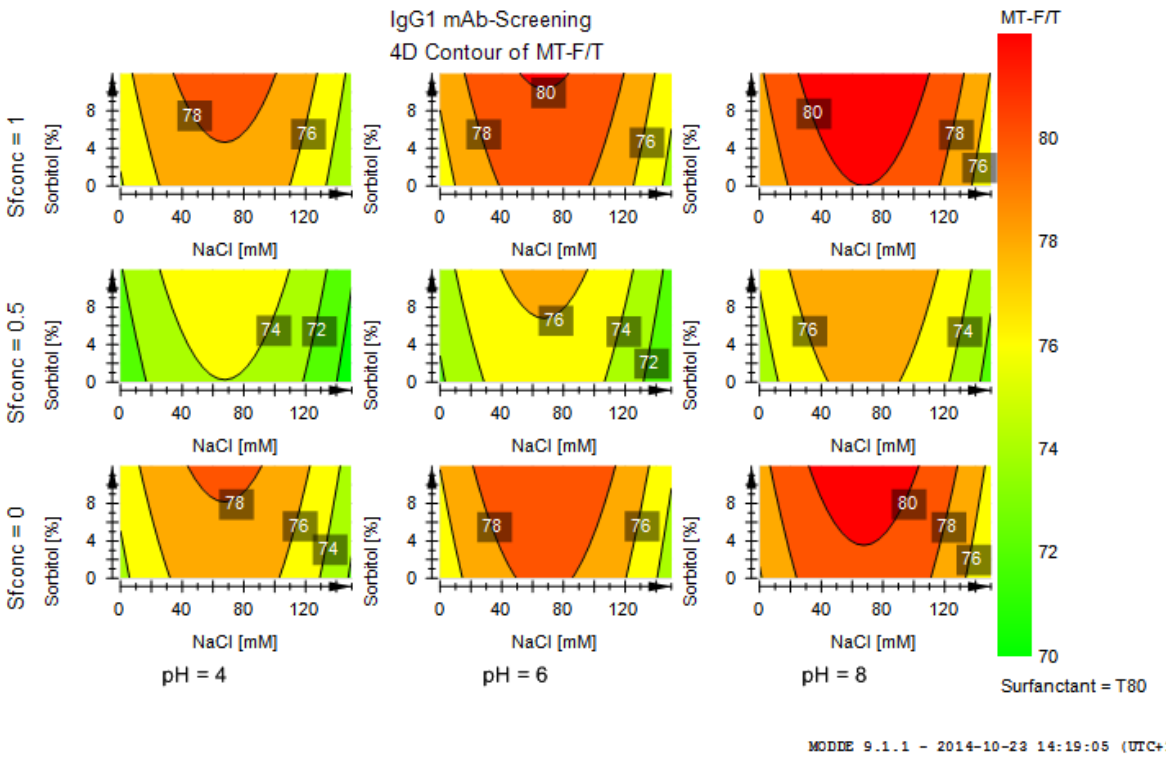


Figure 3: The obtained melting temperatures of the Freeze/Thaw- stressing screening were illustrated using 4D Contour-plot. The colour bar shows the increasing melting temperature. The highest melting temperature was reached with no salt, a high sorbitol and PTS concentration at pH 6. Surfactant type and concentration have a remarkable impact on the melting temperature of pre-stressed formulations.

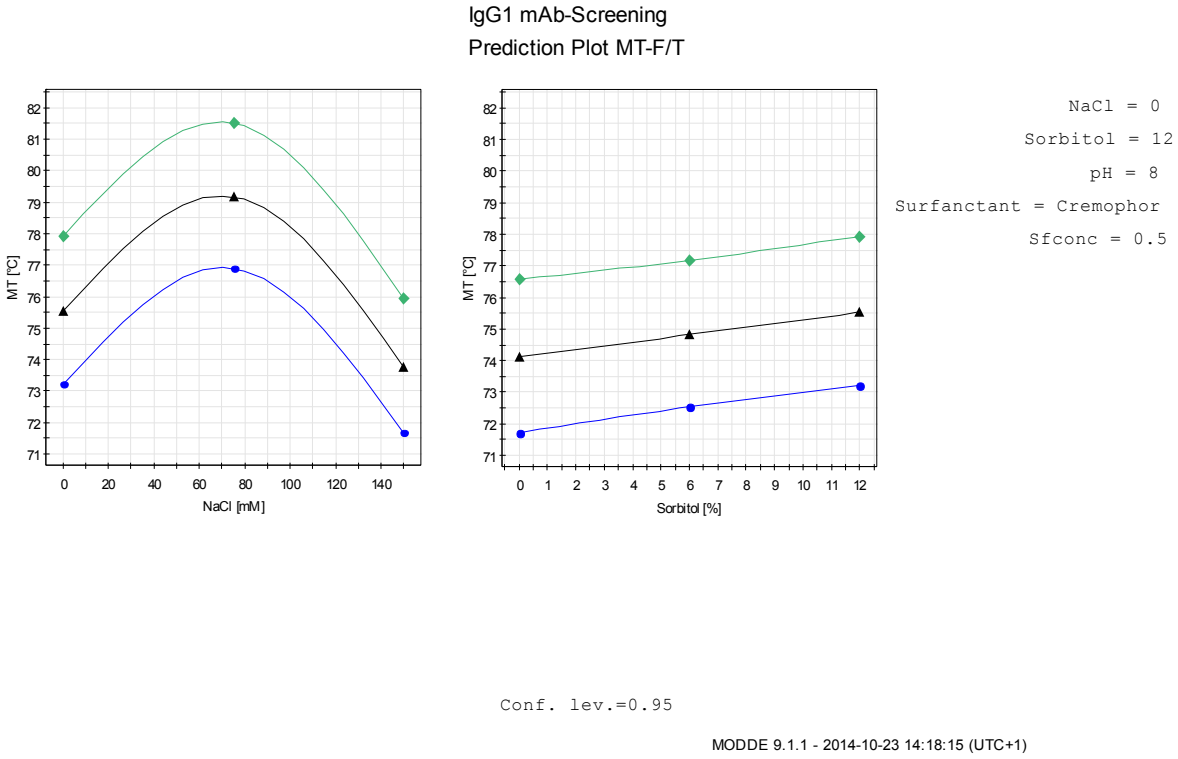


Figure 4: The obtained melting temperatures of the Freeze/Thaw-stressing screening were illustrated using a prediction plot. The melting temperature is plotted against the ion strength and the sorbitol concentration. The black line shows the melting temperature. The green and the blue lines show the upper and lower confidence levels. A high sodium chloride concentration as well as a low sorbitol concentration leads to a lower melting temperature. Using CR-EL or PTS, as well as a higher pH value, led to higher melting temperatures of the pre-stressed formulations.

3.1.1.2 Discussion of thermostability screening of F/T-stressed protein formulations by DSF

The results of the F/T-stressed screening study do not differ particularly from the thermostability screening study seen in 3.1.1.1. The most influencing excipients used in IgG1 mAb formulations were salt and sorbitol. Thermal stable formulations could be generated under moderate sodium chloride and high sugar concentrations.

Already implemented studies by Kueltzo et al. (2008) showed that the pH value has the most influence on the melting temperature of F/T-stressed formulations. The aggregation of the used mAb decreases with increasing pH. In this study, the authors supposed that aggregation at low pH value was a reason of potential mechanisms involving both the formation of aggregation-prone conformational states as well as adsorption to and denaturation at various interfaces. They also decline, that the usage of surface-active excipients should solve the problem of interfacial denaturation [25].
Nevertheless, this work showed no remarkable influence of interfacial-active surfactants, like polysorbate 80, on the melting temperature.

However, the experimental set up for F/T-Cycles has to be improved. Despite ten repeated cycles of freezing and thawing, the melting temperatures of all formulations were relatively high (see Figure 3).

3.1.2 Particle concentration screening by Micro-Flow Imaging (MFI)

MFI analysis was carried out using the screening setup seen in Table 1. Freeze/Thaw-stressing combines a number of stressing factors leading to protein denaturation and/or aggregation, which will lead to the increase of particle concentration, measured by MFI [12].
Exposure of the protein to the liquid - air interface or shear stress caused by manufacturing and transport play a major role for protein destabilization. This could lead to aggregation of the protein, which can be measured by MFI [12].
The experimental setup was generated using MODDE 9.1.1 d-optimal design (see Table 1).
All samples were measured in duplicates and averaged (see 2.1.4.2).
The obtained particle concentrations were used as response factors in the experimental screening setup and illustrated using contour and prediction plots (see Figure 5, Figure 6).

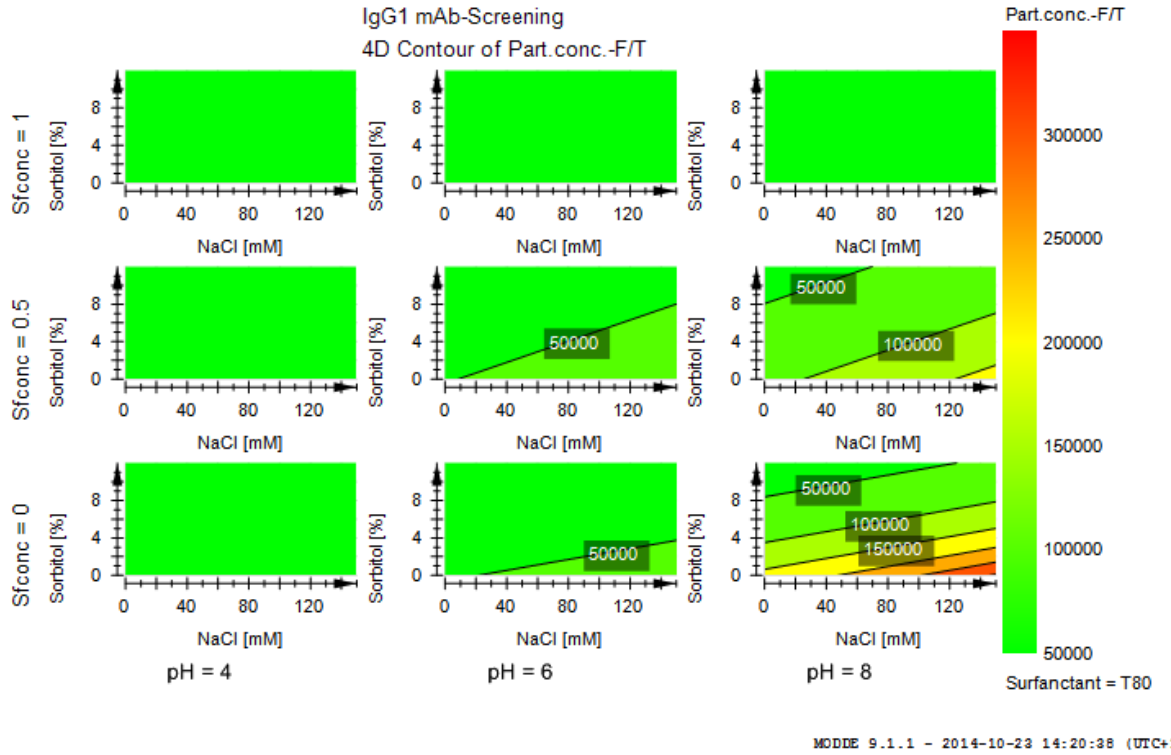


Figure 5: The obtained particle concentrations of the Freeze/Thaw- stressing screening were illustrated using 4D Contour-plot. The colour bar shows the increasing particle concentration. The lowest particle concentration was reached with no salt, a high sorbitol concentration and no surfactant. The most influencing factors on the particle concentration of Freeze/Thaw-stressed formulations are sorbitol and sodium chloride concentration and the pH value. Sodium chloride and pH value should be low, sorbitol concentration should be high.

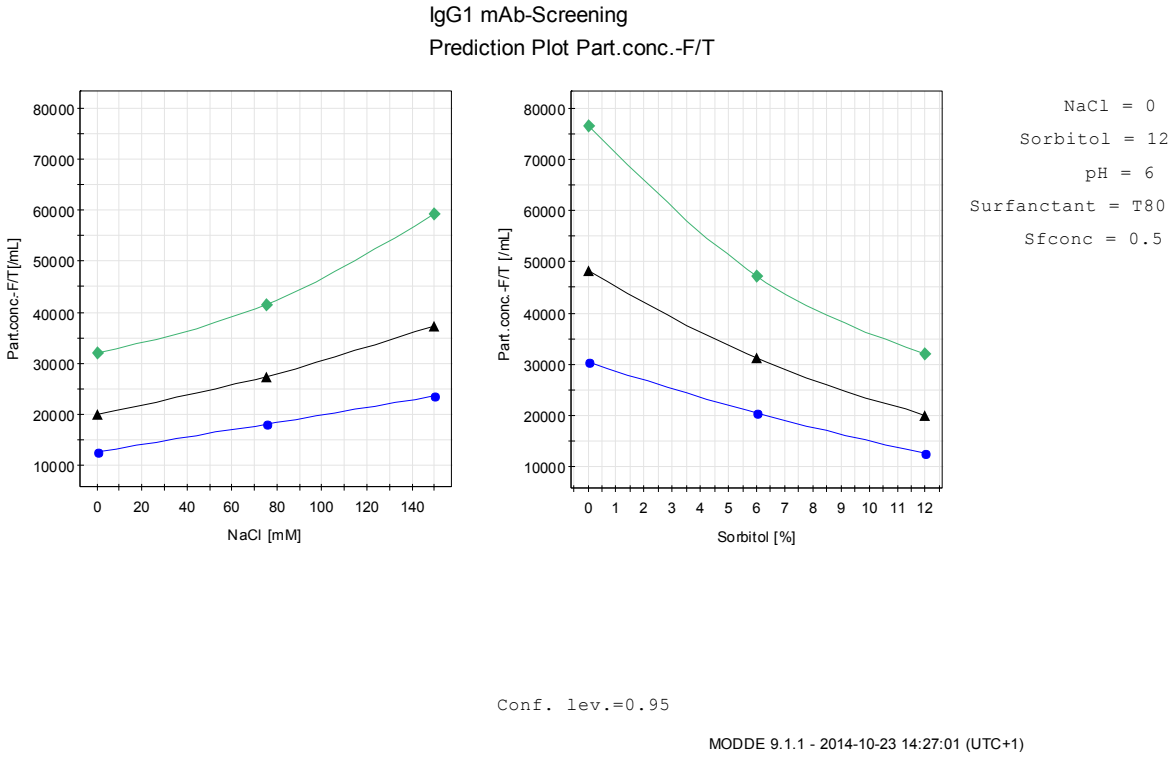


Figure 6: The obtained particle concentrations of the Freeze/Thaw-stressing screening were illustrated using a prediction plot. The particle concentration is plotted against the ion strength and the sorbitol concentration. The black line shows the particle concentration. The green and the blue lines show the upper and lower confidence levels. A low sodium chloride concentration as well as a high sorbitol concentration leads to fewer particles. Surfactant type and concentration as well as the pH value have no remarkable influence on the particle concentration.

