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Comparison of different protein concentration techniques within preformulation development

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ABSTRACT

Highly concentrated antibody solutions are of increasing importance in the pharmaceutical industry. During production highly concentrated solutions are usually prepared by tangential flow filtration (TFF). Since this technique is often not applicable in the early phase of formulation development, where the available amounts of protein are commonly very small, small scale techniques like dialysis or ultrafiltration with stirred cells or centrifugal filters have to be employed. In this study the small scale techniques were compared to tangential flow filtration, with regard to the quality and stability of the concentrated products. The achievable concentration of a protein, when starting from a model antibody solution with 10 mg/ml, was also assessed. Concentrations above 100 mg/ml could be obtained with all techniques, however with different product qualities. The stability of the highly concentrated solutions (100 mg/ml) was analyzed by turbidity measurements, size exclusion chromatography (SEC), SDS-PAGE and isoelectric focusing (IEF) after storage at 25 and 40 °C for 8 weeks. Solutions prepared by dialysis exhibited the smallest degree of instability, whereas those manufactured by centrifugal filtration revealed the best comparability to products obtained by tangential flow filtration with regard to the results of isoelectric focusing, turbidity measurements (UV–vis) and size exclusion chromatography. Stability differences were observed within all analytical methods, primarily after storage and not directly after the concentration process.

1. Introduction

Over the last decade monoclonal antibodies have gained increasing therapeutic importance in fields like cancer, immune mediated diseases and transplant rejection (Berger et al., 2002; Harris, 2004). Therapy with monoclonal antibodies often requires single doses of several mg/kg, with i.v. injection or infusion being the preferred route of administration (Untch et al., 2003). Most diseases, which are treated with antibody based pharmaceuticals, are chronic and require continuous or even lifelong therapy. With regard to patient convenience and healthcare costs it would be of great benefit if these therapeutics could be self-administered by the patient. This would reduce the frequency of medical interventions by a physician or at a hospital. The availability of highly concentrated antibody solutions that can be administered by the *i.m* or *s.c.* route is essential for this. Therefore, the development of corresponding formulations is becoming a major focus in the area of protein pharmaceuticals (Daugherty and Mrsny, 2006). Further advantages of highly concentrated antibody solutions include reduced storage and logistical costs due to the smaller volumes, as well as the easier handling during manufacturing. Potential disadvantages of such formulations are a lower bioavailability and a higher risk of immunogenicity (Shire et al., 2004), as well as stability problems due to protein self-association and aggregation (Saluja and Kalonia, 2008).

The formulation of highly concentrated protein solutions is not an easy task. A frequent complication is an increase in viscosity at higher concentrations. The viscosity can be reduced by excipients (Kanai et al., 2008; Salinas et al., 2010) which can, for example, increase the ionic strength of the formulation (Liu et al., 2005). While the stability against mechanical stress, such as shaking, increases with protein concentration, the storage stability at elevated temperatures decreases in highly concentrated protein solutions (Treuheit et al., 2002). This may lead to the formation of turbid solutions containing aggregates (Daugherty and Mrsny, 2006).

The manufacturing of highly concentrated antibody solutions can be performed using different techniques. For industrial

Abbreviations: TFF, tangential flow filtration; SEC, size exclusion chromatography; IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; AUC, area under the curve; PES, polyethersulfone; RC, regenerated cellulose; PBS, phosphate buffered saline; PI, isoelectric point.

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production, ultrafiltration via tangential flow filtration (TFF) is the standard method as large volumes of solution can be processed (Russell et al., 2007; Rosenberg et al., 2009). However, these comparatively large sample volumes are usually not available during the early phases of pharmaceutical development. Therefore, small scale techniques like ultrafiltration employing stirred cells or centrifugal filters, as well as dialysis-based setups, are used as alternatives in pre-formulation development (Shire et al., 2004; Phillips and Signs, 2004).

Although all these techniques concentrate protein solutions with the aid of filter membranes, there are several differences in the details of each process. In tangential flow filtration, the sample flows tangentially over the membrane with mechanic recirculation. The tangential flow reduces sticking of protein to the membrane and avoids a "dead end" filtration situation (Rubin and Christy, 2002). "Dead end" filtration can lead to blocking of the membrane and the formation of aggregates, due to extremely high concentrations of protein which build up in the gel and polarization boundary layer near the membrane (Rosenberg et al., 2009). The small scale techniques circumvent this problem in different ways. Ultrafiltration in stirred cells is driven by gas pressure under continuous stirring of the solution to avoid high protein concentrations close to the membrane. In centrifugal filter tubes, the filter membrane is positioned in an inclined manner to avoid "dead end" filtration. To achieve appropriate concentrations with a dialysis setup, the dialysis membrane, which encloses the sample, is brought in contact with a hyperosmotic powder leading to the transport of solvent from the protein solution into the powder.

A major problem of concentrating protein solutions by these techniques is the occurrence of shear stress that may lead to protein aggregation (Mahler et al., 2005; Bee et al., 2009). Shear stress may occur due to pumping, centrifugation or stirring, as required in the ultrafiltration methods (Maruyama et al., 2001; Kiese et al., 2008). Slight shear forces may also result from the efflux during the dialysis process. The presence of air–water-interfaces or interfaces between other hydrophobic surfaces and the solution might also trigger protein aggregation and thus destabilization of the solution during the concentration process (Maa and Hsu, 1998).

The destabilizing forces can occur to different extents with the different techniques. For a later large-scale production process, employing tangential flow filtration as the concentration method, it would be important to know which of the small-scale laboratory methods can be employed in pre-formulation development and causes similar stress on the formulation as does tangential flow filtration. It was thus the aim of this study to compare the three small-scale methods with tangential flow filtration with respect to the quality and stability of the concentrated products. The efficiency of the different techniques (speed of concentration and final concentration achievable), as well as the influence of different parameters like the material and molecular weight cut-off of the membrane on the concentration process, were also of interest.

2. Materials and methods

2.1. Materials

A solution of monoclonal IgG antibody (molecular weight: 152 kDa) in PBS (phosphate buffered saline; Dulbecco's PBS, PAA Laboratories GmbH, Germany, pH 7.2) provided by Merck Serono was used as model protein. The concentration was 10 mg/ml, except for the study concerning the influence of the membrane material (2 mg/ml).

