Dissertation zur Erlangung des Doktorgrades

der Fakultät für Chemie und Pharmazie

der Ludwig-Maximilians-Universität München

Filtration and novel polymeric containers for the improved quality of biotech drug products

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Benjamin Patrick Werner

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München, Deutschland

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Objectives

Biopharmaceuticals are powerful therapeutics to treat or even cure severe diseases like cancer or autoimmune diseases. However, adverse effects, in particular immune reactions caused by protein particles have been reported. Hence, the formation of protein particles and their administration to patients has to be prevented. The aim of this thesis is to offer and evaluate approaches that minimize the number of such protein particles in biopharmaceuticals. To this end, bedside filtration as well as novel polymeric primary packaging materials are considered and investigated for their potential to improve the quality of liquid biotech drugs.

In order to characterize and validate the two suggested approaches, this thesis has five objectives and is structured accordingly in five chapters:

- Research on the current status of bedside filtration of worldwide approved biopharmaceuticals with regard to the dosage form, time of filtration, filter membrane and pore size.

- Investigation of an expansion of bedside filtration to a broad variety of biopharmaceuticals with different concentrations and molecular weights. Thereby technical aspects such as filter types, filtration effectiveness, filter cleanliness, protein loss or syringeability shall be considered.

- Analysis of three antivascular epidermal growth factor biopharmaceuticals for ocular use regarding their silicone oil and protein particle levels.

- Evaluation of the suitability of a novel silicone and tungsten free polymer syringe system for the long-term storage of biopharmaceuticals in comparison to siliconized glass syringes.

- Comparison of innovative monolayer and multilayer plastic vials to standard glass vials with regard to product quality and long-term stability of biopharmaceuticals.

Chapter 1

Particle contamination of parenteralia and in-line filtration of proteinaceous drugs

Chapter 1 is published in the International Journal of Pharmaceutics.

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Abstract

Protein drug products play an important role in the treatment of severe diseases. However, due to the instability of these complex molecules, protein aggregates can form which can compromise drug safety and efficacy including immunogenic reactions. In-line filtration during the administration of these drugs can serve as a final safeguarding step to protect patients from risks associated with proteinaceous particles. A unique analysis of more than 300 marketed protein drug products revealed that already around 16 % of all these products are filtered during preparation or administration. Further, the research revealed that no standardized filtration practice exists. Broad variances regarding filter membrane or pore size are found and sometimes no specifications are mentioned at all. The benefits as well as possible negative impacts of filtration like filter shedding, extractables or drug adsorption are critically assessed. Several proposals to improve the current filtration practice and to expand the number of in-line filtered protein drug products are made. The suggestions include the demand for the specific usage of one filter membrane type, the establishment of a filtration routine for unfiltered protein drugs and a statistical analysis between filtered and non-filtered products with similar formulations to identify possible differences in the immunogenicity rate.

Disclaimer

Although the authors have done a careful piece of work by reviewing the current status of the filtration of selected drugs, they cannot be responsible for the completeness of the data, for any omissions or errors, the timeliness of data, or for any consequences that may arise. The reader is advised to consult the newest professional information, like a package insert, available for the product of interest.

Keywords

particulate matter, particle, protein, biopharmaceutical, in-line filtration, immunogenicity

1.1. Introduction

1.1.1 Regulatory specifications

Biopharmaceuticals have been rising for many years and their share on the total drug market will increase in the future to up to 20 % (Walsh, 2014). They are commonly administered intravenously or subcutaneously (Jiskoot et al., 2012). Hence, protein drug products have to comply with the criteria set forth for parenteralia in the United States and European Pharmacopeia (European Directorate For The Quality Of Medicine, 2011c; The United States Pharmacopeial Convention, 2011a). One major aspect is that biopharmaceuticals need to be practically free from visible particles (European Directorate For The Quality Of Medicine, 2011c; The United States Pharmacopeial Convention, 2011a) and must not exceed limits for subvisible particles (European Directorate For The Quality Of Medicine, 2011a; Pharmaceuticals and Medical Devices Agency, 2011; The United States Pharmacopeial Convention, 2011b). Particles become visible above approximately 50 µm, and are well detected by the unaided eye at sizes of about 100 µm (Das, 2012; den Engelsman et al., 2011; Doessegger et al., 2012). At the moment particle number limits in the subvisible range are only defined for particles larger than 10 and 25 µm (European Directorate For The Quality Of Medicine, 2011a; Pharmaceuticals and Medical Devices Agency, 2011; The United States Pharmacopeial Convention, 2011b). However, in the recent years it has become common practice by the authorities to request data for particle sizes below 10 µm for this drug product class (Wang et al., 2012) and (The United States Pharmacopeial Convention, 2015). The European Pharmacopeia also contains a monograph called “Monoclonal antibodies for human use”, which allows the presence of protein particles in protein drug products (European Directorate For The Quality Of Medicine, 2011b). But these particles are only tolerated if they are well characterized and the data is accepted by the regulatory authorities (Doessegger et al., 2012; European Directorate For The Quality Of Medicine, 2011b). Nevertheless, these product inherent particles have to be reduced to a minimum (Doessegger et al., 2012; European Directorate For The Quality Of Medicine, 2011b).

1.1.2 Negative impacts of particles

Particles in parenteral solutions can derive among others from formulation components or other sources like silicone oil, cellulose fibers, cotton, glass microflakes, rubber, plastic or metal (Bethune et al., 2001; Doessegger et al., 2012; Paolo et al., 1990; Shaw and Lyall, 1985; Tran et al., 2006; Waller and George, 1986). Based on estimations of the year 1987, an adult intensive care patient gets more than 10^7 particles with a size larger than 2 µm infused within 24 hours (van Lingen et al., 2004). Ten years earlier, in 1977, Mehrkens *et al.*

found two million particles larger than 2 µm during the same infusion interval (Bethune et al., 2001). Many reports describe the negative impact of particle contamination. For example, a correlation between the frequency of site reactions and the particulate matter is witnessed. A high particle number results in an increased number of adverse effects (Doessegger et al., 2012). Further, particles affect mainly organs like eyes, brain, lungs, heart, kidney, spleen, stomach and intestine (Boehne et al., 2013; Paolo et al., 1990; Puntis et al., 1992; Waller and George, 1986), whereas large particles remain in the lung and small ones are transported within the systemic circulation (Boehne et al., 2013; Langille, 2013; Puntis et al., 1992). If particles are larger than 7 µm, capillary occlusion is a reason why these organs are harmed by particles, because the diameter of the smallest capillary vessel is around 7 µm (Hearse et al., 1986; Shaw and Lyall, 1985; Tran et al., 2006; Waller and George, 1986). Moreover, pulmonary granuloma is associated with the presence of particles in humans (Cant et al., 1988; Lehr et al., 2002; Shaw and Lyall, 1985; Shay et al., 1997; van Lingen et al., 2004). Granuloma formation is also observed for drugs like amphetamines, methadone or methylphenidate, intended to be administered orally but are misused intravenously (Doessegger et al., 2012; Jorens et al., 2009). This further indicates that particles are capable of inducing granuloma. Next, a large number of microthrombi are connected to particles with a size less than 2 µm, which also account for the majority of the particles in intravenous fluids, as Walpot *et al.* note (Lehr et al., 2002; Tran et al., 2006; Walpot et al., 1989).