3.1.2.1 Discussion of particle concentration screening of F/T-stressed protein formulations

Multiple publications have implied that protein aggregates may be a reason for an immune response against biotherapeutics. Although, there is still no evidence from clinical studies that demonstrate the connection between aggregates and immune response. Another question that is still not clear is if it is the mass of the antigen or the number of antigens that is critical to an immune response [26]. Singh et al. (2010) remarked, that particles between 1 and 10 µm, even if present at 10.000 counts/mL, represent about 0.1 µg of material. The risk of an immunological reaction with this amount of material is low. Also the current standard limits or specifications for subvisible particles are monitored in a size range above 10 µm [26]. Looking at the results with respect to particle size, all particles built under F/T-stress were around 10 µm or lower. So looking only at the size of the particles, all measured mAb formulations seem to be stable, which query the influence of used F/T experimental setup. Perhaps, the used freeze-thawing stress conditions were too weak for a forced degradation study.

Another important factor influencing the outcome of particle concentration measurements is the used method. MFI is a well established method to monitor subvisible particles. Before the discovery of this method, the most applied method in that size range was light obscuration. Light obscuration has many limitations and is in contrast to MFI not able to measure the shape and morphology of particles and cannot differentiate between particles from protein aggregation and other sources like air bubbles. Here lies the advantage of MFI, taking images of particles as they flow through the microscope. Therefore, it is possible to distinguish between protein aggregates and other particles. So, including the annotations of Singh et al. (2010), we used a measuring method that can both – measuring particle size and count [26].

Considering all this previous studies, the pharmaceutical industry and regulatory agencies are still interested in reducing particle formation, regardless if there is no direct evidence of danger from clinical studies. There is still the need to develop a common understanding between all interested parties, closing the gap in measuring and controlling subvisible particles as a product quality attribute.

Looking at the ingredients and conditions of the formulations, results from particle concentration screening of F/T-stress do not differ much from the thermostability screening study (see 3.1.1.2). Ion strength and the sorbitol concentration were the most influencing excipients on F/T-stressed formulations.

Aggregation of IgG1 mAb during freeze–thawing increased with decreasing pH, which correlated well with Tm values [25].

In general, the pH value affects many chemical reactions like deamidation, disulfide scrambling, peptide bond cleavage and oxidation [27].
Previous studies showed, that the desired pH value should be well away from the isoelectric point of the protein to keep the net surface charge of the protein high [30].

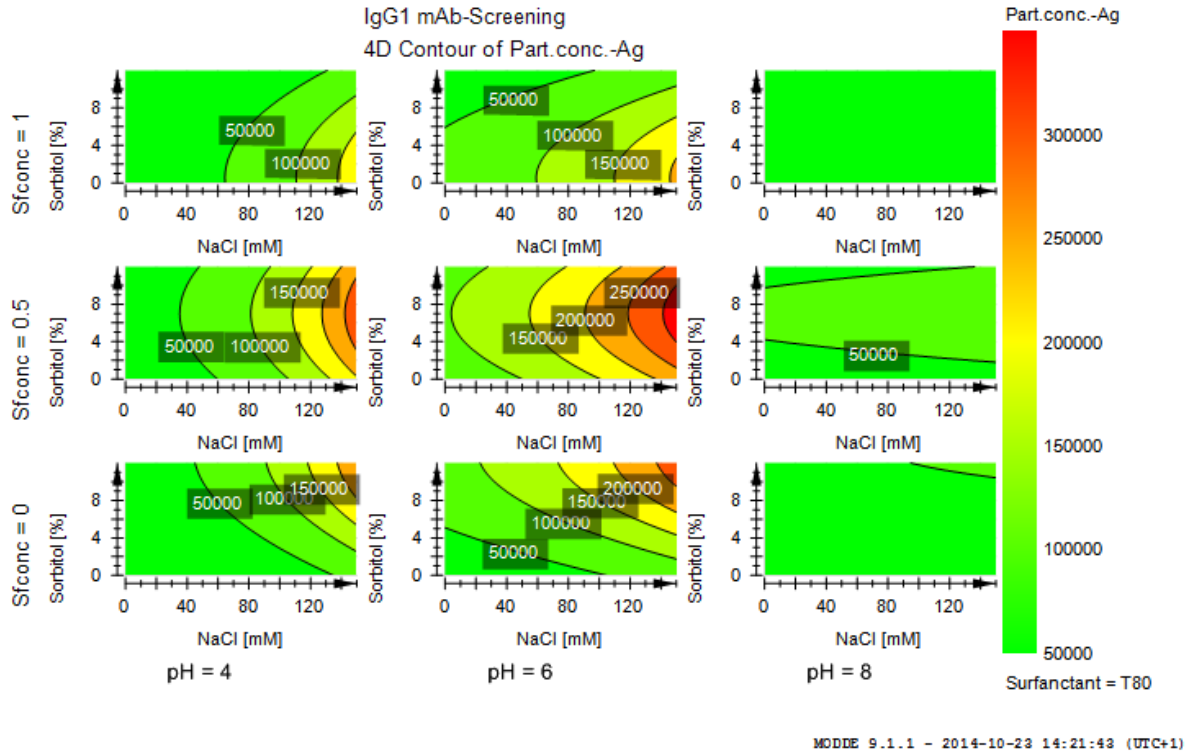


Figure 7: The obtained particle concentrations of the agitation stressing screening were illustrated using 4D Contour-plot. The colour bar shows the increasing particle concentration. The lowest particle concentration was reached witch a high salt, sorbitol and surfactant concentration. The most influencing factors on the particle concentration of agitation stressed formulations are the pH value and the sodium chloride concentration, but the outcome varies a lot.

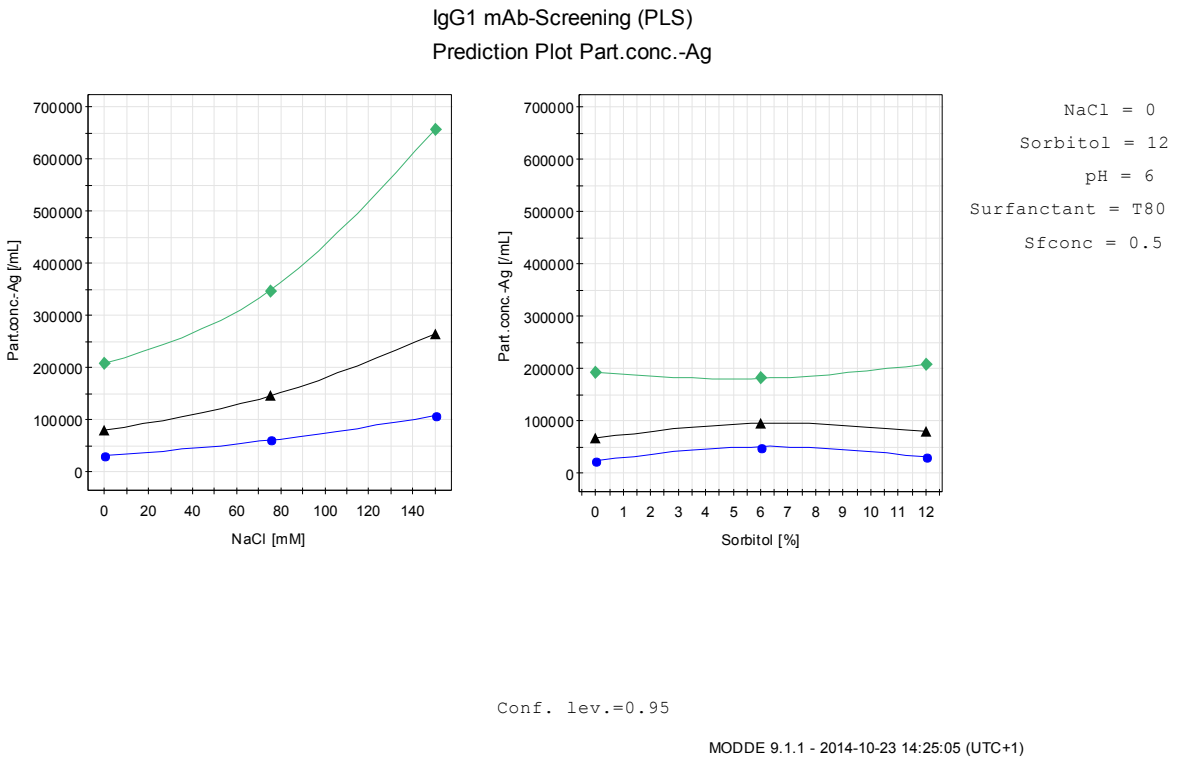


Figure 8: The obtained particle concentrations of the agitation screening were illustrated using a prediction plot. The particle concentration is plotted against the ion strength and the sorbitol concentration. The black line shows the particle concentration. The green and the blue lines show the upper and lower confidence levels. It can be seen, that at the particle concentration increases with the sodium chloride concentration. The sorbitol concentration has no remarkable influence on the particle concentration of agitated samples.

3.1.2.2 Discussion of particle concentration screening of agitated protein formulations

The 4D Counter plot of the agitated formulations illustrate, that sorbitol has less influence on the particle concentration of agitated samples than of F/T-stressed formulations. This was also revealed in previous studies by He et al. (2011).
mAbs are prone to accumulate at interfaces. They react with the liquid-air interface, leading to a rearrangement at the surface which may lead to partial unfolding of the adsorbed protein segments.
Perhaps, the preferential exclusion mechanism of sugar is not strong enough to protect the IgG1 mAb formulation against agitation [22].
In contrast, as seen in Figure 8, the NaCl concentration had a big influence on the particle concentration. He et al. (2011) explained this remarkable dependency with an alteration of the protein net charge. Previous studies showed that increasing salt concentrations reduce mAb net charges. Multiple factors affecting protein stability upon agitation stress are mediated by ions - like protein solubility, net charge, hydrophobicity, charge density distribution on the protein surface, as well as inter- face reactions as the air–liquid interface [28].
Agitation- induced mAb particulation also depends on the pH value. As mentioned in 3.1.2.1, the pH should be well away from the isoelectric point to keep the net surface charge of the protein high [30]. IgG1 mAb has a pI value of 8.6. Hence, the amount of net positive charge should increase as pH decreases [28].
Looking at the 4D Contour plot (Figure 7) the opposite behaviour can be recognized. Particle concentration decreases with increasing pH value. These results are also contradictory to performed thermostability studies, were low pH values led to more stable formulations.

Currently, non-ionic surfactants are the excipients most commonly used to inhibit surface-induced protein aggregation. So it was assumed, that high concentrations of surface-active excipients like polysorbate 80 stabilize IgG1 mAb formulations against agitation stress. Unfortunately, this study shows no remarkable influence of surfactants on the particle concentration of protein.

3.2 OPTIMIZATION EXPERIMENTS

Based on the screening studies, optimized formulation conditions for each stressing method were created using MODDE 9.1.1 (see Table 2, Table 3, Table 4).

3.2.1 Thermostability screening at optimized formulation conditions using Differential Scanning Fluorimetry (DSF)

DSF measurements were implemented as already mentioned in 3.1.1 using the optimization set up seen in Table 2.

For the optimization study, two surfactants – Tween 80 and CR-EL – were applied individually and simultaneously.

The obtained melting temperatures were used as response factors in the experimental optimization setup and illustrated using contour and prediction plots.

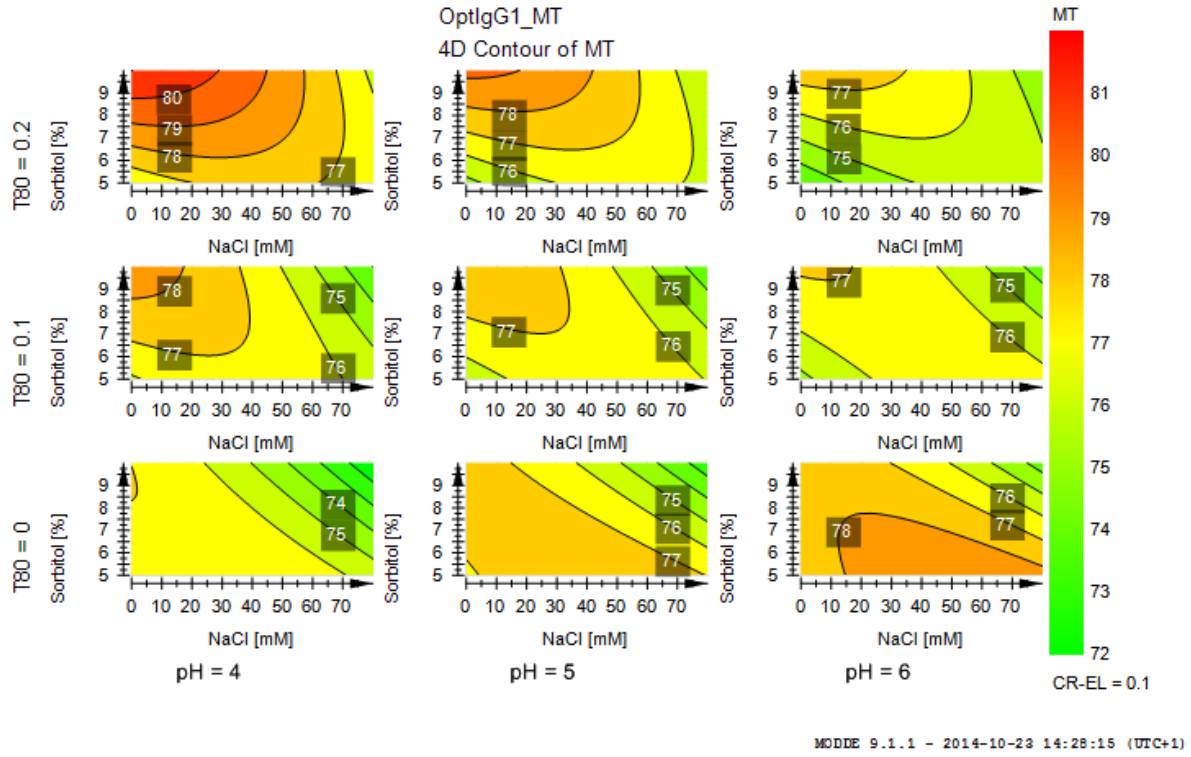


Figure 9: The obtained melting temperatures of optimized thermostability studies were illustrated using 4D Contour-plot. The colour bar shows the increasing melting temperature. It can be seen, that no optimized formulation has a melting temperature lower than 72°C. The highest melting temperature of 78°C was reached at pH 4 with low salt and high surfactant and sorbitol concentration.