2.2. Methods

2.2.1. Ultrafiltration via centrifugal filters

The antibody solution was concentrated using Amicon Ultra filtration tubes (15 ml; Millipore; USA) usually equipped with a 30 kDa regenerated cellulose (RC) membrane. To study the influence of the molecular weight cut-off, 100 and 10 kDa RC-membranes were additionally used. The tubes were filled with 15 ml of the antibody solution and centrifuged at 2000 × *g* and 23 °C in a Multifuge 3SR+ (Heraeus; Germany). The acceleration and the slowdown velocity were set to level 3 of 9 possible levels. To monitor the course of the filtration process centrifugation was stopped at given time points and samples (300 μl) were taken out of the upper area of the tube. Before doing so, the solution was gently shaken to remove protein sticking to the membrane and to homogenize the solution. The protein concentration in these samples was determined by UV-spectroscopy. After sampling, the filtration process was continued. The filtrate (sampled from the lower area of the tube) was tested for protein concentration at the end of the filtration process to control the intactness of the membrane.

The material for the stability study was obtained using a separate centrifugation process, which was stopped after the time required to reach a concentration of 100 mg/ml according to the results of the previously performed concentration study (ca. 110 min).

2.2.2. Ultrafiltration in a stirred cell

An Amicon 8050 stirred cell (Millipore; USA) with a 30 kDa regenerated cellulose membrane (Ultracel YM-30; Millipore, USA) was employed to concentrate the antibody solution. To investigate the influence of the membrane material, a 30 kDa polyethersulfone membrane (Biomax-30; Millipore; USA) was used also. Each concentration process was carried out with a new membrane. Prior to the concentration studies, the membranes were washed inside the cell with 50 ml water for injection and 50 ml PBS using the same process parameters as for the concentration experiments. Subsequently, 50 ml antibody solution was filled into the cell which was placed onto a stirrer platform (Stir SB161; Stuart) and stirred at level 2. The cell was pressurized with 69 kPa nitrogen and the concentration process was carried out at room temperature. After different times, the cell was opened, samples were taken (300 μl) and measured by UV-spectroscopy to determine the concentration. At the end of the process the filtrate was tested as well to confirm the intactness of the membrane.

To obtain the material for the stability study, the concentration process was performed continuously and stopped after a time sufficient to reach a concentration of 100 mg/ml (ca. 210 min).

2.2.3. Dialysis

Slide-A-Lyzer dialysis cassettes (12–30 ml; Pierce; USA) with a regenerated cellulose membrane were employed. For this technique membranes with a 10 kDa molecular weight cut-off were used because 30 kDa membranes were not available. Before use the dialysis cassettes were incubated for 2 min in PBS. Afterwards, they were filled with 30 ml of the protein solution by using a syringe (50 ml; B Braun Melsungen AG; Germany) and a needle (18G, Sterican, B.Braun Melsungen AG, Germany). 10 g of hyperosmotic powder (Spectra/Gel™ Absorbent; Spectrum Laboratories Inc.; USA) was applied onto the outside of the membrane and fixed with aluminium foil. After different times, 300 μl of sample was withdrawn from the cassette using a syringe and a needle. The whole process was performed at room temperature.

The material for the stability study was obtained in a separate process (without intermittent sampling) which was carried

out for a time sufficient to reach a concentration of 100 mg/ml (ca. 1800 min).

2.2.4. Tangential flow filtration (TFF)

Lab scale tangential flow filtration (Millipore; USA) was carried out at room temperature to concentrate the protein solution. The cassette used within the system contained a regenerated cellulose membrane with a cut-off of 30 kDa (Ultracel PLCTK; Millipore; USA). The membrane was first washed with water for injection and tested by determining the volume of the permeating water for 1 min. 200 ml of the antibody solution was used as the starting volume. The trans-membrane-pressure over the membrane was set to 150 kPa with an inlet pressure of 200 kPa and an outlet pressure of 100 kPa. The speed of the stirrer inside the tank was set to level 2. After different times, 300 μl of sample were taken out of the tank and analyzed for protein concentration by UV-spectroscopy. At the end of the process, also the filtrate was measured to check the intactness of the membrane.

A separate concentration process was carried out without intermittent sampling to obtain the solution for the stability study. This process was carried out for ca. 55 min which was sufficient to reach a concentration of 100 mg/ml.

2.2.5. Stability study

For each concentration technique, 0.7 ml protein solution were set to exactly 100 mg/ml with PBS and filtered using a 0.2 μm polyethersulfone membrane, filled into 2 ml vials (Schott Rohrglas GmbH; Germany) and sealed with chlorobutyl rubber stoppers (coated with silicone and crimped with aluminium caps; West Pharmaceutical Service; Germany) under aseptic conditions. The vials had been washed in a dishwasher (Miele Professional G7836CD; Miele & Cie. KG, Germany) with a "particle free" programme and autoclaved. For each concentration technique three vials were stored at 25 °C and 40 °C for 8 weeks. Three additional vials of each concentration technique were analyzed directly after concentrating as start samples.

2.2.6. Concentration determination

The concentration of the solutions was measured at a UV–vis spectrophotometer (NanoPhotometer™ Implen GmbH, Germany) with LabelGuard™ Microliter Cell which allows the measurement of highly concentrated protein samples without dilution (sample volume 4 μl). The measurement wavelength was set to 280 nm minus 320 nm (correction for turbidity of the solution). PBS (pH 7.2) was used as a blank. The protein concentration was calculated from the absorption values according to Lambert–Beer's law using a specific extinction coefficient for the antibody of 1.4 cm^2/mg and the path length of the cell (1 or 0.2 mm). The choice of the path length depended on the concentration of the solutions and was indicated by the spectrophotometer. Between measurements the cell was cleaned with water for injection and PBS.

2.2.7. Turbidity measurements

Turbidity measurements were carried out at 350 nm with a NanoPhotometer™ UV-vis spectrophotometer (Implen GmbH, Germany), in plastic cells with a path length of 10 mm (Brand GmbH & Co KG, Germany). Pure water for injection was used as a blank (Eckhardt et al., 1994). Samples were measured undiluted. Test measurements with formazine solutions of different turbidities indicated that absorption values up to approximately 2.5 are linear. The absorption values of the placebo, PBS buffer at pH 7.2, measured against water were 0.001.