1.1.3 Protein aggregate formation

Beside these non-proteinaceous particles, protein drugs can contribute to the particle burden of a formulation, because they are prone to chemical and physical degradation (Manning et al., 2010). Two very important chemical degradation pathways are deamidation and oxidation. Muromonab-CD3, human growth hormone or insulin are examples of pharmaceutical relevant drugs in which deamidation, a hydrolysis of asparagine or glutamine, is detected. Several factors like the amino acid sequence or pH have an impact. Oxidation processes can occur at any time. Among others light or metals can cause oxidation. Especially histidine, methionine, cysteine, tyrosine or tryptophan are sensitive towards oxidation. Physical degradation can be denaturation, caused for example by temperature or chemicals, or aggregation with different mechanism like chemically modified monomers or surface interfaces (Manning et al., 2010). Even the best formulation and storage condition cannot totally exclude such degradation products (Brange et al., 1992). External factors like temperature, pH, shaking, shearing, etc. can cause particle formation (Wang, 2005). Silicone oil, a non-proteinaceous particle, (Basu et al., 2013; Thirumangalathu et al., 2009) may foster protein aggregation by acting as heterogeneous nuclei (Mahler et al.,

2009; Zölls et al., 2012). Generally, protein aggregates can be dimers or multimers in the lower nanometer range or may assemble to large particles even in the visible range above 100 µm (den Engelsman et al., 2011; Singh et al., 2010).

1.1.4 Immunogenicity of protein particles

In contrast to non-proteinaceous particles, aggregates formed by proteins require additional vigilance. For several monoclonal antibody drug products, for example adalimumab, abciximab, omalizumab or trastuzumab, immunogenic reactions are observed (Getts et al., 2010).

1.1.4.1 Causes for the immunogenic potential of proteins

Not surprisingly proteins with an amino acid sequence differing from the human homologue are immunogenic. Hence, in the case of protein drug products the immunogenicity issue has been detected first by proteins deriving from animal origin, like porcine and bovine insulins (Schellekens, 2002). The response of the immune system towards drugs deriving from animal, microbial or plant origin is rapid and occurs immediately after a single administration. Hereby, neutralizing antibodies are formed. However, immunogenic reactions also take place for recombinant human proteins like human insulin. The reason is a breach of the immune tolerance (De Groot and Scott, 2007; Hermeling et al., 2004). The antibodies, formed by this slow process, disappear either after the end or in the long term of a treatment (Schellekens, 2005a). Several steps have been taken to reduce the immunogenic potential of proteins. The generation of fully humanized proteins, and consequently avoiding proteins based on murine structures, can be one improvement, but is no guarantee for a diminished immune response (Getts et al., 2010). Other approaches are glycosylation, PEGylation, sequence modifications, reduction of process impurities and formulation optimization (Chirino et al., 2004; De Groot and Scott, 2007; Hermeling et al., 2004; Schellekens, 2002). It is of utmost importance to reduce impurities and protein aggregates, because they are the main cause for the breakdown of the immune tolerance (Schellekens, 2005a). The potential of aggregates to induce immune reactions is common knowledge since the 1950s (Rosenberg, 2006). Aggregates may cause an immune response via T cell in- as well as dependent pathway. It is assumed that T cells recognize repetitive patterns on the surface of the aggregates which are similar to the unique epitope arrangement of microbial antigens (Carpenter et al., 2010; De Groot and Scott, 2007; Hermeling et al., 2004). For example, antigenic sites exist for the aggregated, in contrast to the native, species of insulin (Hermeling et al., 2004). A cross-linking of a B cell receptor by an aggregate might trigger a T

cell dependent pathway (De Groot and Scott, 2007). Next, antigen-presenting cells, marking the start of the immune reaction, take up particulate antigens very fast (Rosenberg, 2006).

1.1.4.2 Consequences of anti-drug antibodies

The clinical and biological consequences of the antibody formation against biopharmaceuticals are often negligible. Yet, severe immune responses like anaphylaxis, allergic reactions or serum sickness can also occur, although the use of highly purified products with a high quality diminishes these incidents nowadays. Neutralization of an essential endogenous protein can be life threatening (Carpenter et al., 2009; Hermeling et al., 2004; Kessler et al., 2006; Sharma, 2007). An example of a severe immunogenic reaction is the pure red cell aplasia, resulting from the formation of anti-erythropoietin antibodies (Schellekens, 2005b). Neutralizing antibodies against adalimumab also exist, hereby reducing its efficacy (van Schouwenburg et al., 2013), whereas the formation of non-neutralizing anti-drug antibodies does not influence the efficacy of etanercept (Dore et al., 2007). The potential of protein aggregates to induce immune reactions in other cases is documented as well. For aggregates of insulin (Schernthaner, 1993), interferon alpha (Braun et al., 1997) and human growth hormone antibody formation occurs (Moore and Leppert, 1980). Investigation of different interferon products reveals a correlation between the amount of neutralizing antibodies and aggregate, respectively, particle level of the drug products (Barnard et al., 2013). Also for interleukin-2 neutralizing antibodies are found because the protein drug product is produced as an aggregate with highly immunogenic properties (Rosenberg, 2006). A recent study by Ahmadi *et al.* implies that the clinical immunogenicity profile of the protein drug may influence the potential of present aggregates to induce an immune response (Ahmadi et al., 2015). Trastuzumab, which has a low clinical immunogenicity, is a strong activator of the immune system after the presence of small amounts of aggregated protein (< 3% of total protein content). On the contrary, stressed rituximab, with the native species showing high clinical immunogenicity, does not increase the immune reaction (Ahmadi et al., 2015). But aggregates do not always lead to an immune response. Their properties like secondary structure, solubility or size have an impact on their potential to induce immunogenicity (Freitag et al., 2015; Shomali et al., 2014). For aggregated recombinant human factor VIII no enhanced immunogenicity is observed (Purohit et al., 2006). As this example shows there is no direct correlation between clinical evidence of immune reactions and particles in parenteralia. To establish such a relation is problematic, because several factors including the immune status of the patient, dose, dosage frequency, duration or route of administration as well as other factors influence immunogenicity (Carpenter et al., 2010; Carpenter et al., 2009; Rosenberg, 2006; Schellekens, 2005b).

Further, the larger particles do not need to be more immunogenic than smaller ones (Rosenberg, 2006) or vice versa (Singh, 2011). But a look at the particle count of marketed biologic drug products reveals that particles smaller than 2 µm account for the overwhelming majority of particles, whereas large ones, as monitored according to the pharmacopoeia, represent only a small fraction of the total particle count (Barnard et al., 2013; Singh et al., 2010) Hence, it has to be assumed that particles with a small size are potentially more harmful due to their higher amount in formulations.