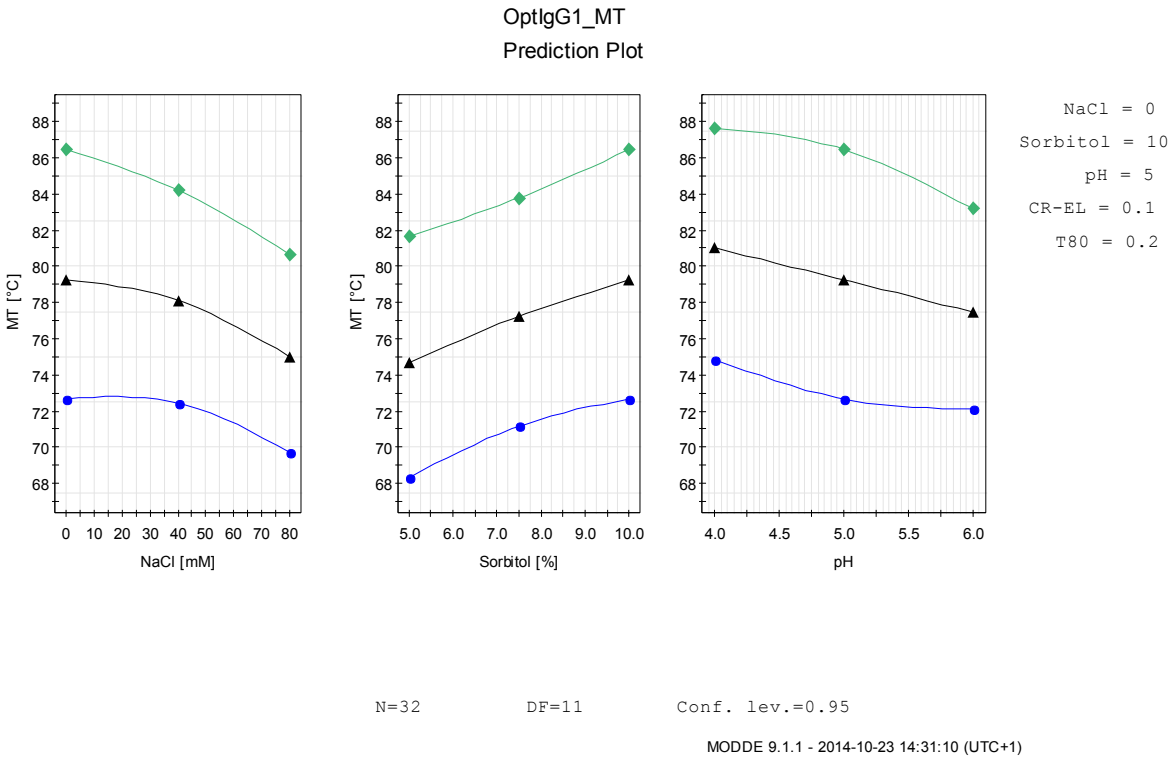


Figure 10: The obtained melting temperatures of optimized thermostability studies were illustrated using a prediction plot. The melting temperature is plotted against the ion strength, the sorbitol concentration and the pH value. The black line shows the melting temperature. The green and the blue lines show the upper and lower confidence levels. A low sodium chloride concentration as well as a high sorbitol concentration leads to a higher melting temperature. A low pH value also leads to high melting temperatures. It can be seen, that optimized formulations can reach melting temperatures above 80°C.

3.2.1.1 Discussion of thermostability screening at optimized formulation conditions using DSF

Optimization via MODDE 9.1.1 leads to thermal stable formulations with melting temperatures above 72°C.

Especially the usage of sorbitol minimizes the negative influence of salt. DSF detects the increase in the fluorescence of CCVJ. CCVJ is a molecular rotor with affinity for hydrophobic parts of the protein, which are exposed as the protein unfolds. The dense packaging of the mAb in presents of sugar may protect the protein from interactions with salts.

The usage of both surfactants – Tween 80 and CR-EL – simultaneously, showed a positive effect on the melting temperatures. As seen in the screening studies (3.1.1), high concentrations of surfactant have a slightly negative impact on the melting temperature. This behaviour was already described by Katakam et al. (1995). Therefore, in the optimization study, lower concentrations of surfactants were appointed. Katakam et al. (1995) also recognized, that surfactants like T80 do not in particular stabilized proteins against thermal stress. Optimization therefore should bring the evidence, that surfactants be inhibiting aggregation but not unfolding [29]. Comparing the results of the optimization study (Figure 9) with previous findings, the assumption was proven right. Even without surfactants, melting temperatures above 78 °C could be achieved.

Contrary to implemented agitation screening study (3.1.2.2), optimization stability study confirmed the results of He et al. (2011). They reported that the pH value of the solution should be well away from the isoelectric point of the protein. As a result, low pH values should lead to more stable formulations with higher melting temperatures [28]. Looking at the prediction plot in Figure 10, also this statement could be proven right. The highest melting temperatures could be reached at pH 4.

3.2.2 Particle concentration measurement of optimized formulations stressed by Freeze/Thaw-Cycles using Micro-Flow Imaging (MFI)

MFI measurement was done seen in 2.1.4.2 using the optimization protocol for F/T-Cycles (Table 3).

Tween 80 and CR-EL were appointed as surfactants, used individually and simultaneously.

Particle concentrations above 50000 particles per mL also reach the maximum count upper limit.

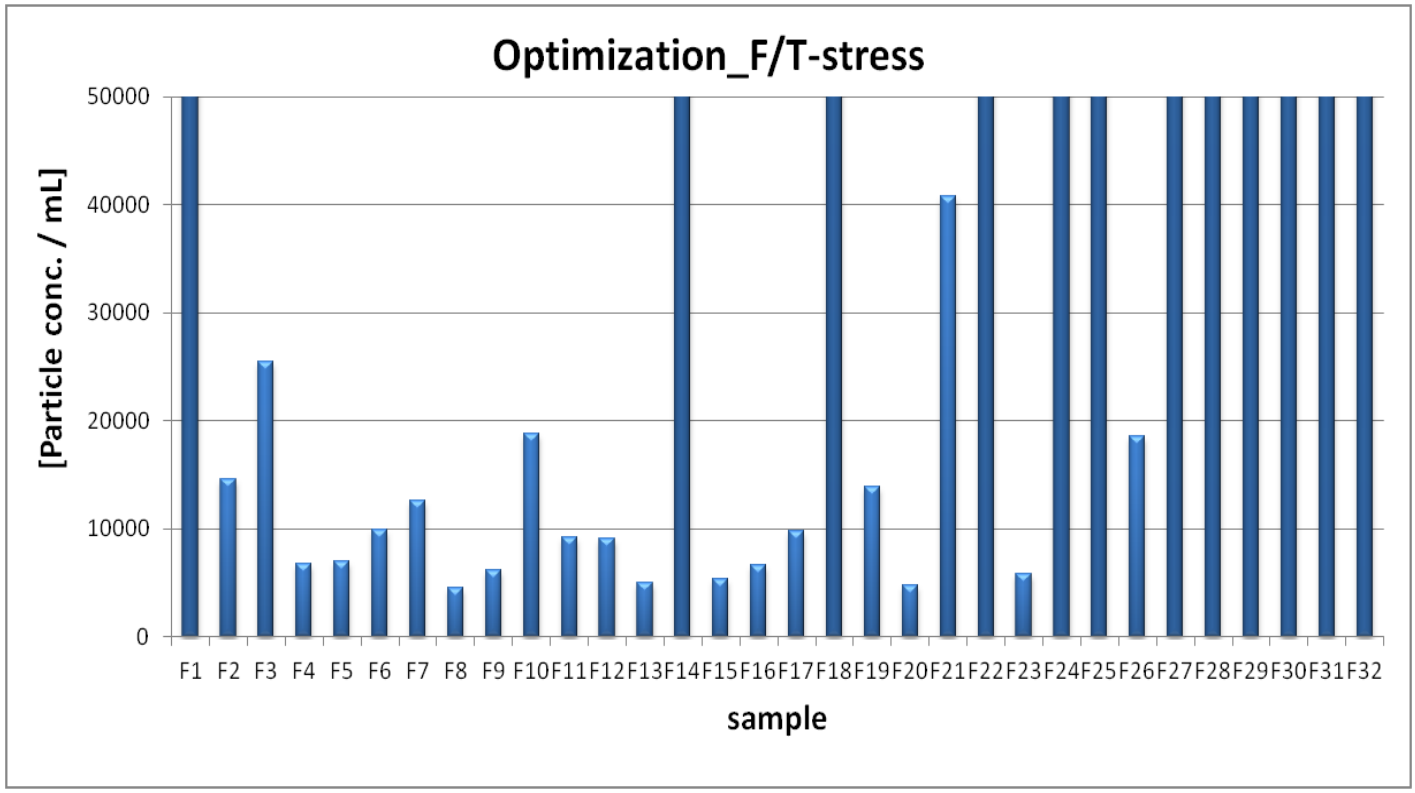


Figure 11: Particle concentration of optimized F/T- stressed formulations. The histogram shows that optimized formulations did not automatically lead to F/T-stressing stable formulations. Formulations with low concentrations of surfactant lead to a high concentration of aggregates. Nevertheless, some optimized formulations reach pharmaceutical suitable numbers.

3.2.2.1 Discussion of Particle concentration measurement of optimized formulations stressed by Freeze/Thaw-Cycles using Micro-Flow Imaging (MFI)

The histogram (Figure 11) shows, that optimization of formulation conditions did not automatically generate aggregation-stable formulations under F/T-stress.
While freezing, physical changes can develop different kind of stresses. To may explain the reason for the observed protein behaviour under F/T-stress I want to look at two stressing behaviours more in detail:

Cold denaturation
Once freezing occurs, the low temperature may induce spontaneous unfolding of the protein, a process termed cold denaturation.
The Gibbs free energy changes during unfolding. This occurs, when the temperature increases or decreases. The obtained negative value of ΔG leads to a transition called cold denaturation, which is accompanied by heat release and entropy decrease.
Cold denaturation has been attributed to an increase in the solubility of the hydrophobic residues in aqueous solutions at low temperatures.
The cold denaturation temperature is dependent on the pH, the concentration of the protein and additives. The effects of commonly used stabilizers – like polysorbates – have not been studied well so far [13].

Freeze concentration
During freezing, ice formation results in an increase in concentration of the solutes in the remaining liquid fraction of the formulation. The concentration and viscosity increases as a result of continued ice crystallization, leading to limited mobility in the high-viscosity system [13].
The high concentration of protein in the remaining liquid fraction increases the possibility of protein – protein interactions, leading to denaturation and/ or aggregation of protein.

In this particular case it seems, that sorbitol and salt protect the IgG1 mAb from cold denaturation, leading to unfolding of the protein. Protection from protein-protein interactions according to freeze concentration seems to be more difficult, explaining the need of additional, non-ionic surfactants, to refrain aggregation.
Looking at the results of F/T-stress optimization experiments, the usage of surfactants like Tween 80 or CR-EL makes it possible to gain fewer particles per mL. The difference between formulations like F8 and F9 and high particle formulations like F14 and F18 is only the usage of surfactants (see Figure 11).

3.2.3 Particle concentration measurement of optimized formulations stressed by agitation using Micro-Flow Imaging (MFI)

MFI measurement was done seen in 2.1.4.2 using the optimization experimental setup for agitation (Table 4).

Tween 80 and CR-EL were appointed as surfactants, used individually and simultaneously.

Particle concentrations above 60000 particles per mL also reach the maximum count upper limit.

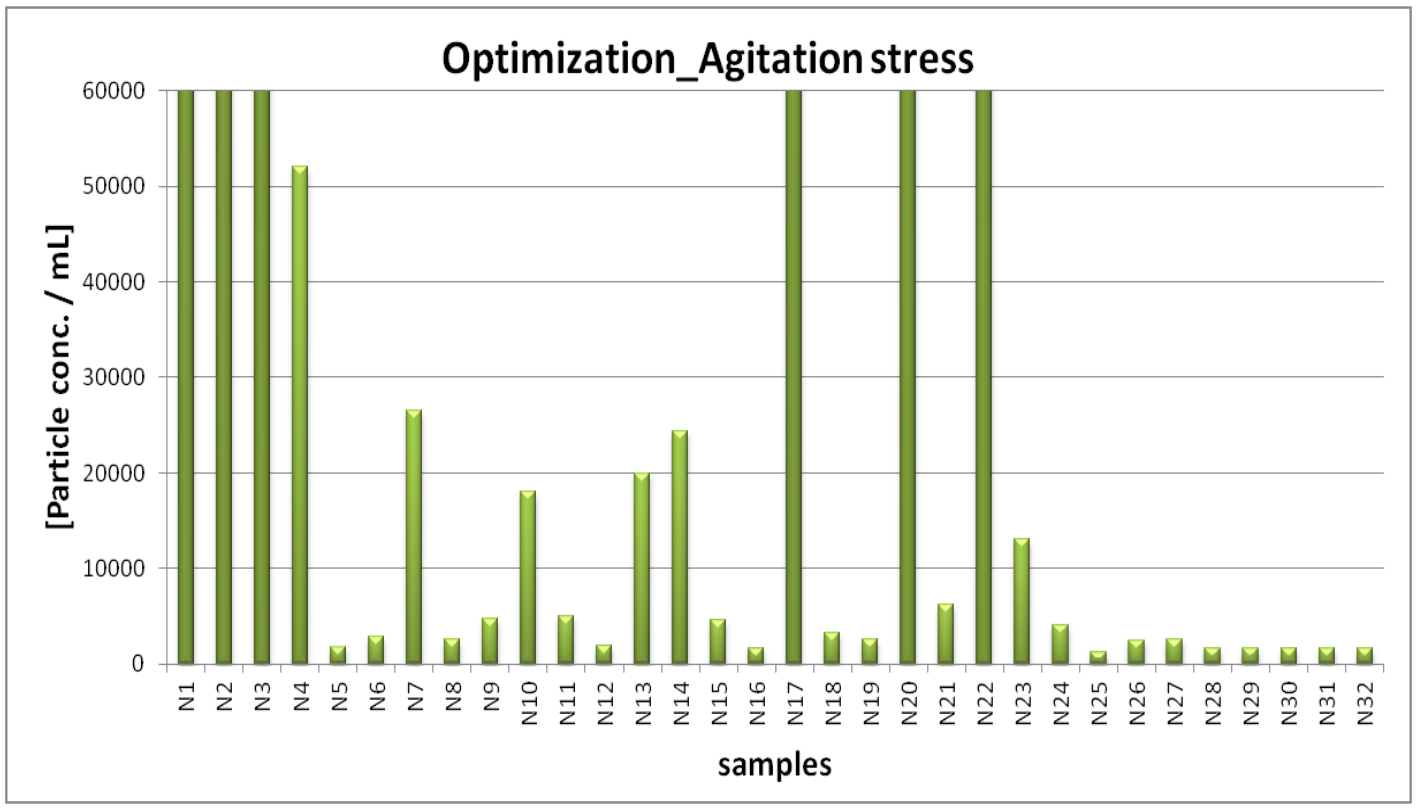


Figure 12: Optimized protein formulations stressed by agitation. The histogram shows, that optimization via MODDE 9.1.1 leads mostly to stable IgG1 mAb formulations.