2.2.8. Size exclusion chromatography (SEC)

The samples were diluted to 5 mg/ml with PBS and centrifuged for 3 min at 8000 rpm (4300 × *g*) (Biofuge fresco; Heraeus Instruments GmbH; Germany) to spin down precipitates. A volume of 20 μl (representing 100 μg antibody) was injected per run. The analysis was performed at room temperature on a Merck-Hitachi HPLC with the following components: D7000 Interface, L7400 UV-Detector, L6000 A Pump, L6200 Intelligent Pump and L7250 programmable auto sampler. The mobile phase used was 0.4 M $NaClO_4$, 0.05 M $NaPO_4$ buffer (pH 7.2), and delivered at a rate of 0.5 ml/min through a TSK-Gel G3000SWXL column 7.8 mm ID × 30.0 cm L (Tosoh Bioscience GmbH; Germany). The column eluate was monitored at 280 nm. Using the HPLC Software EZChrom Elite Client, Version 3.0 (Scientific Software International Inc, USA) the total area under the curve for the antibody monomer was calculated and compared to the monomer content of a standard. Soluble aggregates were calculated by setting all peaks of one run to 100% and calculating the percentage of aggregates. A gel filtration standard (Bio-Rad Laboratories Inc., USA) was injected regularly to assure the performance of the HPLC system. Placebos of the antibody solutions were tested in parallel.

2.2.9. SDS-PAGE

The presence of covalent aggregates was analyzed by non-reducing SDS-PAGE and the formation of degradation products by reducing SDS-PAGE. The protein solution was diluted to 2 mg/ml with PBS (pH 7.2) and centrifuged for 3 min at 8000 rpm (4300 × *g*) (Biofuge fresco; Heraeus Instruments GmbH; Germany) to spin down precipitates. The sample was diluted to 0.04 mg/ml antibody by adding 50 μl tris–glycine–SDS sample buffer (Novex® Tris-Glycine SDS Sample Buffer (2×); Invitrogen; USA), and 30 μl water for injection to 20 μl of sample (2 mg/ml). To achieve reducing conditions, 10 μl of water for injection was replaced by a 100 mg/ml DTT solution (1,4-dithiotreitol; Merck KGaA; Germany). After heating to 95 °C for 4 min, 10 μl of each sample (containing 0.4 μg antibody) was loaded per lane on a tris–glycine-gel (4–20% Tris-Glycine Gel, 1.0 mm × 12 well, Invitrogen, USA) and focused at 300 V, 40 mA and 25 W with a Power Ease 500 power supply (Invitrogen, USA) for 45 min. A standard Coomassie staining protocol including washing, fixing, staining, destaining and drying was used to detect the protein bands. The analysis of the dried gels was performed after scanning the gels with a densitometer (Personal Densitometer SI, Amersham Biosciences, Germany) by use of the software AIDA (Advanced Image Data Analyzer; Version 4.19.029, Raytest GmbH, Germany). In order to determine the molecular weight of the detected bands, a marker (SeeBlue Plus2; Invitrogen, USA) was used. For the non-reducing SDS-PAGE the fraction of monomer was calculated as percentage of the total peak area of one run. For the reducing SDS-PAGE the same was done for heavy and light chain fragments.

2.2.10. Isoelectric focusing (IEF)

Isoelectric focusing was performed to obtain information on the chemical stability of the antibody after storage at elevated temperatures. Focusing was carried out on agarose IEF plates with a pH range of 3–10 (Lonza, Rockland, USA) on a cooling plate (Multiphor2, Amersham Bioscience, Sweden). Voltage (1000 V, 15 W, 25 mA) was generated by a BluePower3000 power supply unit (Serva electrophoresis GmbH, Germany). The samples were diluted to 2 mg/ml with PBS and desalted by washing them with water for injection using Microcon Centrifugal Filters (Ultracel YM-30; regenerated cellulose; Millipore; USA) in a centrifuge (Biofuge fresco; Heraeus Instruments GmbH; Germany) at 8000 rpm. 10 μl (containing 20 μg antibody) was loaded per lane and focused with 1000 V, 25 mA and 15 W for 75 min. The Isoelectric Focusing Calibration Kit (high range pH 5–10.5; GE Healthcare; USA) was used as

a marker. For detection of the lanes a standard Coomassie staining protocol, including fixing, washing, drying, staining, destaining and drying, was used. Subsequently, the gels were analyzed with the Personal Densitometer SI (Amersham Biosciences, Germany) and AIDA software (Advanced Image Data Analyzer; Version 4.19.029, Raytest GmbH, Germany). In the figures, the percentage of the area of each band is plotted against its isoelectric point (PI).

3. Results and discussion

3.1. Influence of membrane parameters on the concentration process

As a first step in the evaluation process of different concentration techniques, the influence of the membrane material and the molecular weight cut-off was studied. Different types of membrane materials can be used for protein concentration purposes (Saxena et al., 2009), with the most commonly employed being polyethersulfone (PES) and regenerated cellulose (RC). To test for potential influences of the membrane material, an antibody solution (2 mg/ml in PBS) was concentrated via stirred cell ultrafiltration using either a polyethersulfone or a regenerated cellulose membrane. The concentration–time profiles (Fig. 1A) indicate that the stirred cell filtration process was not reproducible, with great variability observed between different repetitions. Within 45–65 min, different concentrations of 35–50 mg/ml were achieved, with no clear advantage of one of the two membrane types. The results of size exclusion chromatography (SEC) monomer determination (Fig. 1B) pointed to lower losses in monomer content when polyethersulfone was used as membrane material. Monomer loss was probably due to adsorbance to the membrane or the formation of aggregates. For both membrane materials the formation of insoluble aggregates was observed visually and by spectroscopic turbidity measurements, with no clear difference observed. The fraction of soluble aggregates as determined via SEC (data not shown) was also similar for both membrane types. Therefore, adsorption of monomer to the regenerated cellulose material seems to be the most likely cause for the observed differences in monomer content. Differences in the behaviour of proteins towards different membrane materials have been previously reported (Maruyama et al., 2001). In spite of the slight advantages observed for the polyethersulfone membrane in this experiment, all further studies were carried out with regenerated cellulose membranes since polyethersulfone membranes were not available for all of the techniques under investigation.

The influence of the membrane cut-off was tested with regenerated cellulose membranes with 10, 30 and 100 kDa cut-off (i.e., well below the molecular weight of the antibody under investigation) in centrifugal filters, starting with a protein concentration of 10 mg/ml in PBS. At a centrifugation speed of 2000 × *g*, there were no remarkable differences in the concentration–time profiles for the membranes with different cut-offs (Fig. 2A). Larger pore sizes thus did not lead to a more rapid concentration. Turbidity measurements (data not shown) and the determination of monomer content by SEC (Fig. 2B) also did not reveal differences. In contrast to the above mentioned results with the stirred cell, there was no significant loss in monomer content, in spite of the use of regenerated cellulose membranes, when using the centrifugal filters. This may be due to the higher start concentration or point to more gentle conditions upon centrifugal concentration.