1.1.5 Usage of in-line filters to solve the particle problem

1.1.5.1 Benefits of in-line filtration

A way to overcome negative aspects associated with particles can be the usage of in-line filters, which are incorporated in the infusion set proximal to the patient (Allcutt et al., 1983; Bethune et al., 2001). In-line filters with pore sizes ranging from 0.2 to 0.45 µm reduce the occurrence of phlebitis (Allcutt et al., 1983; Dorris et al., 1977; Falchuk et al., 1985; Gasch et al., 2011; Ortolano et al., 2004; Paolo et al., 1990; Waller and George, 1986). However, a systematic review of randomized controlled trials shows an uncertain benefit regarding the reduction of phlebitis, due to methodological shortcomings and unexplained statistical heterogeneity in studies ranging from 1973 to 1990 (Niel-Weise et al., 2010). Besides, in-line filtration decreases the frequency of systemic inflammatory response syndrome in critically ill children very effectively (Boehne et al., 2010). Another study shows several positive effects of the usage of in-line filters in sick newborn infants. The rates of sepsis, infection, thrombi and necrotizing enterocolitis are reduced (van Lingen et al., 2004). A recent study from 2012 with 807 critically ill children shows a significant positive effect of in-line filtration (Jack et al., 2012). Filter usage decreases complications like sepsis, circulatory failure or acute renal failure, whereas in-line filtration significantly reduces the occurrence of systemic inflammatory response syndrome and the duration of the hospitalization and mechanical ventilation (Jack et al., 2012). A follow up study has investigated the impact of filtration on organ dysfunction and reveals a positive impact of in-line filters regarding the respiratory, renal and hematologic function (Boehne et al., 2013). In-line filters can also diminish the risk of infection especially for immunocompromised individuals (Ennis et al., 1983; Francomb et al., 1994; Gasch et al., 2011).

Hospital pharmacies recommend that depending on the administered solution filtration should be considered (Doessegger et al., 2012). Yet, filters are rarely used beside the preparation of anticancer drugs or for administration of total parenteral nutrition, due to costs and time reasons (Lee et al., 2011). However, the usage of in-line filters in sick newborn infants results in considerable financial and nursing time savings (van Lingen et al., 2004).

Other studies observe a prolonged use of the infusion set and saving time of the nurses for not changing the set, as well as a better recovery of the patient, if in-line filters are applied. Hence, costs and also waste are reduced (Bethune et al., 2001; Gasch et al., 2011; Jack et al., 2012).

1.1.5.2 Reduction of particles with in-line filters and filtration recommendations of institutions

Among others the U.S. Food and Drug Administration and the Intravenous Nurses Society advocate the usage of a final filter for the administration of intravenous drugs (Kuramoto et al., 2006). In Japan, in-line filters are considered to be essential due to their ability to remove insoluble particles (Kuramoto et al., 2006). However, data supporting the usage of final filters are rare and date back to 1979. A more recent study by Kuramoto *et al.* provides evidence regarding particle numbers and not clinical pictures for the usefulness of final filters (Kuramoto et al., 2006). They investigated nine different freeze dried drugs after reconstitution and they reduced the number of particles larger than 5 µm for all products to 0 to 2 particles/5 mL (Kuramoto et al., 2006). Further, intravenous administration sets with an incorporated 15 µm in-line filter significantly reduce the amount of large particles, independent of their origin which can be the parenteral solution or administration set (Paolo et al., 1990).

Beside these in-line filters, filter needles can be used to eliminate impurities. For example filter needles are able to prevent the aspiration of glass particles from opened glass ampoules as shown in several studies (Sabon et al., 1989). However, one other study did not find this positive effect, because penetration of the filter by glass particles occurred during aspiration (Carbone-Traber and Shanks, 1986). The aspiration technique might influence the particle contamination of the aspirated solution (Preston and Hegadoren, 2004).

Yet, the Centers for Disease Control and Prevention are one example that the use of inline filters is not generally recommended. Its decision is based on the fact that total parenteral nutrition solutions are already filtered by the pharmacies before they are used in the hospital (Allcutt et al., 1983; Kuramoto et al., 2006). The U.S. Food and Drug Administration, the National Advisory Group and Standards and Practice guidelines for Parenteral Nutrition and the British Pharmaceutical Nutrition Group Working Party recommend a filtration with a 1.2 µm pore size filter for parenteral nutrition solutions containing a fat emulsion (Ortolano et al., 2004). Non lipid total parenteral nutrition solutions shall be administered with a 0.2 µm filter (Bethune et al., 2001; Driscoll et al., 1996).

Particulate matter has a severe impact, which can lead to organ failure, morbidity and mortality, particularly in individuals with a disturbed microcirculation, including patients after major surgeries, with sepsis, ischemia and other disorders as data implies (Jack et al., 2012; Lehr et al., 2002). Especially intensive care and immunosuppressed patients, neonates and

children may benefit from a reduction of the particle burden, because they all are exposed to a high amount of intravenously administered fluids (Bethune et al., 2001).

Considering the consequences of particle containing drug solutions and the use of filters in the clinical practice, the purpose of this review is to provide a comprehensive overview on the filtration recommendations for commercial protein drug products, because to our knowledge such a broad analysis is so far not available in the literature. Our review shall fill this gap by providing important pieces of practical information with a product independent view. Because in-line filtration is a controversially discussed routine with high relevance, we make several proposals at the end to establish uniform standards for the filtration of protein drug products. Overall, we believe that many immunogenicity issues caused by protein drug products may be eliminated by in-line filtration. Further, it might be worth to consider a change of the overall routine in the field to ensure the highest level of patient safety as possible.

1.2. Filtration of protein drug products

On the one hand, particles possess the potential to reduce the efficacy or safety of the protein drug product or induce immunogenicity (Kessler et al., 2006; Rosenberg, 2006; Schellekens and Casadevall, 2004). On the other hand, the usages of filters show positive effects. Weighing these two options, it might be beneficial, if protein drug products would be filtered prior to administration. Indeed, there exist a number of protein drug products, which are filtered during preparation or administration. To identify these drugs, the Rote Liste (Rote Liste® Service GmbH Frankfurt/Main, 01/2015, 2013), a register for traded drugs in Germany, as well as other sources from the U.S. Food and Drug Administration (U.S. Food and Drug Administration, 2014) or European Medicines Agency (European Medicines Agency, 2014) were analyzed. In the process, more than 150 protein molecules were found and more than 320 different marketed formulations of these molecules were investigated. Some of the described protein drug products are also listed in publications which deal with filtered drugs in general (Chen and Martinez, 1998; Ipema et al., 2012; Turcasso and Bush, 2001; Wolters Kluwer Health / Facts and Comparisons, 2007).

We focused for statistical analysis purposes on all protein drug products registered in the Rote Liste because not all marketed products are approved worldwide. Nevertheless, Table 1 also includes proteins which are filtered in other countries but are not included into the calculation.

The Rote Liste contains 142 drug substances and 314 marketed products plus their various dose variants (Rote Liste® Service GmbH Frankfurt/Main, 01/2015, 2013). The largest class of drug molecules is hormones (27.46 %), followed by IgGs (21.13 %), enzymes (16.90 %),

cytokines (11.27 %), antihemophilic molecules (7.75 %) and others (Rote Liste® Service GmbH Frankfurt/Main, 01/2015, 2013). Considering all the marketed products of these molecules, excluding their dose variants, the shares look slightly different. Products with hormones lead the market with 40.45 %, followed by IgG (11.46 %), immunoglobulins (9.87 %), cytokines (8.60 %), enzymes (8.60 %) and antihemophilic (7.96 %) drugs before other classes (Rote Liste® Service GmbH Frankfurt/Main, 01/2015, 2013).