3.2.3.1 Discussion of particle concentration measurement of optimized formulations stressed by agitation using Micro-Flow Imaging (MFI)

Optimization of agitated-stressed protein formulations – similar to F/T-stressed formulations – did not automatically lead to stable formulations.
In the absence of non-ionic surfactants, aggregation due to agitation could not be prevented (see Figure 12).

The effect of non-ionic surfactants for protein stabilization

Most pharmaceutical proteins are surface-active, leading to loss of native structure and aggregation. Surfactants are added to the formulations to protect proteins from surface-induced damage by competing with proteins for adsorption sites on surfaces. Furthermore, they can protect proteins against surface-induced aggregation by binding to hydrophobic regions of the protein molecule, leading to decreasing intermolecular interactions [32].

3.3 STORAGE EXPERIMENTS

The stability of a protein is also limited by time. Transport or long-time storage have a major contribution on protein properties. Pharmaceutical companies have the responsibility to ensure that their products are stable to their “beyond-use” date. This date is defined as the time by which the drug must be used to avoid potential ineffectiveness or the risk of an immunologic reaction of the patient [33]. So, pharmacists aspire to extend the shelf-life of pharmaceutical products.

Several formulation conditions were selected for accelerated stability studies in climate chambers at 37°C and 40% relative humidity and at 25°C and 60% relative humidity. The selected formulations spanned a range from optimal conditions to very unstable conditions based on the initial screening assays. Extensive biophysical characterization was done at time points of zero, one week, two weeks, two months and three months. The methods employed included SEC (Size-Exclusion-Chromatography), Nanoparticle-Tracking-Analysis (NTA), CD-Spectroscopy and Micro-Flow Imaging (MFI).
With the help of NTA, SEC and MFI a particle size range of 1 nm up to 70 µm can be characterized.
CD-Spectroscopy was only implemented at time point zero and after 3 months. The goal of the CD measurement was only to show the effect of a long-time storage on the protein formulations.

3.3.1 Particle formation of stored protein formulations using Micro-Flow Imaging (MFI)

MFI measure particle size and distribution of liquid protein formulations in the micrometer range.

For each measure point, 500 µL of sample was measured in duplicates and averaged. MFI measure the particle concentration per mL sample. The particle concentration of each formulation at each measuring point was illustrated using a histogram. Particle concentrations above 1 million particles per mL were set as worst case formulations. These formulations were also the only formulations that had particle concentration above 80000 particles per mL, what was the reason to set 80000 particles per mL at upper limit.

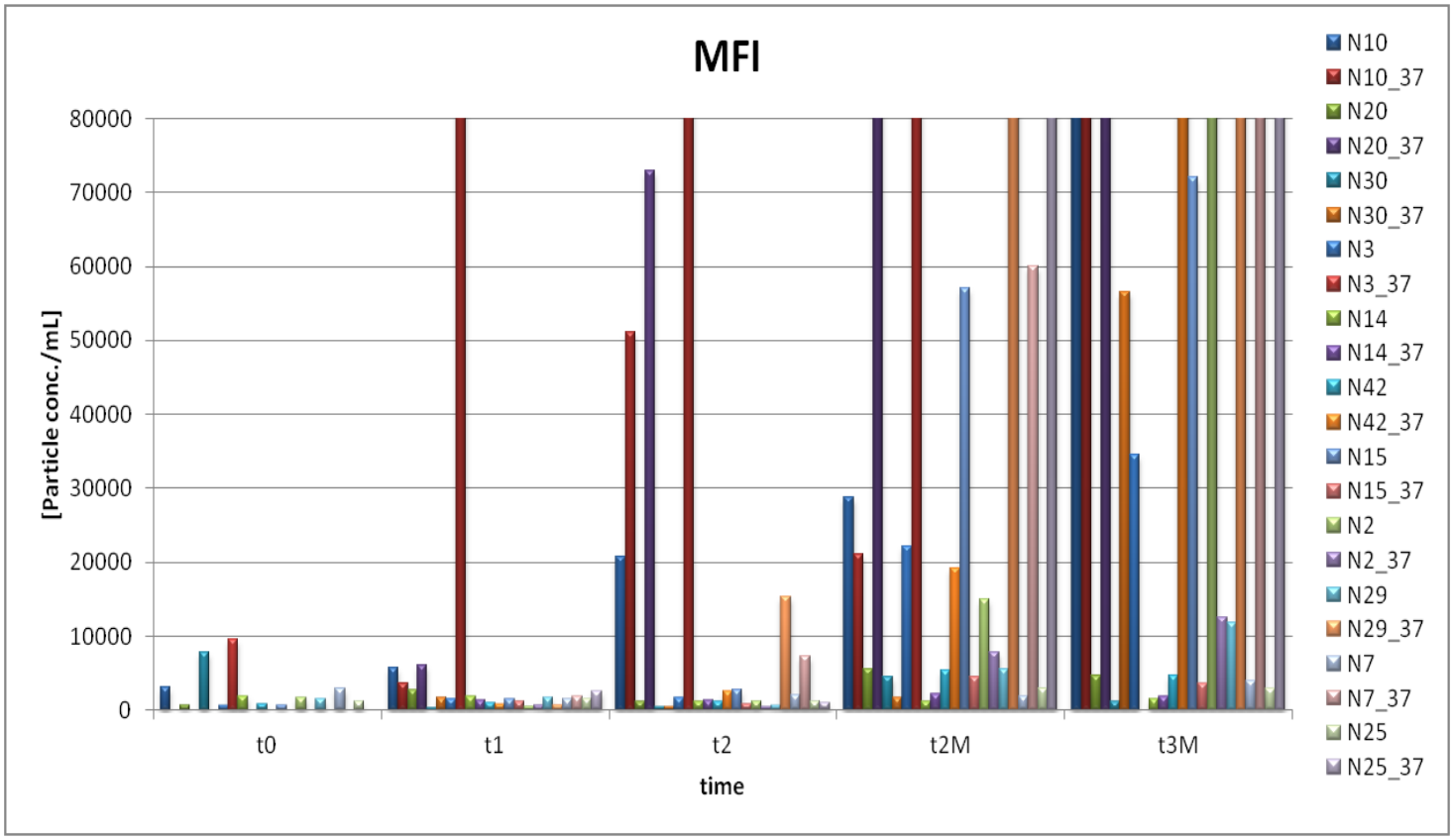


Figure 13: MFI measurement of stored protein formulations. The particle concentration per mL is plotted against the time points of sampling. It can be seen, that the particle concentration increases with storage time. Furthermore, storage at 37°C at 40% humidity as more negative influence on the particle concentration than storage at 25°C at 60% humidity. With time of storage the formulation composition has a reduced influence on the particle formation.

3.3.1.1 Discussion of particle formation of stored protein formulations using Micro-Flow Imaging (MFI)

The histogram in Figure 13 shows, that aggregation continuously increase with storage time. Furthermore, formulations that showed already low aggregation behaviour in previous screening studies also showed good resistance against aggregation under unfavourable storage conditions. As was expected, storage at 37°C at 40% humidity more likely leads to aggregation than storage at 25°C at 60% humidity.

3.3.2 Particle formation of stored protein formulations using Nano-Tracking-Analysis (NTA)

Micro-Flow Imaging is a suitable technique of measuring particle size and size distribution, but it is limited to the micro scale. Nano-Tracking-Analysis (NTA) makes it possible to accurately determine size down to 10 nm and an upper limit around 1-2 µm for particles in aqueous solutions [15].
Nanoparticles play an important and growing role of influencing the properties and stability of many pharmaceutical products [14].
NTA utilizes the properties of both light scattering and Brownian motion in order to obtain particle size distributions of samples in liquid suspension. A laser beam is passed through a prism edged glass flat within the sample chamber into the liquid formulation.
The particles in suspension in the path of this beam scatter light in such a manner that they can be easily visualized. The camera subsequently captures a video file of particles moving under Brownian motion within 90 sec.

The image analysis software then determines the average distance moved by each particle in the horizontal and vertical direction. This value allows the particle diffusion coefficient (Dt) to be determined and from which, if the sample temperature T and solvent viscosity µ are known, then the sphere-equivalent hydrodynamic diameter d of the particles can be identified using the Stokes-Einstein equation [14]:

$$Dt = \frac{TkB}{3\pi\mu d}$$

kB = Boltzmann Constant
μ = viscosity
T = Temperature

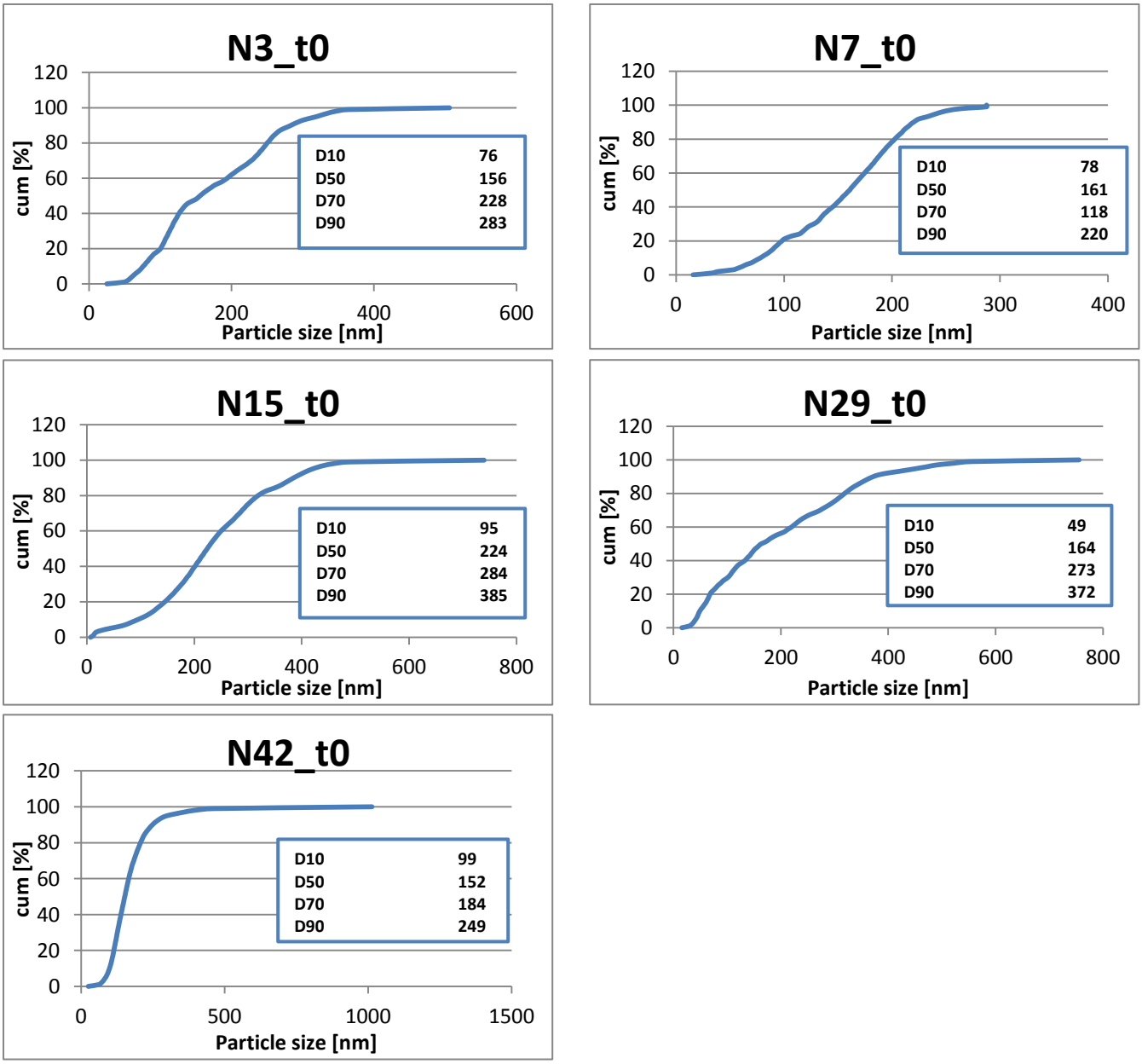
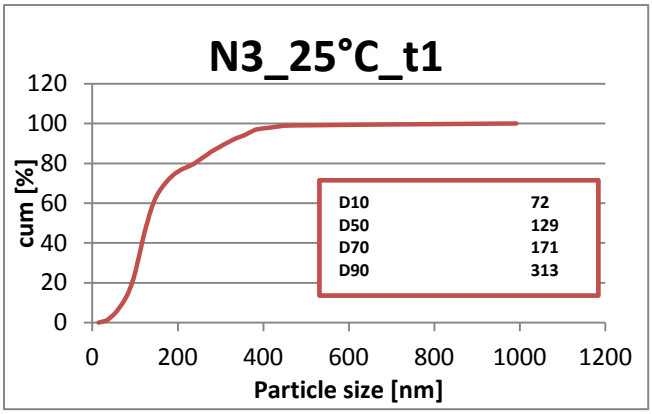
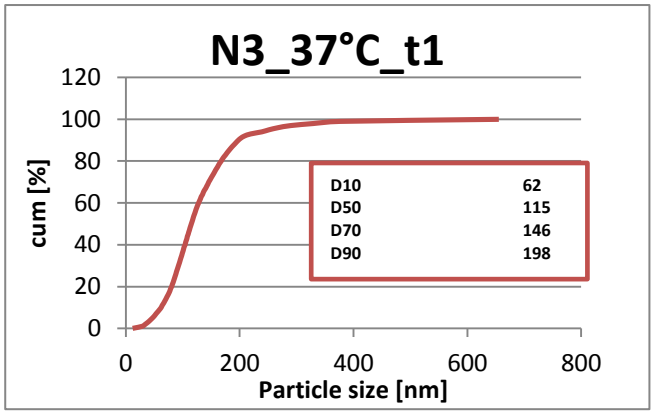
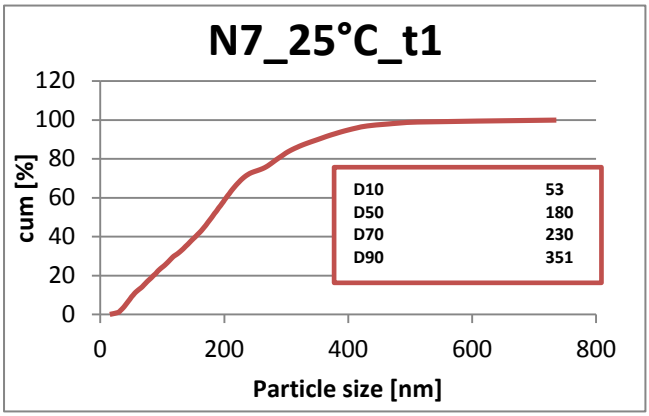
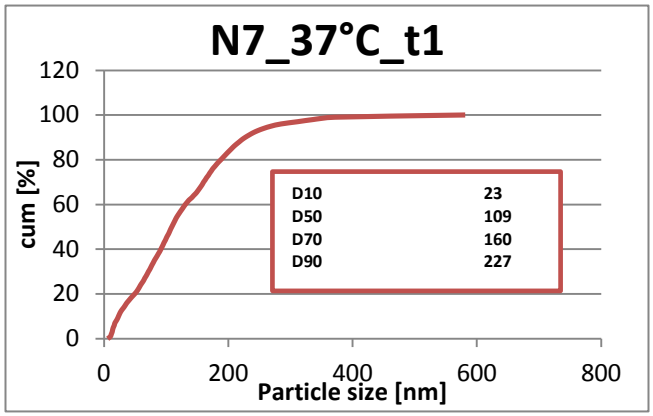