Marginal differences between the membranes with different cut-offs were observed concerning the presence of soluble aggregates and degradation products (Fig. 2C and D). The fraction of soluble aggregates was slightly higher after concentration with 10 and 100 kDa membranes than by the 30 kDa membrane. The 100 kDa membrane led to a decrease in the fraction of degraded protein (Fig. 2D). This might point to an escape of some degraded protein through the large pores of the 100 kDa membrane.

Although the observed influences of the molecular weight cut-off were not very significant, the use of membranes with 100 kDa cut-off is not recommendable when concentrating solutions of antibodies with a molecular weight of approximately 150 kDa. Also a molecular weight cut-off of 10 kDa seems to be less favourable. As a consequence, all experiments described below were carried out with a membrane with a cut-off of 30 kDa.

3.2. Feasibility study

In this study the goal was to investigate which concentrations can be reached with different concentration techniques starting from an antibody solution with 10 mg/ml formulated in PBS and aiming at a final concentration of at least 100 mg/ml. In addition, differences between the techniques were studied.

The concentration–time profiles (Fig. 3) revealed that concentrations above 100 mg/ml could be achieved with all of the tested techniques. The most rapid concentration was observed with tangential flow filtration, whereas dialysis took a very long time to reach high protein concentrations. The results obtained with the stirred cell were more reproducible than those described in Section 3.1, possibly due to the higher start concentration employed (10 mg/ml instead of 2 mg/ml). Since the time required for concentration depends on the process parameters the optimisation of the process parameters might lead to similar concentration times for tangential flow filtration, centrifugal filters and stirred cell techniques. In contrast, the dialysis process cannot be accelerated to a great extent due to the slow process of osmosis. Moreover, the handling of the dialysis process was more inconvenient and the outlier in the dialysis data probably indicates a leakage of one of the devices.

In this study, the highest concentration was achieved by use of the centrifugal filters. However, there were no indications of saturation of the curves for any of the investigated techniques. In each case the concentration process was stopped when the smallest processible volume of antibody solution for the respective technique was reached. Therefore, the other techniques theoretically could lead to the same concentrations achieved by centrifugal filtration if they were given more time and if a higher volume of starting solution was used. Tangential flow filtration, for example, has the ability to reach very high concentrations (e.g., >100 mg/ml) (Shire et al., 2004). In the study presented here, the start volume was limited to only 200 ml to maintain the character of a small scale study as much as possible. When higher start volumes are used the achievable concentration in tangential flow filtration is only limited by the high viscosity or instability of the highly concentrated antibody solution (Shire et al., 2004; Salinas et al., 2010).

The other techniques used within this experiment required smaller amounts of material than the tangential flow filtration to reach concentrations above 100 mg/ml. Centrifugal filtration only needed 15 ml to achieve a concentration of 170 mg/ml, the stirred cell started off with 50 ml and the dialysis required only 30 ml. If even higher protein concentrations are required, the stirred cell or the centrifugal filters containing the concentrated protein solutions can be refilled and the process repeated. A combination of different techniques is also possible to achieve the desired protein concentration and volume (Russell et al., 2007). Comparing the techniques and process conditions under investigation, the use of centrifugal filters resulted in the highest protein concentrations and, additionally, required the smallest amount of material.

As already stated the concentration techniques used may impose mechanical stress on the protein solutions. Therefore,

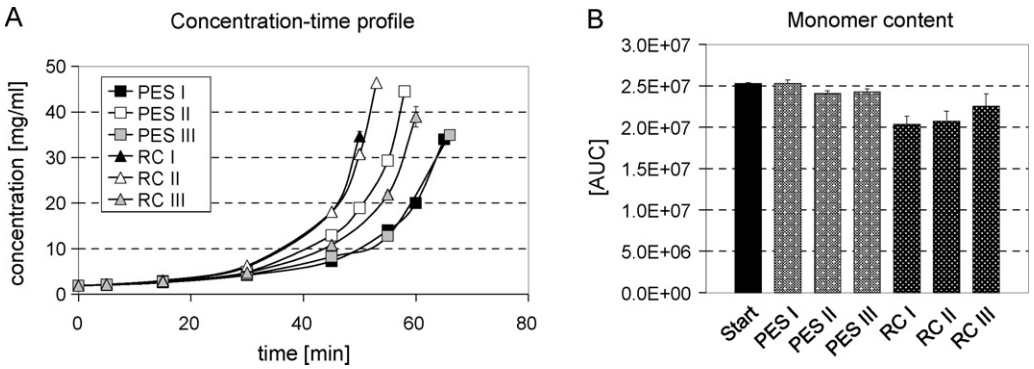

Fig. 1. (A) Concentration–time profiles of antibody solutions during stirred cell processing and (B) monomer content of the corresponding antibody solutions before and at the different end points of the concentration process. Each membrane type was investigated 3 times; PES: polyethersulfone, RC: regenerated cellulose.

the concentrated formulations were investigated with regard to stability issues. All solutions displayed an increase in turbidity during processing (Fig. 4). Solutions processed in the stirred cell showed the highest turbidity, whereas the use of centrifugal filters generated the lowest turbidity and the highest reproducibility. Agitation inside the stirred cell thus seems to exert the highest stress under the conditions applied. Stirring of antibody solutions is a well-known cause of aggregation (Kiese et al., 2008; Lahlou et al., 2009). Moreover, the longer processing time to achieve the required concentration with the stirred cell, compared to the use of the tangential flow filtration or centrifugal filters (Fig. 3), might contribute to the higher amount of precipitants observed.