For parenteral administration the dosage forms solution, lyophilisate or suspension are interesting. The leading dosage form is solution (54.78 %), followed by lyophilisate (33.12 %) and suspensions (7.64 %) (Figure 1) (Rote Liste® Service GmbH Frankfurt/Main, 01/2015, 2013). The percentage of tablets, capsules and gels are summarized as other forms in Figure 1.

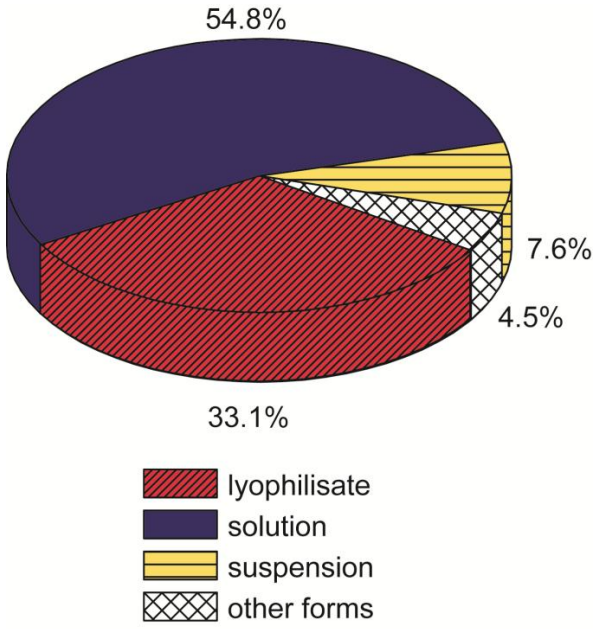

Figure 1: Share of the dosage forms of all marketed protein products listed in the Rote Liste® (Rote Liste® Service GmbH Frankfurt/Main, 01/2015, 2013).

In the following, the most prominent examples of protein drugs with their respective filtration instruction are reviewed. As the reader will realize, the instructions are in parts very detailed, in other aspects, e.g. filter type or pore size surprisingly wide recommendations are provided. Overall, a general picture of the current routine emerges. Table 1 displays a condensed overview of the filtered drug products.

Table 1: Overview about proteins which are filtered during preparation (P) or administration (A). Their dosage form (lyophilisate (L) or solution (S)) as well as recommended filter pore sizes or filter membranes are stated as far as declared by manufacturers or publications. The table provides further important, but not all information about the indication, (soluble) protein concentration and several data about administration. Abbreviations: h = hour; min = minutes; max. = maximum; i.v. = intravenous; s.c. = subcutaneous; i.m. = intramuscular.

Fusion proteins

Drug product (Trade name) Indication (Company)	Dosage form	Protein concentration Route of administration Dosage and frequency of administration Citation	Filter pore size recommendations	Filter type recommendations	Filter usage during P or A
abatacept (Orencia) Adult rheumatoid arthritis / Juvenile idiopathic arthritis (Bristol-Myers Squibb)	L / S	after reconstitution: 25 mg/mL weight dependent dose initial dose, 2 and 4 weeks after initial dose, then every 4 weeks thereafter over 30 min i.v. prefilled syringe:125 mg/mL initial i.v. dose (weight dependent), within a day 125 mg s.c., afterwards weekly 125 mg s.c.. S.c. only for patients > 18 years (Orencia - Anhang 1 - Zusammenfassung der Merkmale des Arzneimittels, accessed 08/13; Orencia (abatacept) [Package insert], 12/2011)	0.2 µm to 1.2 µm infusion set with filter (only infusion)		A
aflibercept (Eylea™) Neovascular & age-related macular degeneration (Regeneron Pharmaceuticals, Inc.)	S	40 mg/mL 2 mg/0.05 mL monthly for the first 3 months, followed by every 2 months by ophthalmic intravitreal injection (Eylea™ (aflibercept) Injection [Package insert], 11/2011)	19 gauge x 1.5-inch, 5 µm filter needle		P

ziv-aflibercept (Zaltrap®) Metastatic colorectal cancer (sanofi-aventis U.S. LLC)	S	stock solution: 25 mg/mL final concentration: 0.6 to 8 mg/mL. every 2 weeks 4 mg/kg over 1 h (Zaltrap® (ziv-aflibercept) [Package insert], 08/2012)	0.2 µm	polyethersulfone but no polyvinylidene fluoride or nylon filter	A
belatacept (Nulojix) Prophylaxis of kidney rejection (Bristol-Myers Squibb Company)	L	25 mg/mL 10 mg/kg over 30 minutes on day 1 & 5, end of week 2, 4, 8 & 12 5 mg/kg on end of week 16 and every 4 weeks thereafter (Nulojix (belatacept) [Package insert], 04/2013)	0.2 µm to 1.2 µm infusion set with filter		A

Antibodies or antibody fragments

Drug product (Trade name) Indication (Company)	Dosage form	Protein concentration Route of administration Dosage and frequency of administration Citation	Filter pore size recommendations	Filter type recommendations	Filter usage during P or A
abciximab (ReoPro®) Prevention of cardiac ischemic complications (Centocor B.V. Leiden)	S	2 mg/mL i.v. bolus with 0.25 mg/kg 10 to 60 min before PCI start; thereafter continuous i.v. infusion of 0.125 µg/kg/min (to max. 10 µg/min) for 12 h dose is different for unstable angina (ReoPro® (abciximab) [Package insert], 11/2005)	0.2 µm or 5 µm (preparation) 0.2 µm or 0.22 µm (administration)	polyvinylidene fluoride filter (Millipore SLGV025LS or SLSV025LS) or Abbott #4524 or equivalent	P or A
cetuximab (Erbitux®) Head, neck, colorectal cancer (ImClone LLC)	S	2 mg/mL initial dose: 400 mg/m^2 over 2 h weekly dose: 250 mg/m^2 over 1h use infusion or syringe pump max. infusion rate of 10 mg/min (Erbitux® (cetuximab) [Package insert], 08/2013)	0.22 µm		A