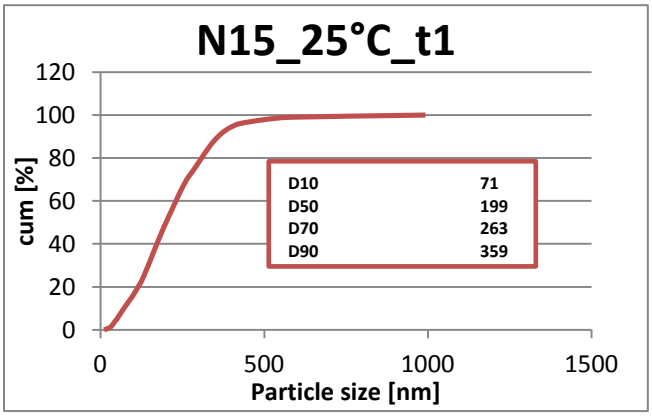
Figure 14: The distribution of particles in the nanoscale was diagrammed using a cumulative plot. The D values represent the percentage of particles below this size. These plots show the obtained particle distribution at time point zero.

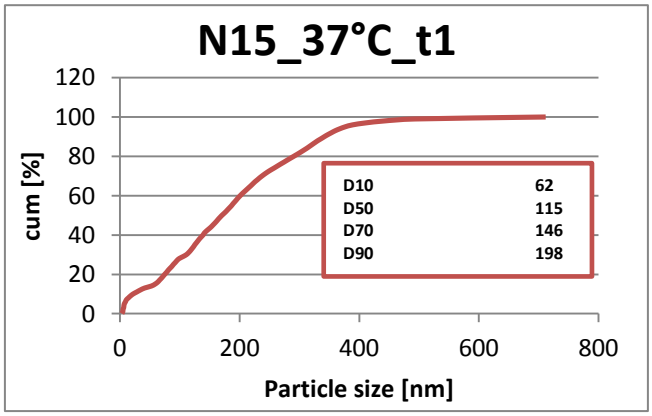

N3_25°C_t1
cum [%]
Particle size [nm]
D10 72
D50 129
D70 171
D90 313

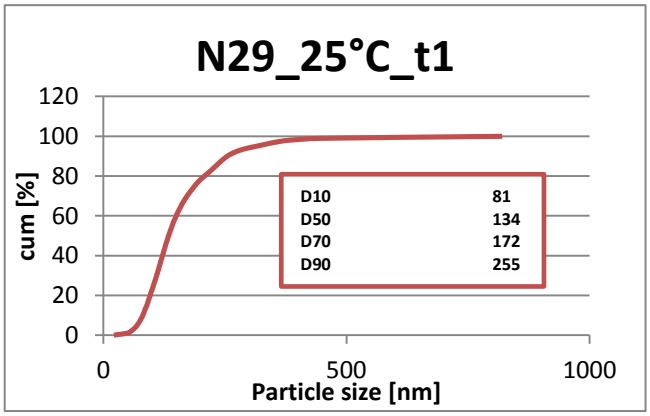

N3_37°C_t1
cum [%]
Particle size [nm]
D10 62
D50 115
D70 146
D90 198

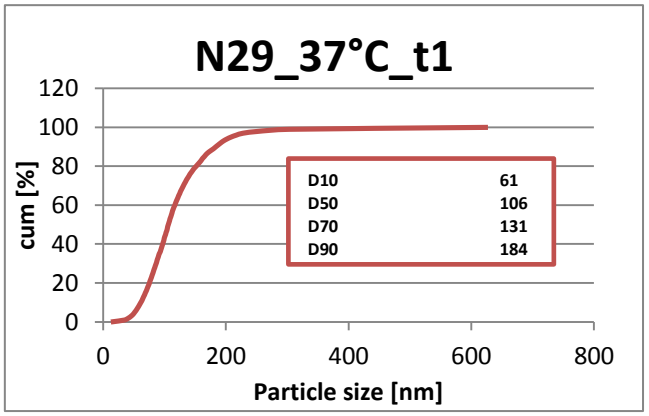

N7_25°C_t1
cum [%]
Particle size [nm]
D10 53
D50 180
D70 230
D90 351


N7_37°C_t1
cum [%]
Particle size [nm]
D10 23
D50 109
D70 160
D90 227


N15_25°C_t1
cum [%]
Particle size [nm]
D10 71
D50 199
D70 263
D90 359


N15_37°C_t1
cum [%]
Particle size [nm]
D10 62
D50 115
D70 146
D90 198


N29_25°C_t1
cum [%]
Particle size [nm]
D10 81
D50 134
D70 172
D90 255


N29_37°C_t1
cum [%]
Particle size [nm]
D10 61
D50 106
D70 131
D90 184

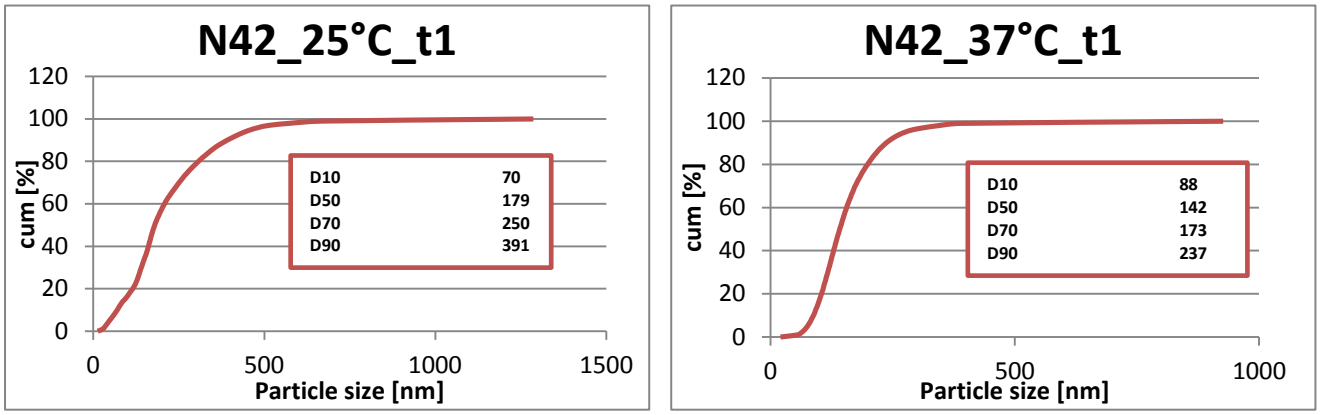
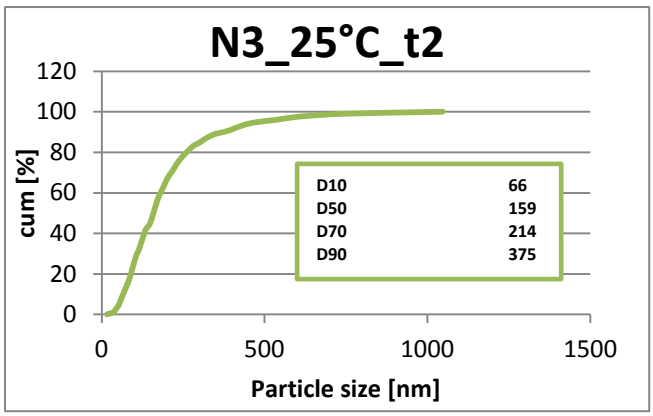
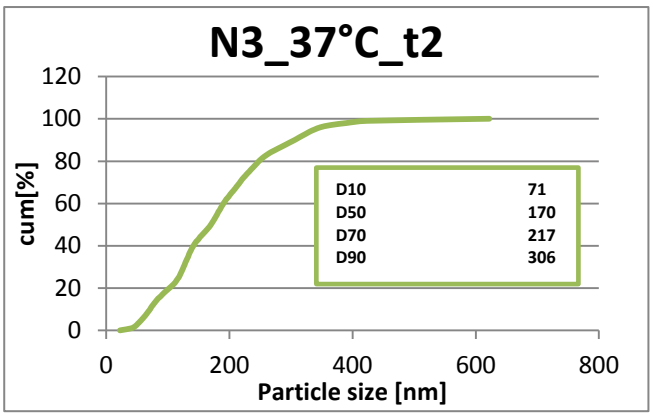
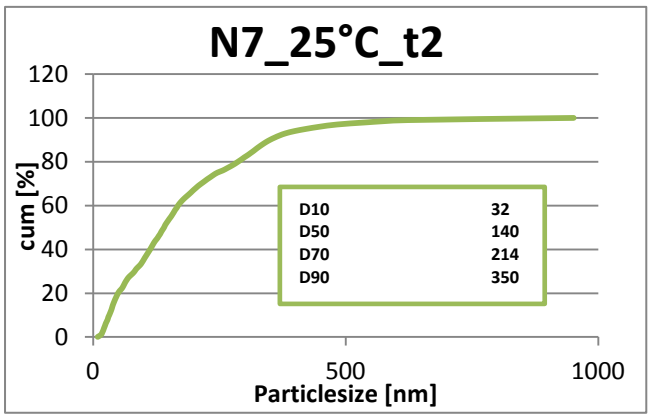
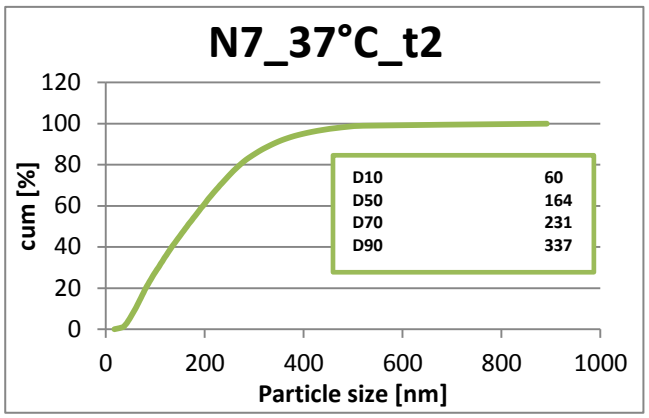


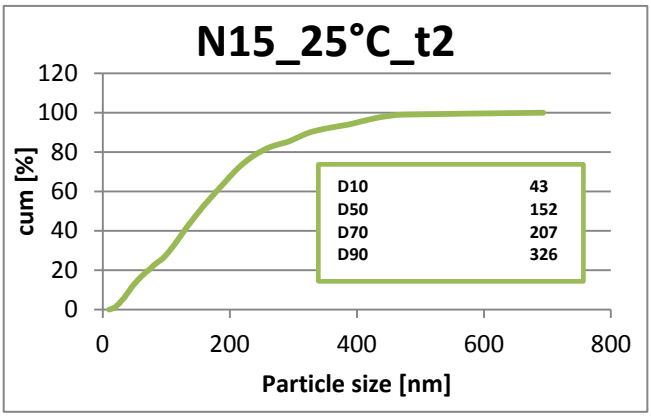
Figure 15: The distribution of particles in the nanoscale was diagrammed using a cumulative plot. The D values represent the percentage of particles below this size. These plots show the obtained particle distribution after one week of storage.