A decrease in monomer content was observed by SEC for all the investigated techniques at the end of the concentration process (Fig. 5). The smallest decrease was found for the centrifugal filters, even though the protein concentration achieved with this technique was the highest. The standard deviations were, however, quite high and, therefore, it was difficult to accurately rank the techniques. A reason for the high standard deviations might be the dilution step required during sample preparation for SEC (the 100 mg/solution had to be diluted to 5 mg/ml). Such dilution

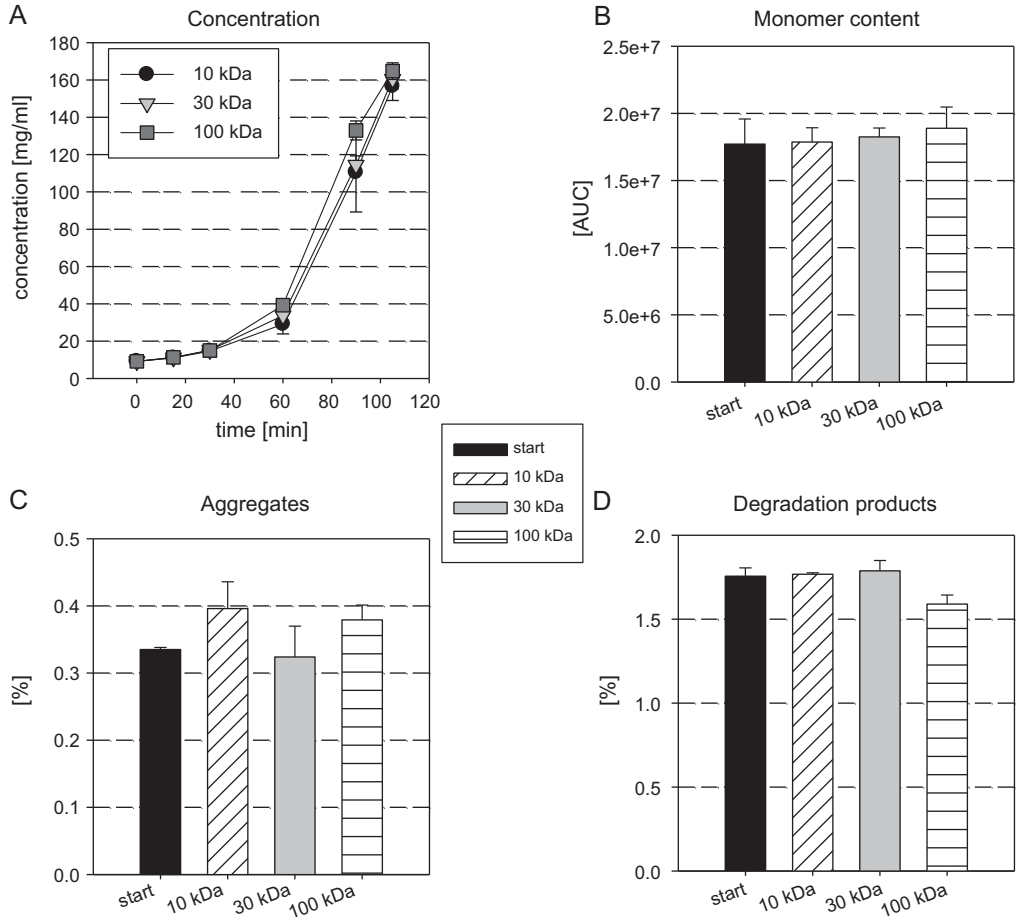

Fig. 2. (A) Concentration–time profiles during protein concentration by centrifugal filters with different molecular weight cut-offs, (B) monomer content, (C) aggregates and (D) degradation products of the corresponding antibody solutions before and after the concentration process as determined by SEC. Each experiment was performed in triplicate.

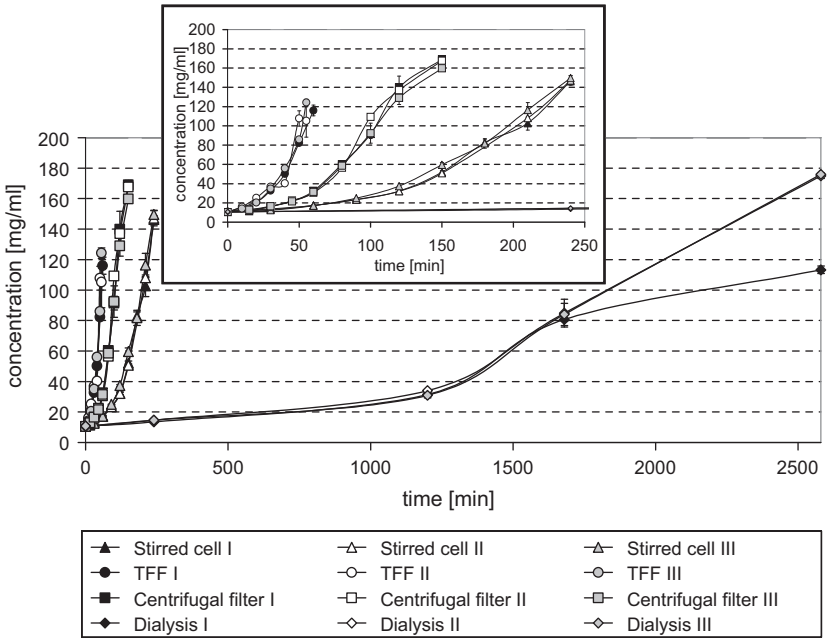

Fig. 3. Concentration–time profiles of the antibody solutions concentrated by the different techniques; concentration with each technique was performed 3 times; inset: enlarged time scale for the beginning of the concentration process.