digoxin immune Fab (Digibind®) Digoxin antidote (GlaxoSmithKline Australia Pty Ltd.)	L	38 mg/4mL dosage depends on intoxication level i.v. over 30 min (Digibind (Digoxin immune Fab) [Product information], 08/2008)	0.22 µm		A
gemtuzumab ozogamicin (Mylotarg®) (withdrawn) CD33 positive acute myeloid leukemia (Wyeth Pharmaceuticals Inc.)	L	1 mg/mL 9 mg/m^2 over 2 h use line only for gemtuzumab ozogamicin (Mylotarg® (gemtuzumab ozogamicin for Injection) [Package insert], 04/2010; U.S. Food and Drug Administration, 2010)	0.22 µm or 1.2 µm polyethersulfone (Supor®) 1.2 µm acrylic copolymer hydrophilic (Versapor®) 0.8 µm cellulose mixed ester (acetate and nitrate) 0.2 µm cellulose acetate filter	A	
Yttrium-90 ibritumomab tiuxetan Non-Hodgkin lymphoma (Spectrum Pharmaceuticals, Inc.)	S	3.2 mg/2 mL platelets concentration dependent: 0.4 mCi/kg or 0.3 mCi/kg Y-90 ibritumomab tiuxetan on day 7, 8, or 9 after rituximab treatment test the whole infusion set including the syringe and filter after application for any remaining activity to ensure complete drug delivery (Zevalin®) (Fink-Bennett and Thomas, 2003; Hagenbeek and Lewington, 2005; Zevalin - Anhang 1 - Zusammenfassung der Merkmale des Arzneimittels, accessed 08/2013; Zevalin® (ibritumomab tiuxetan) [Package insert], 11/2011)	0.2 µm	wet filter with normal saline prior administration to reduce any residual activity of the drug on the filter flush the line with at least 10 mL normal saline after injection to prevent an inadequate dosing due to drug retention by the filter	A

infliximab (Remicade®) Arthritis, psoriasis, Crohn's disease, ulcerative colitis & others (Janssen Biologics B.V. Leiden)	L	10 mg/mL rheumatoid arthritis: 3 mg/kg all other indications: 5 mg/kg over 2 h Initial dose, 2 & 6 weeks after initial dose, all 6 to 8 weeks afterwards depending on disease progression (Remicade® (infliximab) [Package insert], 06/2013)	≤1.2 µm	infusion set with in-line filter	A
ipilimumab (Yervoy®) Metastatic melanoma (Bristol-Myers Squibb Company)	S	5 mg/mL final concentration 1 to 2 mg/mL 4 times 3 mg/kg over 90 min every 3 weeks flush i.v. line after administration with an isotone sodium chloride solution or with a 5 % dextrose injection (Bristol-Myers Squibb, 2011; Knauth et al., 2010; Yervoy® (ipilimumab) [Package insert], 05/2013)	0.2 µm to 1.2 µm polyethersulfone 0.2 µm nylon filter	A	
muromonab-CD3 (Orthoclone® Okt 3) Treatment of different allograft rejections (Janssen-Cilag GmbH)	S	1 mg/mL 5 mg/day as bolus over ≤ 1 min for 10 to 14 days children dose differs (Orthoclone® Okt 3 [Package insert], 04/1998)	0.2 or 0.22 µm		P
ofatumumab (Arzerra) Chronic lymphocytic leukemia (GlaxoSmith-Kline)	S	20 mg/mL concentrate first dose: 300 mg following doses: 2 g 8 times weekly, break for 4/5 weeks before 4 times weekly infusion rate starts with 12 mL/h but changes with duration and frequency (Arzerra - Anhang 1 - Zusammenfassung der Merkmale des Arzneimittels, accessed 08/2013)	0.2 µm		A

panitumumab (Vectibix®) Metastatic colorectal carcinoma (Amgen Inc.)	S	20 mg/mL every 2 weeks: 6 mg/kg over 60 min (≤ 1000 mg) or 90 min (> 1000 mg) with infusion pump (Vectibix® (panitumumab) [Package insert], 06/2013)	0.2 or 0.22 µm		P
ranibizumab (Lucentis®) Different ophthalmic diseases (Genentech Inc.)	S	10 mg/mL (ranibizumab 0.5 mg) or 6 mg/mL (ranibizumab 0.3 mg) monthly ophthalmic intravitreal injections of 0.5 mg (0.05 mL) or 0.3 mg (0.05 mL) (disease dependent) (Lucentis® (ranibizumab injection) [Package insert], 02/2013; Montero et al., 2012)	5 µm, 19 gauge filter needle supplied by manufacturer	filter needle prevents injection of rubber particles into the eye	P
siltuximab (Sylvant®) Multicentric Castleman disease (Janssen Biotech Inc.	L	20 mg/mL every 3 weeks: 11 mg/kg over 1 h use of a polyvinyl chloride or polyurethane administration set (Sylvant® (siltuximab) [Package Insert], 06/2014)	0.2 µm	polyethersulfone	A
tositumomab and Iodine I^{131} tositumomab (Bexxar) Non-Hodgkin lymphoma (GlaxoSmith-Kline)	S	Tositumomab 14 mg/mL Iodine I^{131} tositumomab solution: different concentrations available day 0: 450 mg protein over 60 min followed by 5 mCi I-131 and 35 mg protein over 20 min day 6 or 7: 75 cGy total body dose day 7 to 14: 450 mg protein over 60 min followed by therapeutic dose Ci and 35 mg protein over 20 min (Bexxar (tositumomab and iodine I 131 tositumomab) [Package insert], 05/2013)	0.22 µm	use same filter for non- and labeled antibody otherwise possible drug loss up to 7 %	A

trastuzumab emtansine (Kadcyla®) Metastatic breast cancer (Genentech Inc.)	L	20 mg/mL every 3 weeks until disease progression or high toxicity: 3.6 mg/kg first dose over 90 min subsequent ones over 30 min (Kadcyla® - Anhang 1 - Zusammenfassung der Merkmale des Arzneimittels, accessed 07/2014; Kadcyla® (ado-trastuzumab emtansine) [Package insert], 2014)	0.22 µm	polyethersulfone USA: filter is always applied Germany: filter usage only if diluted with a 0.9 % sodium chloride solution, but not with a 0.45 % solution	A

Immune globulin

Drug product (Trade name) Indication (Company)	Dosage form	Protein concentration Route of administration Dosage and frequency of administration Citation	Filter pore size recommendations	Filter type recommendations	Filter usage during P or A
antithymocyte globulin (Atgam®) (Pharmacia & Upjohn Company Division of Pfizer Inc.)	S	information in publications not available (Atgam® [Package insert], 11/2005)	0.2 to 1 µm		A
Thymoglobulin®* Anti-human-thymozyten-immunglobulin (Genzyme Corporation)	L	5 mg/mL daily for 7 to 14 days: 1.5 mg/kg initial dose: over ≥ 6 h subsequent dose: over ≥ 4 h (different instructions in Germany) (Thymoglobulin Anti-thymocyte Globulin (Rabbit) [Package insert], 09/2008; Thymoglobuline 5 mg/ml [Package insert], 01/2013)	0.22 µm		A