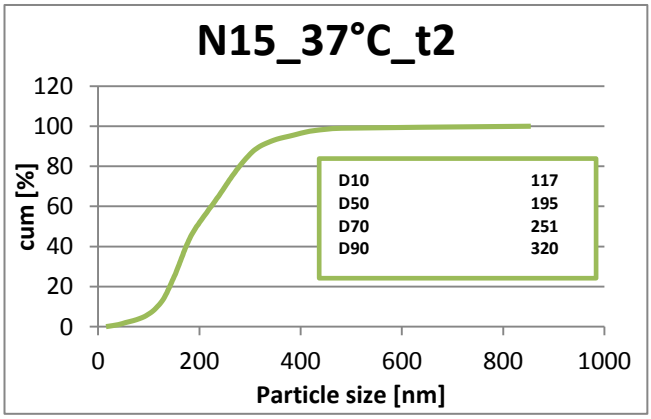

N3_25°C_t2
cum [%]
Particle size [nm]
D10 66
D50 159
D70 214
D90 375

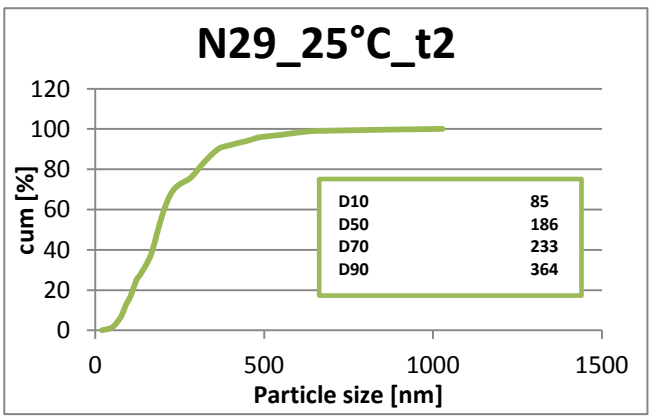

N3_37°C_t2
cum[%]
Particle size [nm]
D10 71
D50 170
D70 217
D90 306

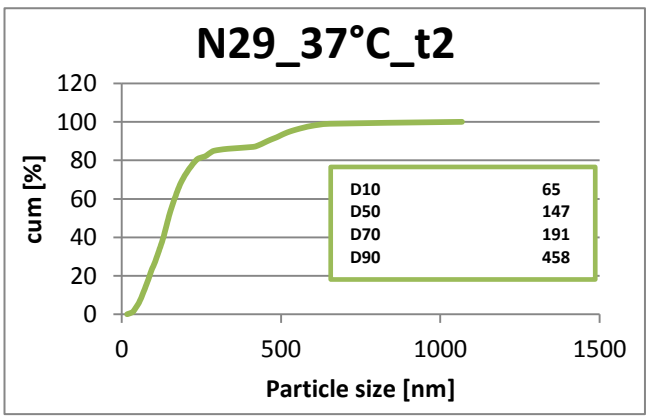

N7_25°C_t2
cum [%]
Particlesize [nm]
D10 32
D50 140
D70 214
D90 350


N7_37°C_t2
cum [%]
Particle size [nm]
D10 60
D50 164
D70 231
D90 337


N15_25°C_t2
cum [%]
Particle size [nm]
D10 43
D50 152
D70 207
D90 326


N15_37°C_t2
cum [%]
Particle size [nm]
D10 117
D50 195
D70 251
D90 320


N29_25°C_t2
cum [%]
Particle size [nm]
D10 85
D50 186
D70 233
D90 364


N29_37°C_t2
cum [%]
Particle size [nm]
D10 65
D50 147
D70 191
D90 458

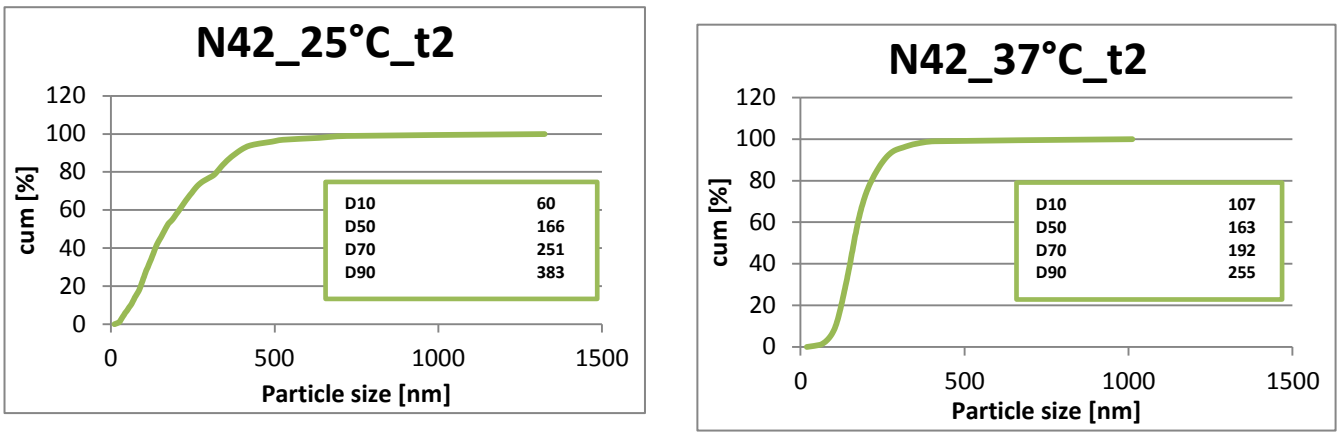
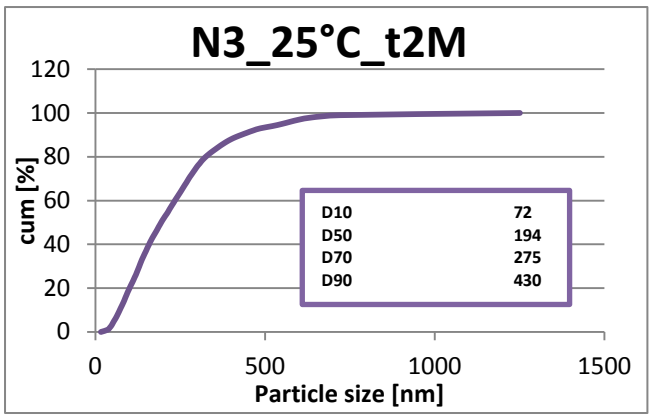
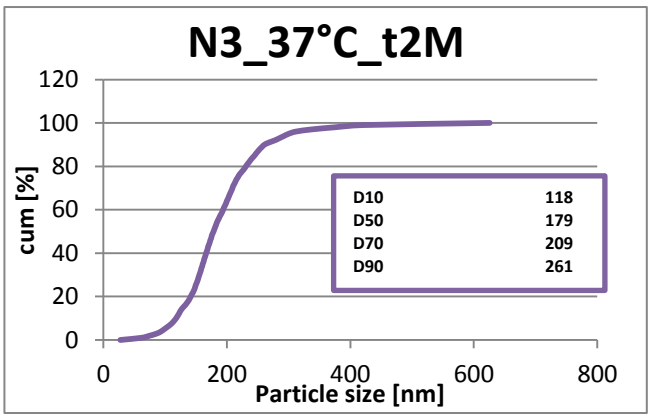
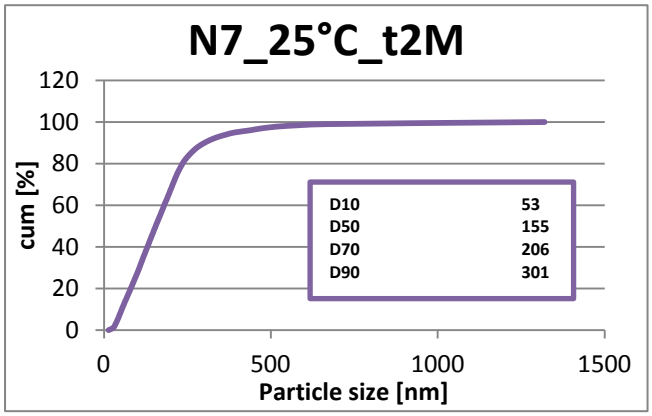
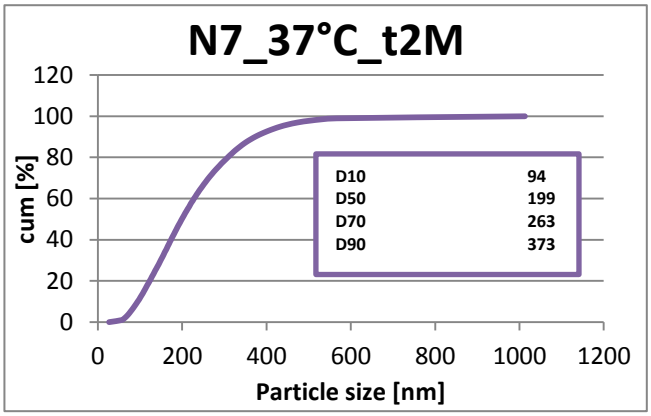


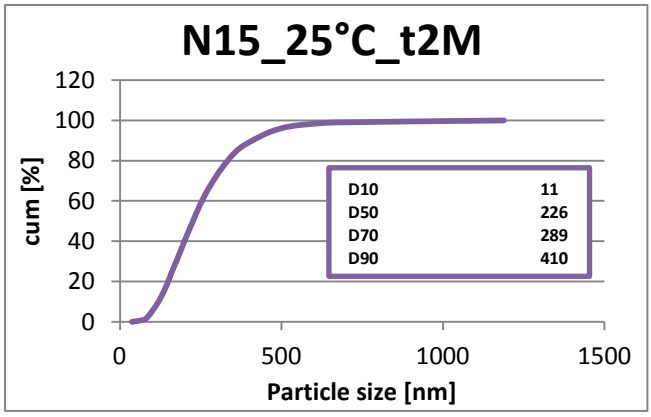
Figure 16: The distribution of particles in the nanoscale was diagrammed using a cumulative plot. The D values represent the percentage of particles below this size. These plots show the obtained particle distribution after two weeks of storage.

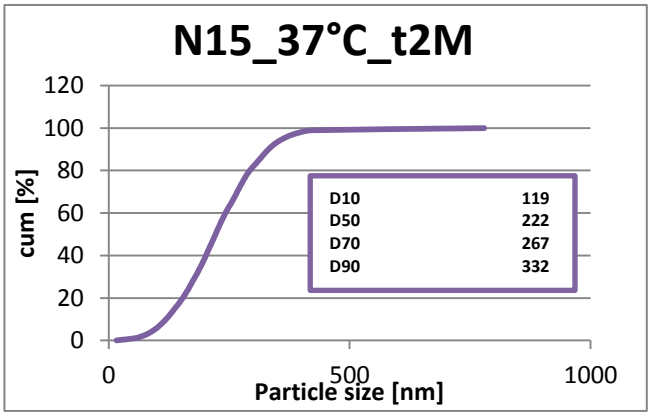

N3_25°C_t2M
cum [%]
Particle size [nm]
D10 72
D50 194
D70 275
D90 430

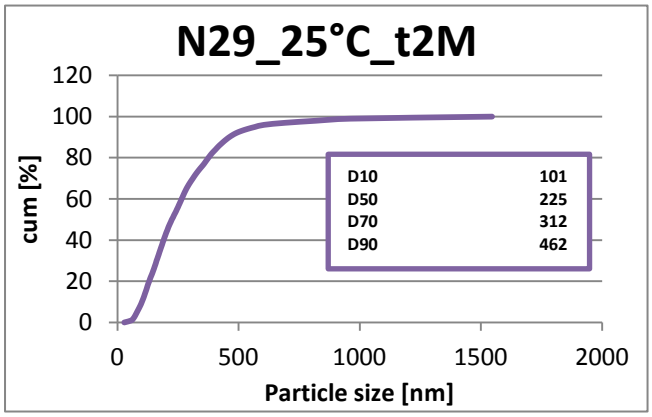

N3_37°C_t2M
cum [%]
Particle size [nm]
D10 118
D50 179
D70 209
D90 261


N7_25°C_t2M
cum [%]
Particle size [nm]
D10 53
D50 155
D70 206
D90 301


N7_37°C_t2M
cum [%]
Particle size [nm]
D10 94
D50 199
D70 263
D90 373


N15_25°C_t2M
cum [%]
Particle size [nm]
D10 11
D50 226
D70 289
D90 410


N15_37°C_t2M
cum [%]
Particle size [nm]
D10 119
D50 222
D70 267
D90 332


N29_25°C_t2M
cum [%]
Particle size [nm]
D10 101
D50 225
D70 312
D90 462

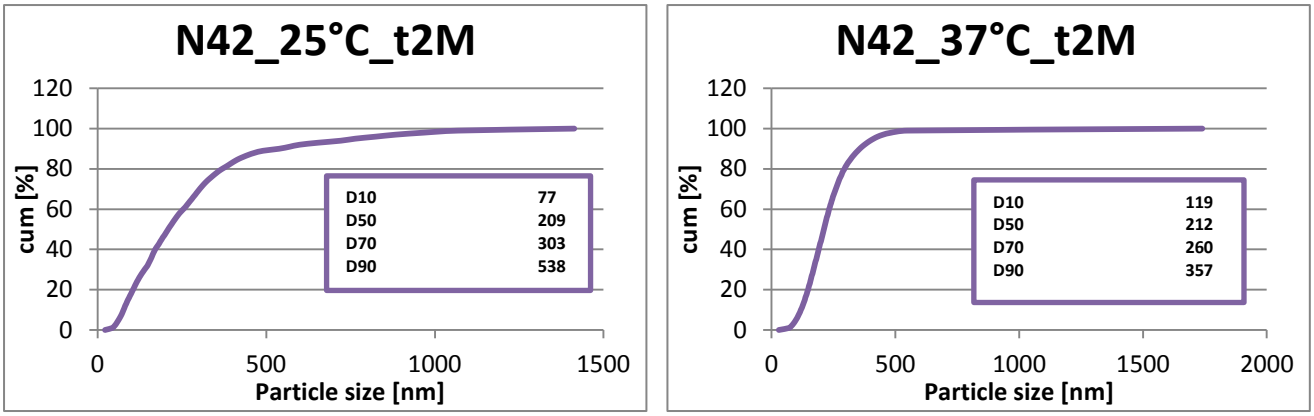
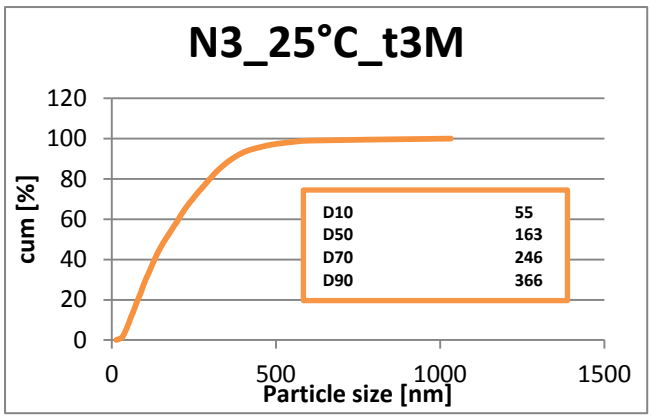
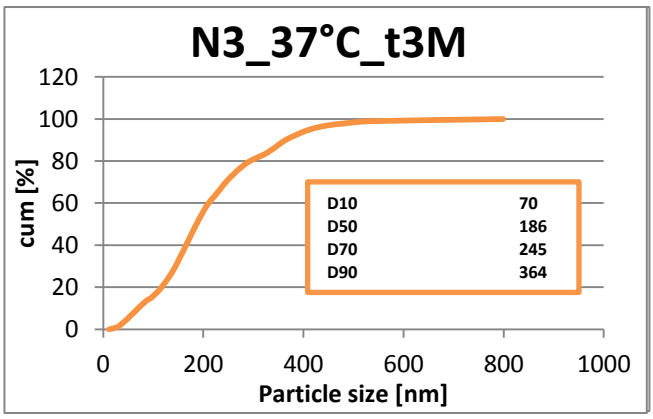
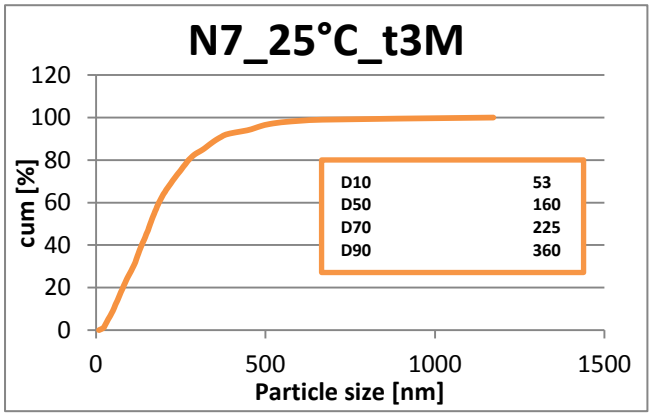
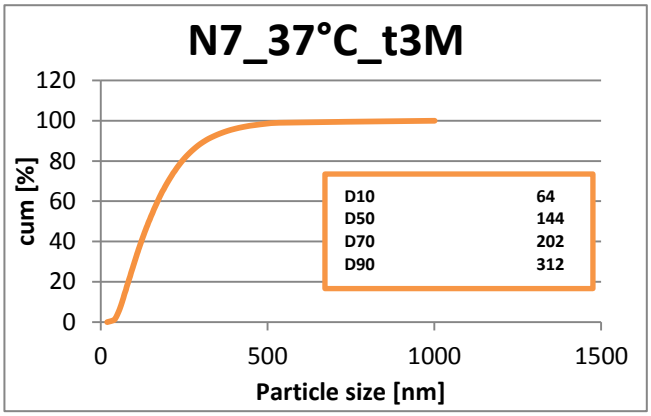