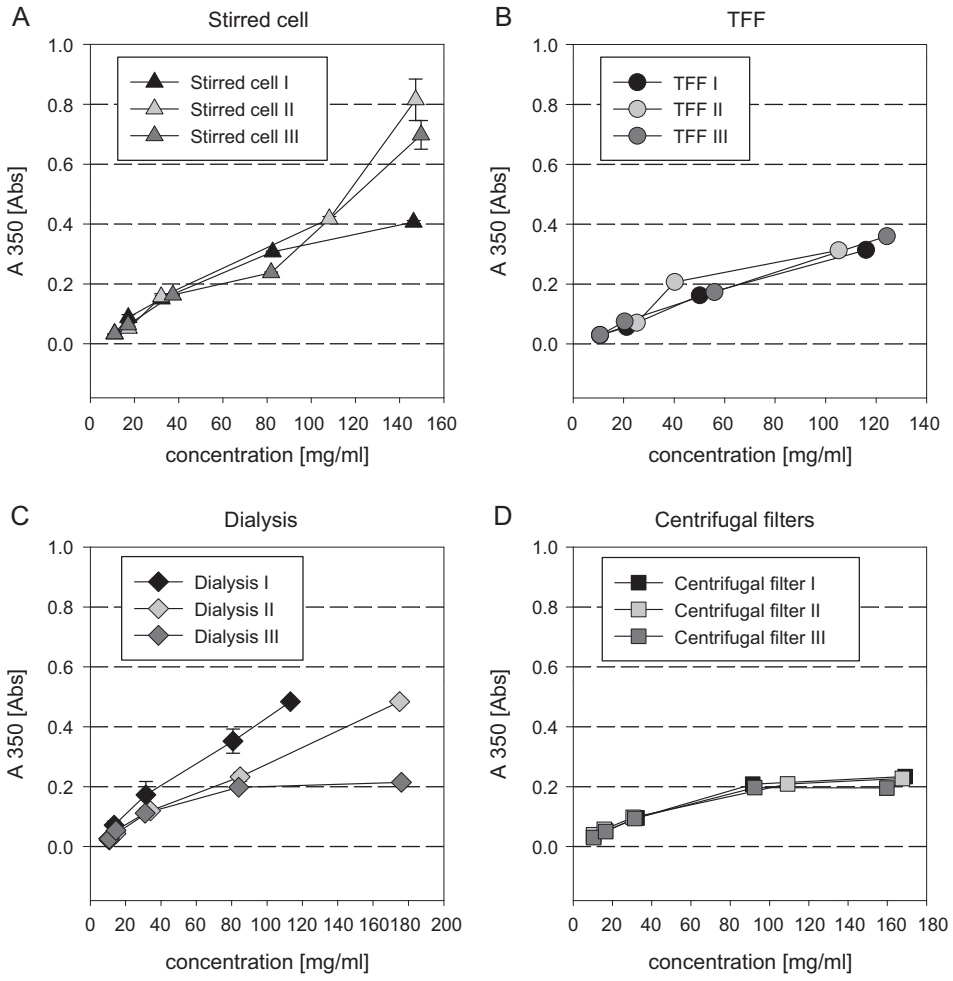

Fig. 4. Turbidities (UV-spectroscopy at 350 nm) of the antibody solutions at different concentrations during processing by the different concentration techniques. (A) Stirred cell, (B) tangential flow filtration (TFF), (C) dialysis and (D) centrifugal filters (the experiments with each concentration technique were performed in triplicate).

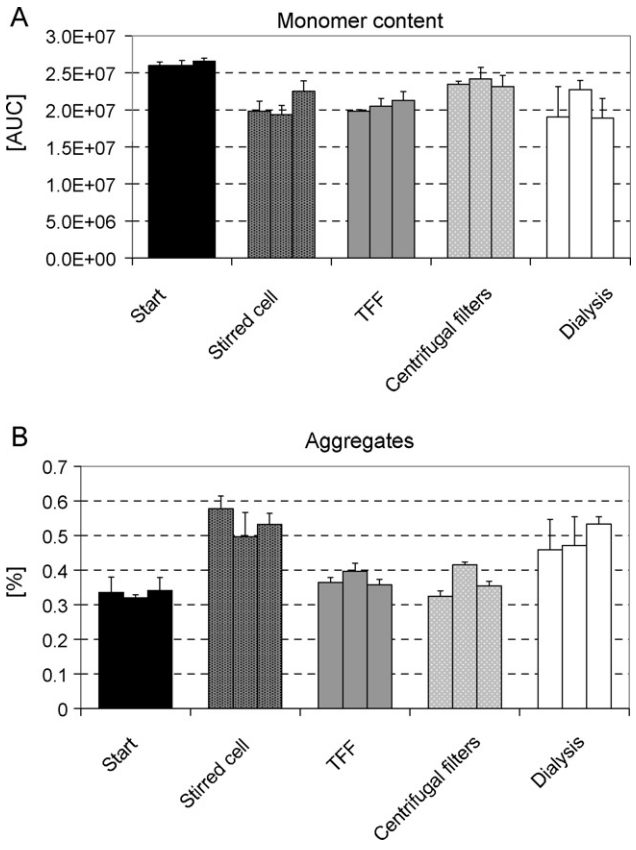

Fig. 5. SEC results for the protein solutions obtained at the endpoint of the feasibility study: (A) monomer content and (B) fraction of aggregates.

processes are a well-known cause of measurement uncertainties. Other analytical methods like Fourier transform infrared spectroscopy, circular dichroism or differential scanning calorimetry (Matheus et al., 2006; Harn et al., 2007) are often performed to avoid this problem.

The fraction of soluble aggregates detected by SEC was highest in the solutions processed in the stirred cell (Fig. 5). Dialysis also caused an increase in the fraction of soluble aggregates. One possible reason might be the shear stress, which was applied by extracting the highly concentrated solution from the dialysis cassette by use of a syringe and a needle. Additionally, a SDS-PAGE was performed, but did not reveal any differences between the concentration techniques (data not shown), indicating that no covalent aggregates were formed.

In summary, the best results concerning the highest achievable concentration, reproducibility and quality of the highly concentrated antibody solution were observed by the use of the centrifugal filters. In addition, this technique was most comparable to tangential flow filtration. The worst result concerning the quality of the concentrated antibody solution was obtained by use of the stirred cell, presumably due to shear stress and an extended air–water-interface, caused by stirring. The dialysis procedure was most tedious to handle and resulted in very long concentration times.

3.3. Stability study

In order to evaluate the quality and storage stability of the solutions processed with the four different techniques at a comparable concentration a further concentration process was carried out with each technique and stopped after reaching a concentration slightly above 100 mg/ml. After dilution to the exact concentration of 100 mg/ml with PBS and filtration to remove insoluble aggregates,