botulism immune globulin IV* (BabyBIG®) Infant botulism caused by toxin types A or B (California Department of Public Health)	L	50 mg/mL 2.0 mL/kg (100 mg/kg) over 0.5 mL/kg/h for 15 min before increase (if no adverse effects) to 1.0 mL/kg/h (BabyBIG® [Package insert], 09/2009; Botulismus-Antitoxin Behring [Package insert], 10/2011)	18 µm	in-line or syringe-tip sterile, disposable filter	A
cytomegalo-virus immune globulin* (CytoGam®) Prophylaxis of cytomegalovirus disease (CSL Behring AG)	S	50 ± 10 mg/mL max. dosage 150 mg/kg 72 h and 8 times every 2 weeks post transplantation initial infusion rate of 15 mg/kg/h increases to 30 and 60 mg/kg/h in 2 steps after certain periods of time (CytoGam® Cytomegalovirus Immune Globulin Intravenous (Human) [Package insert], 03/2007; Cytotect® CP Biotest 100 E/ml, 04/2013)	15 µm 0.2 µm		A
immune globulin IV	S or L	please consult package inserts (Carimune® NF [Package insert], 10/2008; Gammagard Liquid® [Package insert], 06/2012; Gammagard S/D® Immune Globulin Intravenous (Human) IgA less than or equal to 1 µg/mL in a 5% Solution [Package insert], 12/2011; Gammagard S/D® Immune Globulin Intravenous (Human) IgA less than or equal to 2.2 µg/mL in a 5% Solution [Package insert], 12/2011; Gamunex®-C [Package insert], 06/2012; Octagam® [Package insert], 12/2005; Privigen [Package insert], 02/2013; Section S: Blood Product Overview - Gamunex® Intravenous Immune Globulin (IVIG) – 10% CBS IGIV-nex, 07/2009)	15 µm (stated for some immune globulin IV drugs)	filter incorporated into the infusion set and provided by distributer filter usage optional for Gammagard Liquid® use 0.2 to 200 µm filter for Octagam® if administered with infusion set	A

* no filtration in German package insert/information sheet for professionals stated

Hormones

regular insulin diabetes	S	exact product name unknown 12 %, respectively, 4 % insulin binds in the first 150 mL of a 5 % dextrose or normal saline solution steps (Trissel, 2007)		Abbott Ivex integral filter and extension sets: modified cellulose ester filters	A

Enzymes

Drug product (Trade name) Indication (Company)	Dosage form	Protein concentration Route of administration Dosage and frequency of administration Citation	Filter pore size recommendations	Filter type recommendations	Filter usage during P or A
Alpha-1 proteinase inhibitors:					
Prolastin® (Talecris Biotherapeutics Inc.)	L	≥ 20 mg/mL (Prolastin® [Package insert], 06/2008)	5 µm	filter needle supplied by manufacturer	P
Zemaira® Alpha-1-antitrypsin deficiency (CSL Behring LLC)	L	50 mg/mL both drugs: weekly 60 mg/kg over 30 min at a rate of ≥ 0.08 ml/kg/min (Remedy Health Media LLC; Zemaira® [Package insert], 04/2013)	5 µm	in-line filter in an administration set	A
agalsidase beta (Fabrazyme®) Fabry disease (Genzyme Corporation)	L	5 mg/mL every two weeks 1 mg/kg infusion rate ≤ 0.25 mg/min (15 mg/h) (Fabrazyme® (agalsidase beta) [Package insert], 2010)	0.2 µm	filter needles are not recommended for preparation	A

alglucosidase alfa (Myozyme®) Pompe disease (Genzyme Corporation)	L	5 mg/mL every 2 weeks 20 mg/kg over 4 h with pump several steps for infusion volumes and rates (Myozyme® (alglucosidase alfa) [Package insert], 2012)	0.2 µm	filter needles are not recommended for preparation	A
asparaginase (Elspar®) Acute lymphoblastic leukemia (Lundbeck)	L	5000 I.U/mL i.m. or 2000 I.U./mL i.v. 3 times weekly 6000 I.U./m^2 i.m. single injection max. 2 mL; multiple injection sites by volume > 2 mL infusion over ≥ 30 min use reconstituted asparaginase immediately, otherwise a small amount of gelatinous fiber-like particles can form (Elspar® (asparaginase) [Package insert], 07/2013; Ipema et al., 2012; Trissel, 2007)	5 µm	no reduction of drug potency with a 5 µm filter, but possible drug loss with a 0.2 µm filter	A
elosulfase alfa (Vimizim™) Mucopoly-saccharidosis type IVA (BioMarin Pharmaceutical Inc.)	S	1 mg/mL weekly 2 mg/kg over ≥ 3.5 to 4.5 h depending on volume with a pump duration and patient weight dependent rate (Vimizim™ (elosulfase alfa) [Product Monograph], 2014)	0.2 µm	infusion set with an incorporated filter	A
galsulfase (Naglazyme®) Mucopoly-saccharidosis VI (BioMarin Pharmaceutical Inc.)	S	1 mg/mL weekly 1 mg/kg over ≥ 4 h with pump volume and duration dependent rates (Ipema et al., 2012; Naglazyme® Dosing & Administration Guide, 2011)	0.2 µm	polyvinyl chloride infusion set with an incorporated filter	A

idursulfase (Elaprase®) Hunter syndrome	S	2 mg/mL weekly 0.5 mg/kg over 3 h rate can be accelerated/decreased depending on hypersensitivity reactions (Elaprase® (idursulfase) [Package insert], 06/2013)	0.2 µm	infusion set with an incorporated filter	A
imiglucerase (Cerezyme®) Gaucher disease (Shire Human Genetic Therapies Inc.)	L	40 U/mL patient individual dosage between 3 times weekly 2.5 U/kg to 60 U/kg once every 2 weeks over 1 to 2 h (Cerezyme® (imiglucerase for injection) [Package insert], accessed 08/2013)	0.2 µm		A
laronidase (Aldurazyme) Mucopoly-saccharidosis I & others (Genzyme Corporation)	S	2.9 mg/5 mL weekly 0.58 mg/kg over 3 to 4 h at a rate of ≤ 200 µg/kg/h use low protein binding container (Aldurazyme (laronidase) [Package insert], 04/2013)	0.2 µm	no usage of filter needles due to possible agitation use low protein binding infusion set with in-line filter	A
Protein C (Ceprotin®) Congenital Protein C deficiency (Baxter Healthcare Corporation)	L	100 IU/mL circumstance dependent dose maintenance dose is 45 to 60 IU/kg every 6 or 12 h infusion rate of 2 mL/min or if body weight < 10 kg ≤ 0.2 mL/kg/min (Ceprotin® [Package insert], 12/2011)		filter needle	P
streptokinase thrombolytic enzyme	L	information in publications not available (Chen and Martinez, 1998; Trissel, 2007; Wolters Kluwer Health / Facts and Comparisons, 2007)	≥ 0.8 µm		P
taliglucerase alfa (Elelyso™) Gaucher disease (Pfizer Labs Division of Pfizer Inc.)	L	200 Units/5 mL weekly 60 units/kg over ≥ 60 min adult rate: 1.2 mL/min (max. 2.2 mL/min) pediatric rate: 1 mL/min (max. 2 mL/min) (Elelyso™ (taliglucerase alfa) [Package insert], 09/2014)	0.2 µm		A

urokinase (Abbokinase®) Lysis of pulmonary emboli (Abbott Laboratories)	L	50000 I.U./mL weight dependent concentration initial rate: 90 mL/h for 10 min rate for 12 h period: 15 mL/h (Abbokinase® [Package insert], 10/2002)	≤ 0.45 µm	cellulose membrane filter	A
velaglucerase alfa (Vpriv®) Gaucher disease (Shire Human Genetic Therapies, Inc.)	L	100 Units/mL weekly 15 to 60 units/kg over 60 min (Vpriv® (velaglucerase alfa for injection) [Package insert], 08/2013)	0.2 µm		A