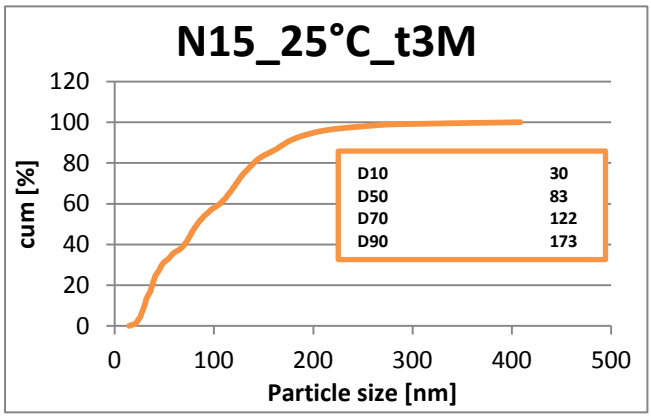
Figure 17: The distribution of particles in the nanoscale was diagrammed using a cumulative plot. The D values represent the percentage of particles below this size. These plots show the obtained particle distribution after two months of storage

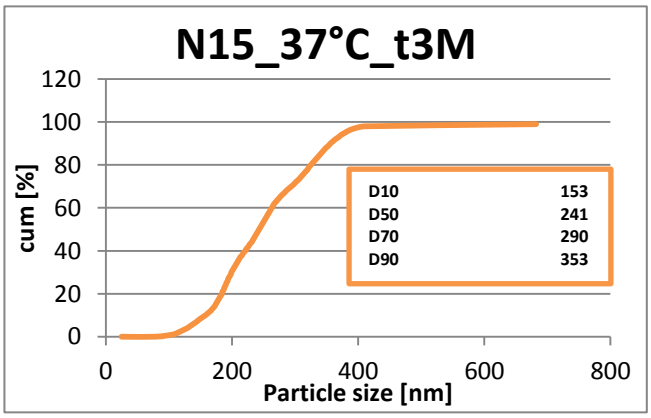

N3_25°C_t3M
cum [%]
Particle size [nm]
D10 55
D50 163
D70 246
D90 366

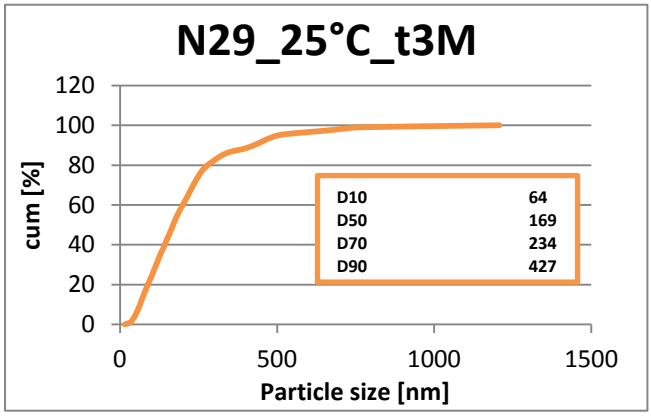

N3_37°C_t3M
cum [%]
Particle size [nm]
D10 70
D50 186
D70 245
D90 364

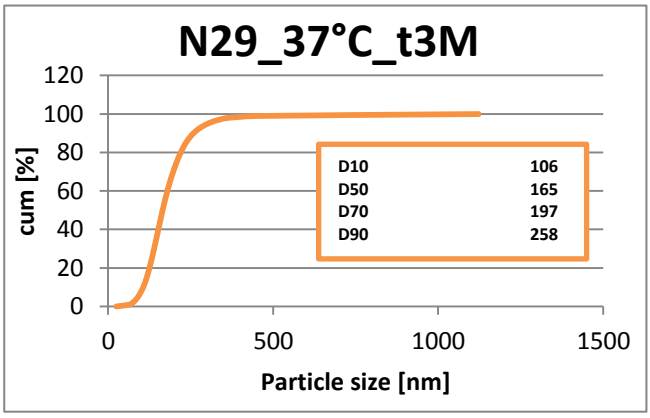

N7_25°C_t3M
cum [%]
Particle size [nm]
D10 53
D50 160
D70 225
D90 360


N7_37°C_t3M
cum [%]
Particle size [nm]
D10 64
D50 144
D70 202
D90 312


N15_25°C_t3M
cum [%]
Particle size [nm]
D10 30
D50 83
D70 122
D90 173


N15_37°C_t3M
cum [%]
Particle size [nm]
D10 153
D50 241
D70 290
D90 353


N29_25°C_t3M
cum [%]
Particle size [nm]
D10 64
D50 169
D70 234
D90 427


N29_37°C_t3M
cum [%]
Particle size [nm]
D10 106
D50 165
D70 197
D90 258

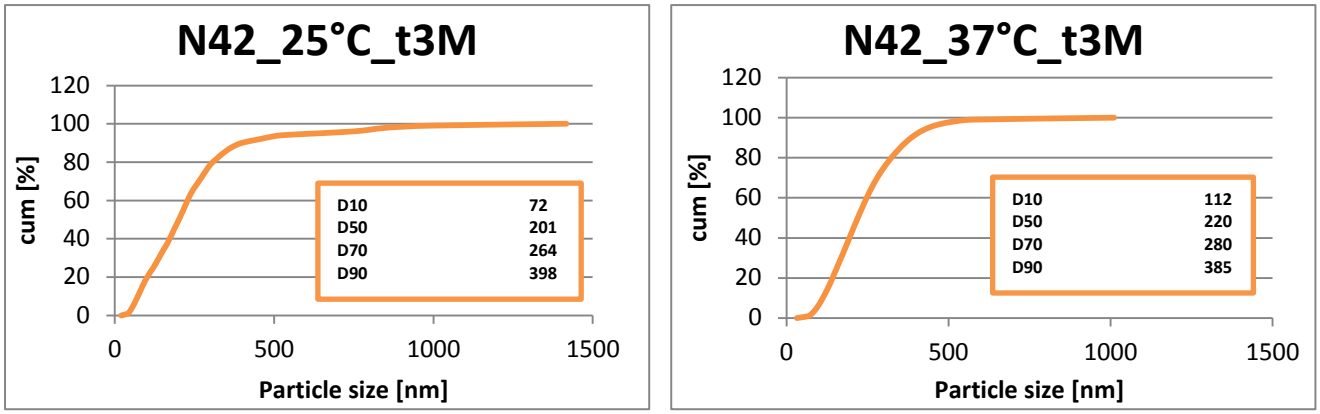


Figure 18: The distribution of particles in the nanoscale was diagrammed using a cumulative plot. The D values represent the percentage of particles below this size. These plots show the obtained particle distribution after three months of storage.

3.3.2.1 Discussion particle formation of stored protein formulations measured by NTA

NTA was illustrated using a cumulative data plot. The pharmaceutical important D-values (D10, D50, D70 and D90) were highlighted beside the plot.

Nanosight Analysing Software is not suitable of detecting particle formation due to aggregation in the microscale. So, NTA was used to find differences in particle size and distribution of particles between 1–1000 nm due to possible denaturation of protein or other ingredients.

Unfortunately, there was only a slight increase of particle size recognizable. With time of storage, the D90 value increases, but there was no big trend remarkable.

Fragmentation of IgG1 mAb or other ingredients seems to be no consequence of wrong storage.

3.3.3 Protein aggregation and denaturation analysis using Size – Exclusion – Chromatography (SEC)

Size-exclusion chromatography (SEC) is a chromatographic method in which molecules in solution are separated by their size, not by molecular weight. It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. SEC is a widely used polymer characterization method because of its ability to provide good Mw results for polymers.

SEC is a relative technique that requires column calibration in order to determine statistical average molecular weights and the molecular weight distribution of polymers [19], [20], [21].

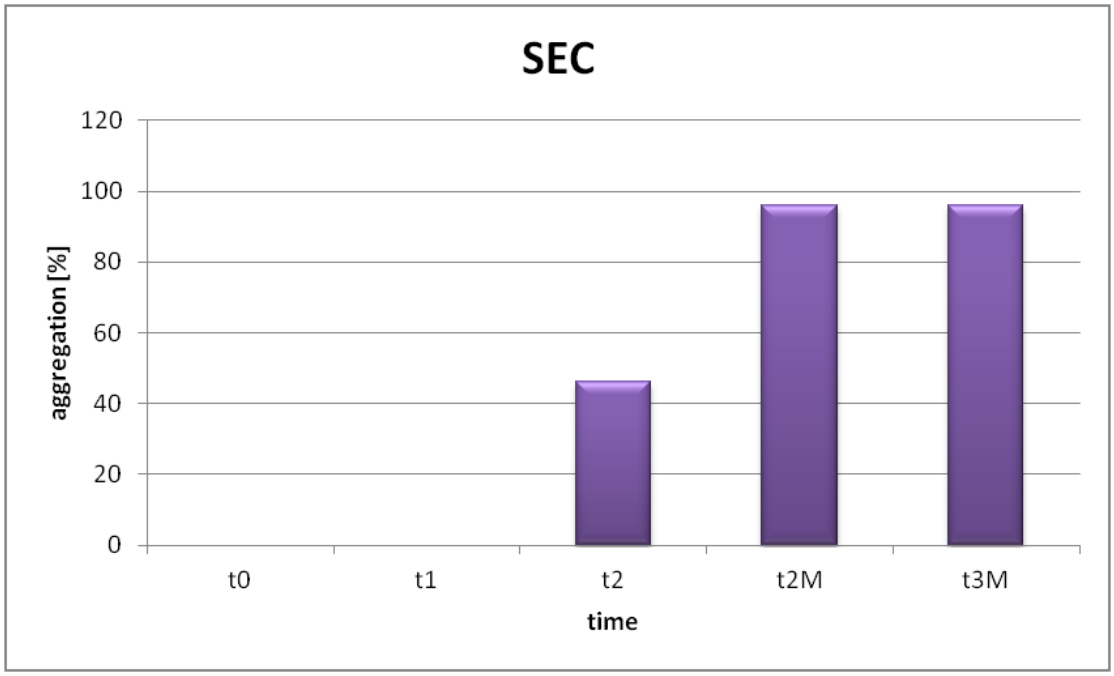


Figure 19: Formation of aggregates during storage. The histogram shows, that with time of storage the percentage of aggregates increases.

3.3.3.1 Discussion of protein aggregation and denaturation using SEC

As mentioned above, SEC separates molecules by size. The protein solution passes through the packed column. Smaller molecules move slower through the column because they are restrained by the packing material. Therefore, building of aggregates should lead to a lower retention time than the native protein. As mentioned in 2.1.4.4.4, each formulation ingredient was measured separately to find out their retention time. For IgG1 mAb the retention time was 12 min. In the final formulation another peak around 9 min was dominant, which can be explained by dimerization or protein–excipient interaction. Therefore, both peaks were appointed as IgG1 mAb in its native form.

All peaks with a lower retention time were appointed as aggregates, all peaks with a higher retention time were appointed as denaturation of protein.

To get a good overview of the behaviour of IgG1 mAb under storage, all retention times that stands for building of aggregates were pooled together and the percentage of aggregates were compared to the percentage of IgG1 mAb still remained in the native form. This percentage over time was illustrated using a histogram (Figure 19).

The histogram shows that storage under two weeks does not lead to the formation of aggregates regardless of whether the samples were stored under 25°C or 37°C. Between two weeks and two months of storage the most aggregates were developed. After two months of storage under awkward conditions, nearly 99% of protein was already aggregated. This demonstrates that IgG1 mAb is really sensitive against unfavourable storage conditions. Therefore, the right storage of protein formulations is seemingly very important and need more attention in future projects.

3.3.4 Conformational changes of protein structure measured by Circular Dichroism spectroscopy (CD spectroscopy)

Circular Dichroism (CD) is the difference in the absorption of left-handed circularly polarised light (L-CPL) and right-handed circularly polarised light (R-CPL) and occurs when a molecule contains one or more chiral chromophores (light-absorbing groups) [18].

Circular Dichroism (CD) is a valuable tool to study the conformation of proteins.
It is a form of light absorption spectroscopy that measures the difference in absorbance of right- and left-circularly polarized light [17].
Different secondary structures of proteins (a-helix, b- sheets, b-turns and unordered) give characteristic CD spectra between 260 and approximately 180 nm [16].