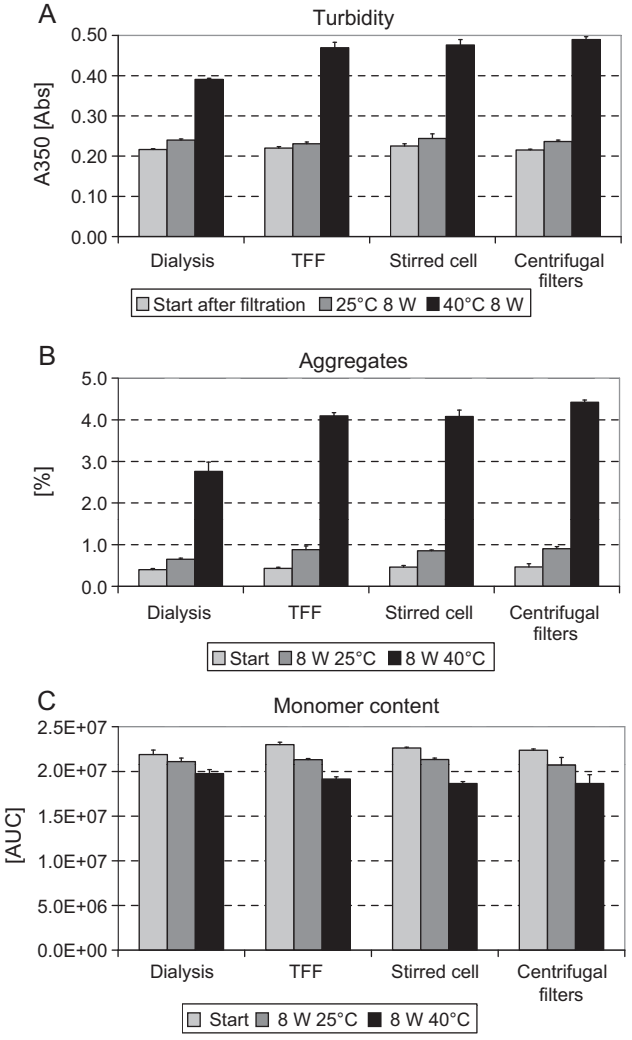

Fig. 6. Analysis results for filtered samples (100 mg/ml) directly after the concentration process and after storage for 8 weeks (w) at 25 °C or 40 °C. (A) Turbidity (UV-spectroscopy at 350 nm), (B) soluble aggregates (SEC) and (C) monomer content (SEC).

one fraction of the filtered solution was analyzed directly and two other fractions after storage for 8 weeks at 25 °C or 40 °C.

Directly after filtration the solutions obtained by the different techniques were of comparable turbidity; a slight increase in turbidity was observed after storage at 25 °C (Fig. 6A). In contrast, samples stored at 40 °C displayed a pronounced increase in turbidity indicating the formation of aggregates. The dialysed solution increased the least in turbidity during storage at 40 °C. The aggregates that formed during storage at 40 °C were obviously very small in size, because large aggregates were not observed visually under cold light, although an increase in opalescence was evident (data not shown).

By SEC, virtually the same ranking of the solutions was generated with regard to the fraction of soluble aggregates as in the turbidity measurements (Fig. 6B). However, the differences occurring over time and at different storage temperatures were, more pronounced in the SEC results than in the turbidity measurements. The lowest fraction of aggregates was again observed for the dialysed solutions but using SEC this difference compared to the other techniques also became evident after storage at 25 °C.

The aggregate formation observed during turbidity and SEC measurements was also reflected in a decrease in the absolute monomer content (determined by SEC) (Fig. 6C). The monomer

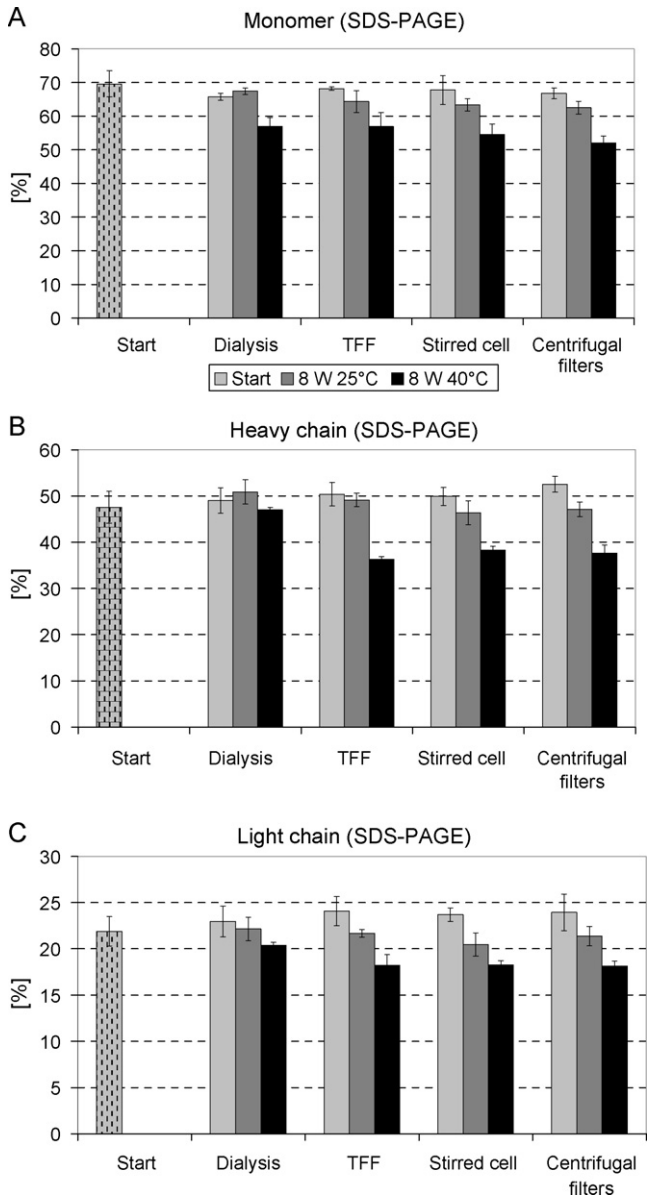

Fig. 7. Results of SDS-PAGE obtained on filtered samples (100 mg/ml) directly after the concentration process and after storage for 8 weeks at 25 °C or 40 °C. (A) Monomer fraction according to non-reducing SDS-PAGE, (B) heavy chain fraction according to reducing SDS-PAGE and (C) light chain fraction according to reducing SDS-PAGE. The dotted grey columns on the left (start) show the results before the concentration process.