Cytokines

molgramostim (Macrogen™) Cancer chemotherapy/ bone marrow transplantation (Zenotech Laboratories Limited)	L	$4.4x10^6$ I.U./mL (equivalent to 400 mg) cancer: 5-10 µg/kg s.c for 7 to 10 days transplantation: 10 µg/kg i.v. 4 to 6 h; max. duration 30 days (Molgramostim Macrogen™ [Package insert], 01/2006)	0.2 µm (only infusion not s.c.)		A

Antihemophilic drugs
Drugs for blood coagulation
For all antihemophilic drugs: disease progression depending dose and frequencies

Drug product (Trade name) Indication (Company)	Dosage form	Protein concentration Route of administration Dosage and frequency of administration Citation	Filter pore size recommendations	Filter type recommendations	Filter usage during P or A
Advate® (Baxter Healthcare Corporation)	L	250/500/1000/1500/2000/3000/4000 I.U./5 mL 250/500/1000/1500 I.U./2 mL bolus infusion: ≤ 5 min (max. infusion rate 10 mL/min) (Advate 250 IU powder and solvent for solution for injection - ANNEX I - Summary of product characteristics, 03/2009; Advate [Package insert], 06/2013, 07/2012)		filter device	P
AlphaNine® SD (Grifols Biologicals Inc.)	L	500/1000/1500 I.U. FIX/10 mL (AlphaNine® SD [Package insert], 01/2013)		Mix2Vial™ filter transfer device	P
Helixate FS® (CSL Behring LLC)	L	250/500/1000 I.U./2.5 mL 2000/3000 I.U./5mL patient dependent injection rate over 1 to 15 min (Helixate FS [Package insert], 05/2012)		Mix2Vial™ filter transfer device	P
Hemofil-M® (Baxter Healthcare Corporation)	L	250/500/1000/1700 I.U./10 mL injection rate: 10 mL/min use of disposable plastic syringes due to sorption problems with all-glass syringes (Hemofil M [Package insert], 11/2010)		filter needle	P
Humate-P® (CSL Behring GmbH)	L	VWF:RCo/vial 600 I.U. / FVIII/vial 250 I.U. / 5 mL VWF:RCo/vial 1200 I.U. / FVIII/vial 500 I.U./ 10 mL VWF:RCo/vial 2400 I.U. / FVIII/vial 1000 I.U./15 mL infusion rate: ≤ 4 mL/min (Humate-P® [Package insert], 05/2012)		Mix2Vial™ filter transfer device	P

Koate®-DVI (Kedrion Biopharma Inc.)	L	250/500 I.U./5mL 1000 I.U./10 mL syringe injection or drip infusion patient dependent rate over 5 to 10 min (Antihemophilic Factor (Human) Koate-DVI [Package insert], 08/2012)		filter needle	P
Kogenate FS® (Bayer HealthCare LLC)	L	250/500/1000/2000/3000 I.U. patient dependent injection rate over 1 to 15 min (Kogenate FS [Package insert], 01/2013)		filter needle **AND** in-line filter (provided with administration set) otherwise use a compatible filter	P **AND** A
Monarc-M™ (Baxter Healthcare Corporation)	L	injection rate: ≤ 10 mL/min use of disposable plastic syringes due to sorption problems with all-glass syringes (Monarc-M™ [Package insert], 04/2005)		filter needle	P
Monoclate-P® (CSL Behring LLC)	L	100 I.U./ mL or 150 I.U./ mL injection rate: 2 mL/min use of disposable plastic syringes due to sorption problems with all-glass syringes (Monoclate-P® [Package insert], 10/2010)	5 µm	filter needle or vented filter spike	P
NovoEight® (Novo Nordisk A/S)	L	250/500/1000/1500/2000/3000 I.U. corresponds to 62.5/125/250/375/500/750 I.U./mL slow injection over 2 to 5 min (NovoEight® - ANNEX I - Summary of product characteristics accessed 07/2014; NovoEight® Antihemophilic Factor (Recombinant) [Package insert], accessed 07/2014)	25 µm	vial adapter	P

Human Prothrombin Complex					
Beriplex® P/N 250/500/1000 (CSL Behring GmbH)	L	total protein amount: 6 to 14 mg/mL max. dose: 5000 I.U. injection rate: 8 mL/min (Gebrauchsinformation: Information für den Anwender Beriplex® P/N 250/500/1000, 01/2013)		Mix2Vial™ filter transfer device	P
octaplex® (Octapharma Pharmazeutika Produktionsges. m.b.H)	L	total protein amount: 13 to 41 mg/mL injection rate: at beginning 1 mL/min followed by 2 to 3 mL/min (Product Monograph octaplex®, 08/2014)		Mix2Vial™ filter transfer device	P
Antihemophilic drugs registered in Germany (17 of 25 are filtered)					
Beriate® 250/500/1000/ 2000 (CSL Behring GmbH)	L	100 I.U./ml or 200 I.U./ml slow injection (Beriate® 250/500/1000/2000 Fachinformation, 05/2014; Beriate® [Package insert], 12/2011)		Mix2Vial™ filter transfer device	P
Berinin® P 300/600/1200 (CSL Behring GmbH)	L	120 I.U./ml injection or infusion rate: ≤ 2 mL/min (Gebrauchsinformation: Information für den Anwender Berinin® P 300/600/1200, 08/2011)		Mix2Vial™ filter transfer device	P
Fanhdi® (Grifols Deutschland GmbH)	L	250/500/1000 I.U./10 mL or 1500 I.U./15 mL injection rate: 3 ml/min (max. 10 mL/min) (Fanhdi® [Package insert], 09/2011)		filter	P
Fibrogammin® 250/1250 (CSL Behring GmbH)	L	250 I.U./4 mL or 1250 I.U./20 mL injection or infusion rate: ≤ 4 mL/min (Fibrogammin® 250/1250 Gebrauchsinformation und Fachinformation, 05/2013)		Mix2Vial™ filter transfer device	P
Haemonine® 250/500/1000	L	50 I.U./mL or 100 I.U./mL injection rate: 2 to 3 mL/min (max. 5 mL/min)		transfer system with integrated filter	P

(Biotest Pharma GmbH)		(Gebrauchsinformation: Information für den Anwender Haemonine® 250, 03/2009; Haemonine® 500/1000 Fachinformation, 04/2013)			
Immuseven (Baxter Deutschland GmbH)	L	60 I.U./ml injection rate: 2 ml/min (Immuseven Fachinformation, 08/2013)		filter needle	P
Mononine® 1000 I.U. (CSL Behring GmbH)	L	100 I.U./ml bolus injection: 2 ml/min infusion over hours or days (Mononine® 1000 I.E. [Package insert], 08/2007)		filter transfer set 20/13	P
Octanate® 250/500/1000 (OCTAPHARMA GmbH)	L	Octanate 250: 50 I.U./ml Octanate 500: 100 I.U./ml Octanate 1000: 200 I.U./ml injection rate: 2 to 3 mL/min (Octanate 250/500/1000 [Package insert], 05/2011)		filter	P
Recombinate® 1000 (Baxter Healthcare Corporation)	L	100 I.U./mL injection rate: 10 mL/min (Gebrauchsinformation: Information für den Anwender Recombinate Antihämophilie Faktor (rekombinant) 1000, 07/2012; Recombinate® [Package insert], 12/2010)		BAXJECT II	P