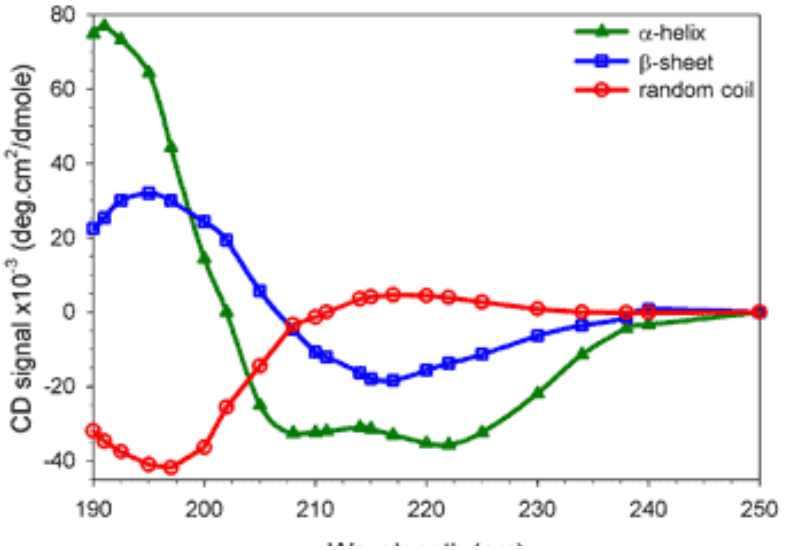
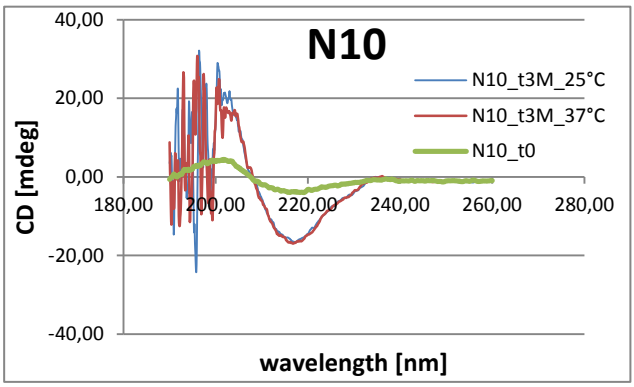
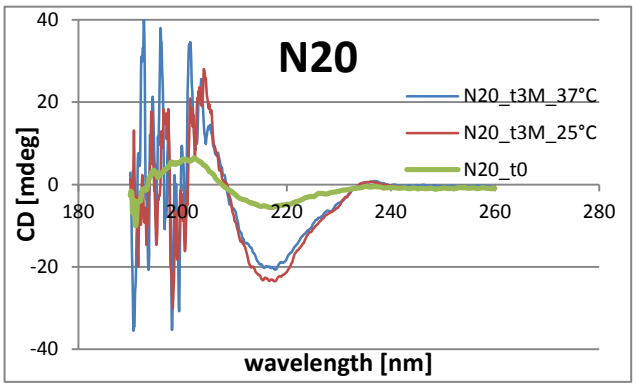
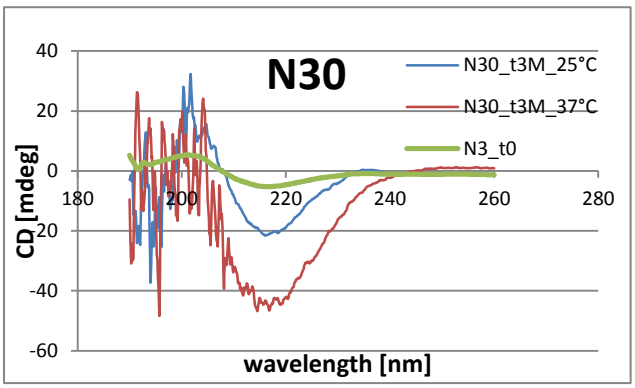
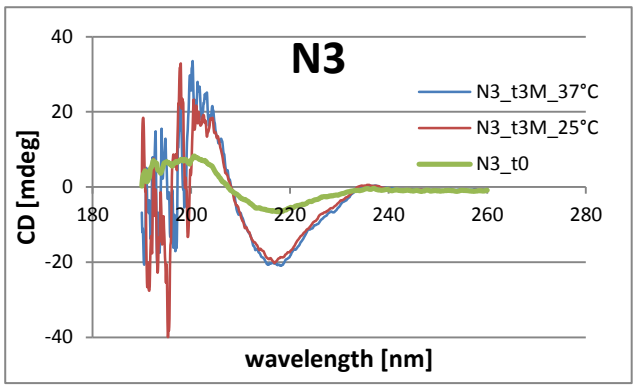


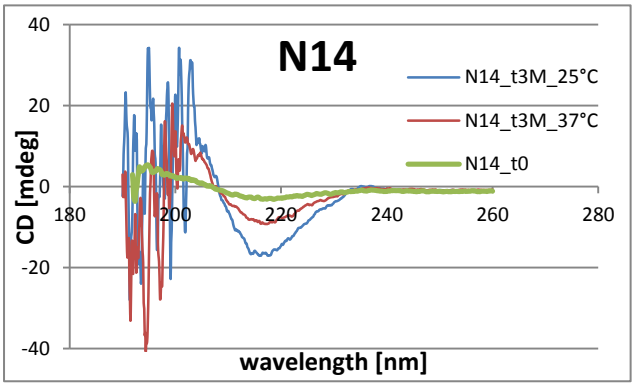
Figure 20: CD signals for pure α-helix, β-sheet and random coil structures used as characterization standard.

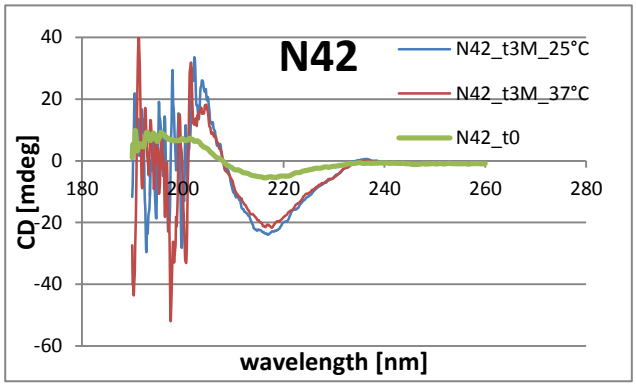

N10
N10_t3M_25°C
N10_t3M_37°C
N10_t0
CD [mdeg]
wavelength [nm]

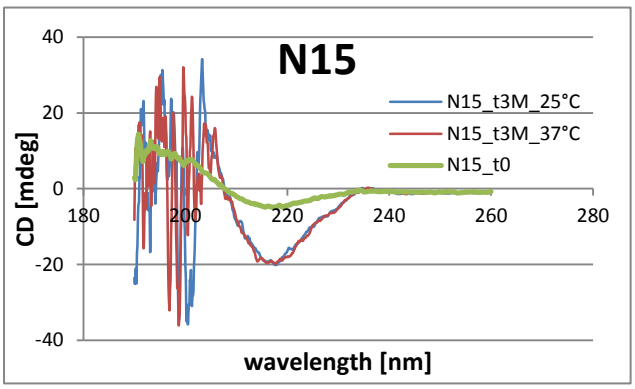

N20
N20_t3M_37°C
N20_t3M_25°C
N20_t0
CD [mdeg]
wavelength [nm]

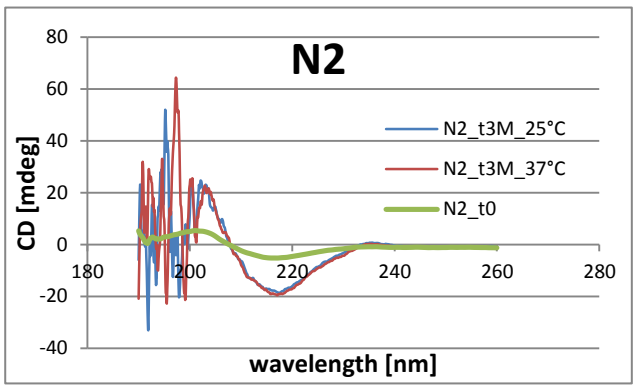

N30
N30_t3M_25°C
N30_t3M_37°C
N3_t0
CD [mdeg]
wavelength [nm]


N3
N3_t3M_37°C
N3_t3M_25°C
N3_t0
CD [mdeg]
wavelength [nm]


N14
N14_t3M_25°C
N14_t3M_37°C
N14_t0
CD [mdeg]
wavelength [nm]


N42
N42_t3M_25°C
N42_t3M_37°C
N42_t0
CD [mdeg]
wavelength [nm]


N15
N15_t3M_25°C
N15_t3M_37°C
N15_t0
CD [mdeg]
wavelength [nm]


N2
N2_t3M_25°C
N2_t3M_37°C
N2_t0
CD [mdeg]
wavelength [nm]

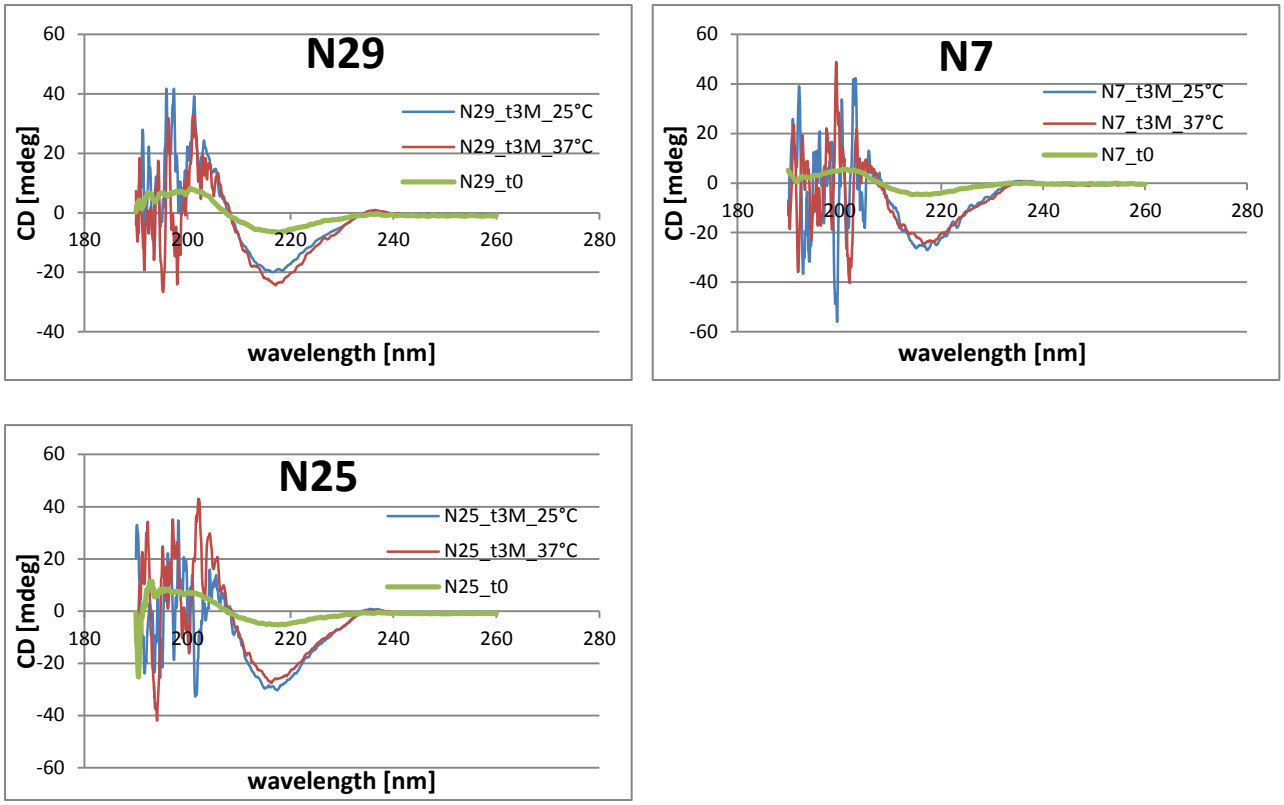


Figure 21: CD spectra of storage experiment. The spectra show, that the signal after storage strongly flutters. Protein aggregates locate on the glass cuvette and make it difficult for the light to pass.

3.3.4.1 Discussion of conformational changes of protein structure measured by CD spectroscopy

CD spectroscopy was done to demonstrate eventual conformational changes of the protein during storage time. Therefore, the samples were measured before and after storage. As can be seen in Figure 21, all formulations act similar. After storage, the secondary structure of IgG1 mAb does not differ from the conformation of the protein before storage. But due to aggregation, the light beam is not able to pass the solution unimpeded leading to a shiver of signal. So, it seems that the mAb does not unfold. It aggregates as a dense pack due to protein interactions.

4 CONCLUSIO

The goal of this master thesis was to take Quality by Design (QbD) to the next level. The Design of Experiments (DoE) software MODDE 9.1.1 can design and handle multiple qualitative and quantitative factors, telling you which factor has the most influence and revealing the impact on selected combinations of factors. MODDE 9.1.1 creates your design space for low risk operations and summarizes all statistical results in clear, accessible graphs. In my work I wanted to base the formulation development on a rational approach combining different accelerated stress conditions.

The common practice is to stress your protein in different formulations, leading to different optimal formulation conditions for each stressing factor. Our overall approach is to bundle the outcome of different independent stressing experiments in one multivariate model that will predict a global optimal formulation for the target protein. Therefore, my work set its focus on simplifying the commonly used empirical study procedure using high-throughput methods and finding the robust areas of the most common stressing methods – temperature, agitation, F/T-Cycles – using DoE. For the used excipients, this work also included an investigation of potential new surfactants as excipients for pharmaceutical protein drug formulation development.

Furthermore, I selected several formulation conditions for accelerated stability studies in climate chambers, using NTA, SEC, CD and MFI for biophysical characterization.

I was able to demonstrate that with the help of DoE and high-throughput methods, it is possible to simplify the experimental setup for protein formulation development at a high level, still receiving stress-stable formulations that can be gracefully used in pharmacy. I could also demonstrate that the used excipients differ in the stabilization support against different stressing conditions. I could also show increased stabilization behaviour by using more than one surfactant in a formulation.

For IgG1 mAb the storage experiments showed, that storage at room temperature or above could have a high negative impact on protein stability, especially notable in the micrometer scale. The unfavorable storage conditions led to aggregation by protein-protein or protein-excipient interactions.

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