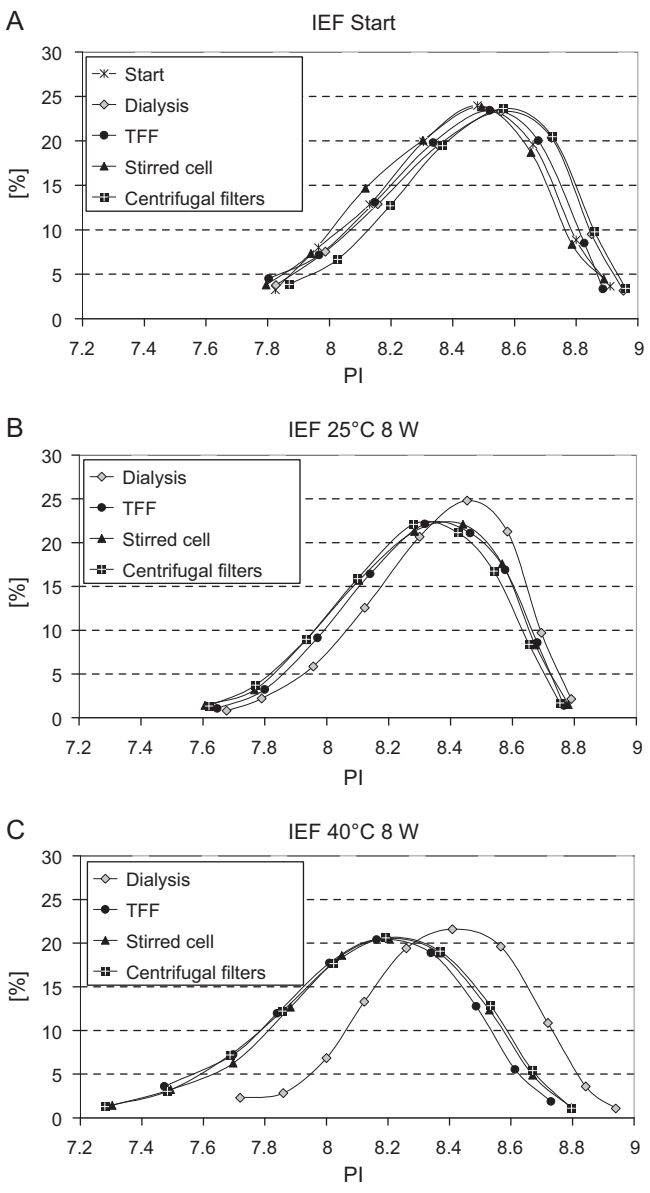

Fig. 8. Isoelectric focusing (IEF) results obtained on 100 mg/ml antibody solutions concentrated by different techniques. (A) Before or directly after the concentration process, (B) after storage for 8 weeks at 25 °C and (C) after storage for 8 weeks at 40 °C.

content decreased with increasing storage temperature and the decrease was higher after using tangential flow filtration, stirred cell or centrifugal filters than after using dialysis. It is likely that dialysis created less stress for the protein and a smaller fraction of monomers were converted to aggregates.

To detect the nature of the aggregates SDS-PAGE, under reducing and non-reducing conditions, was carried out (Fig. 7). The non-reducing SDS-PAGE indicated a monomer decrease with increasing storage temperature without a clear difference between the tested techniques (Fig. 7A). Obviously, an increase in covalent aggregates occurred with increasing storage temperature for all techniques in the same manner. The reduced SDS-PAGE, however, revealed a difference between dialysis and the other techniques (Fig. 7B and C). After dialysis a higher fraction of light and heavy chains remained intact than with the other techniques. The aggregates formed according to the results of non-reducing SDS-PAGE thus seemed to be partly different in their nature causing different results in the reduced SDS-PAGE.

The higher stability of the solution produced by dialysis was also reflected in the isoelectric focusing (IEF) results (Fig. 8). Before and directly after the concentration process, the isoelectric point (PI) curves were very similar, regardless of the concentration technique applied. Therefore, the different concentration processes did not have a direct effect on the isoelectric point. Storage of the solutions, in particular at an elevated temperature, resulted in a shift towards smaller isoelectric points for all techniques except for dialysis, which caused only a small isoelectric point shift.

These observations might be explained by the following hypothesis: Antibodies concentrated by dialysis were only very weakly stressed, compared to the other techniques. Directly after the concentration process this difference was not observable (similar instabilities compared to the other techniques were observed within the feasibility study). However, the concentration time during dialysis was, much longer and, therefore, the proteins were

exposed to less shear stress. Due to this reduced stress the antibodies were less likely to partially unfold than during the concentration process with the other techniques. In addition, deamidation of the antibodies may occur to a greater extent at elevated temperatures (Cleland et al., 1993). A smaller fraction of partially unfolded antibodies after dialysis might have caused a lower degree of deamidation, resulting in a less pronounced shift of the IEF curves. This hypothesis might also explain the higher amount of aggregates after storage at elevated temperatures in general, because higher temperatures result in an increased mobility of the antibodies within the solution (Jaenicke, 1991). Consequently, the partially unfolded antibodies collide more often to form aggregates.

Interestingly, aggregate formation and isoelectric point shifts were detected only after storage and not directly after the concentration process. This kind of "memory effect" was earlier observed with regard to the influence of pH (Vakos et al., 2000). In the present study, it appeared after storage at elevated temperatures and was apparently induced by an initial shear stress during the concentration process, however with no detectable effect directly after the concentration process. In conclusion, shear stress during the concentration process may have an influence on the long-term stability of a highly concentrated antibody solution.

Overall dialysis generated the best stability results, but they were not comparable to those of tangential flow filtration. The aim of this study was to find a small scale technique which is most comparable to the technique used later in production, i.e., tangential flow filtration. Therefore, taking this into account, the centrifugal filters have to be ranked as the technique exhibiting the best comparability.

In the studies presented here, simple, buffered antibody solutions were investigated, which are not as stable as a completely formulated product. This may be an important reason for the formation of a high number of aggregates during storage. However, concentrating final formulations is difficult because excipients, like polysorbates, which are added to the formulations to prevent aggregate formation are also concentrated during this process (Mahler et al., 2008). Usually, a protein solution would be brought into its final formulation after the concentration process and prior to storage. In the present study the storage of simple, buffered protein solutions was performed intentionally, because it facilitated the discrimination between the influences of the different techniques and allowed the identification of the technique with the lowest stress level.

4. Conclusion

The comparison of the small scale concentration techniques including centrifugal filters, stirred cell, dialysis and tangential flow filtration revealed that concentrations higher than 100 mg/ml could be achieved with all techniques. However, the concentration times and qualities of the resulting solutions were quite different. Dialysis was very time consuming and the stirred cell concentration method led to a high number of insoluble aggregates. The stability of 100 mg/ml solutions, concentrated by the different techniques, was also very different what only became evident after storage at 25 °C and 40 °C. Directly after concentrating, similar instabilities were observed between the solutions obtained by the different concentration techniques. The solution concentrated by dialysis displayed much less instability after storage than the solutions concentrated by the other techniques. A potential reason might be less shear stress during dialysis compared to the other techniques. The results after dialysis were, however, not comparable to those of tangential flow filtration. In contrast, the results after centrifugal filtration were more comparable to the tangential flow filtration results, showed the highest reproducibility and required the smallest volume of protein solution, which is very favourable for a small scale evaluation. Although this evaluation of different small scale techniques was performed with a specific monoclonal antibody, it is likely that the techniques will perform in a similar way with solutions of other antibodies. This assumption will need to be confirmed in the future, in particular with regard to the influences on chemical stability observed in this study.

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