Antithrombin substances
Inhibitor of blood coagulation

Drug product (Trade name) Indication (Company)	Dosage form	Protein concentration Route of administration Dosage and frequency of administration Citation	Filter pore size recommendations	Filter type recommendations	Filter usage during P or A
Thrombate III® (Grifols Therapeutics Inc.)	L	500 I.U./10 mL individual doses over 10 to 20 min (Antithrombin III (Human) Thrombate III® [Package insert], 10/2012)		filter needle	P

recombinant antithrombin (ATryn®) (GTC Biotherapeutics Inc.)	L	1750 I.U./10 mL individual doses 15 min initial dose followed by continuous infusion (ATryn® [Package insert], 11/2010)	0.22 µm	infusion set with filter	A

Plasma protein Albumin
Hypovolemia, hypoalbuminemia, cardiopulmonary bypass surgery & others
Dosage is patient dependent

Drug product (Trade name) Indication (Company)	Dosage form	Protein concentration Route of administration Dosage and frequency of administration Citation	Filter pore size recommendations	Filter type recommendations	Filter usage during P or A
Buminate® 5 % (Baxter Healthcare Corporation)	S	5 g/100 mL of albumin instructions printed on administration set container (Buminate® 5% [Package insert], 09/2009)	≤ 15 µm	integrate in-line filter in administration set	A
Flexbumin® 25 % (Baxter Healthcare Corporation)	S	25 g/100 mL of albumin (Flexbumin® 25% [Package insert], 09/2009)	≤ 15 µm	integrate in-line filter in administration set	A
Plasbumin®-5* (Grifols Therapeutics Inc.)	S	Plasbumin®-5: 2.5g/50 mL or 12.5g/250 mL of albumin infusion rate: max. 5 mL/min (Gebrauchsinformation und Fachinformation Plasbumin® 20 [Package insert], 10/2011; Plasbumin®-5 [Package insert], 12/2011)		filter needle	A

* no filtration in German package insert stated

For a small number of proteins an explicit non-filtration statement exists, These proteins are etanercept (Enbrel® (etanercept) [Package insert], 06/2013), Gamunex® intravenous immune globulin (Section S: Blood Product Overview - Gamunex® Intravenous Immune Globulin (IVIG) – 10% CBS IGIV-nex, 07/2009), rasburicase (Elitek® (rasburicase) [Package insert], 10/2009), palifermin (Kepivance™ (palifermin) [Package insert], 02/2013), aldesleukin (Proleukin® (aldesleukin) [Package insert], 07/2012) and sargramostim (Leukine Sargramostin [Package insert], 06/2002). Possible adsorption of the drug onto the filter might be possible for sargramostim (Trissel, 2007).
Proteins which shall not be filtered are listed in Table 2.

Table 2: The following listed protein drug products should not be filtered during preparation or administration

NO filtration of the following drug products
etanercept (Enbrel®) (Immunex Corporation)
Gamunex® intravenous immune globulin (Talecris)
rasburicase (Elitek®) (Sanofi-Aventis U.S. LLC)
palifermin (Kepivance™) (A. Menarini Australia Pty Ltd)
aldesleukin (Proleukin®) (Prometheus Laboratories Inc.)
sargramostim (Leukine Sargramostin) (Berlex Laboratories Inc.)

Table 3 shows a large group of products for which no data on filtration is available.

Table 3: For a large number of proteins a filtration recommendation cannot be found. The list is not complete.

No filtration recommendation available	
adalimumab (AbbVie Inc.)	insulin lispro (Lilly USA, LLC)
alipogen tiparvovec (uniQure biopharma B.V.)	interferon alfa-2a (Roche)
anakinra (Swedish Orphan Biovitrum AB (publ))	interferon beta-1a (Biogen Idec.)
basiliximab (Novartis Pharmaceuticals Corporation)	interferon alfa-2b (Schering Corporation)
belimumab (GlaxoSmithKline Manufacturing SpA)	interferon beta-1b (Novartis Pharmaceuticals Corporation)
bevacizumab (Genentech Inc.)	interferon gamma-1b (Vidara Therapeutics Inc.)
brentuximab vedotin (Seattle Genetics Inc.)	lenograstim (Chugai Pharmaceuticals Co. Ltd)
canakinumab (Novartis Pharmaceuticals Corporation)	liraglutid (Novo Nordisk A/S)

Table 3 continued	
catridecacog (Novo Nordisk A/S)	lutropin alfa (EMD Serono Inc.)
certolizumab pegol (UCB Inc.)	mecasermin (Ipsen Pharma)
choriogonadotropin alfa (EMD Serono Inc.)	methoxy polyethylene glycol-epoetin beta (Roche Products Pty Limited)
conestat alfa (Pharming Group N.V.)	moroctocog alfa (Pfizer Limited)
corifollitropin alfa (N.V. Organon)	natalizumab (Biogen Idec Inc.)
darbepoetin alfa (Amgen Inc.)	nonacog alfa (Pfizer Limited)
denosumab (Amgen Inc.)	ocriplasmin (ThromboGenics Inc.)
desirudin (Canyon Pharmaceuticals Inc.)	omalizumab (Genentech Inc.)
dornase alfa (Genentech Inc.)	palivizumab (MedImmune LLC)
dibotermin alfa (Medtronic BioPharma B.V.)	pegfilgrastim (Amgen Inc.)
eculizumab (Alexion Pharmaceuticals Inc.)	peginterferon alfa-2a (Genentech Inc.)
epoetin alfa (Janssen-Cilag Pty Ltd)	peginterferon alfa-2b (Schering Corporation)
epoetin beta (Roche Pharma AG)	peginterferon beta-1a (Biogen Idec Inc.)
epoetin theta (ratiopharm GmbH)	pegloticase (Crealta Pharmaceuticals LLC)
epoetin-zeta (Biosimilar) (HOSPIRA Enterprises B.V.)	pegvisomant (Pharmacia & Upjohn Co)
eptacog alfa (recombinant Factor VII) (Novo Nordisk A/S)	pertuzumab (Genentech Inc.)
eptotermin alfa (Olympus Biotech International Limited)	rituximab (Genentech Inc.)
factor VIII	romiplostim (Amgen Inc.)
filgrastim (G-CSF) (Amgen Inc.)	simoctocog alfa (Octapharma AB)
follitropin alfa (EMD Serono Inc.)	somatropin (Pharmacia & Upjohn Co)
follitropin alfa/Lutropin alfa (Merck Serono Europe Limited)	tasonermin (Boehringer Ingelheim Internation GmbH)
follitropin beta (Merck Sharp & Dohme Corp.)	teduglutid (Hospira Inc.)
glucagon (Novo Nordisk Pharmaceuticals Pty Ltd)	tenecteplase (Genentech Inc.)
golimumab (Janssen Biotech Inc.)	teriparatid (Lilly USA LLC)
insulin aspart (Novo Nordisk Canada Inc.)	thyrotropin alfa (Genzyme Canada)
insulin degludec/ insulin aspart (Novo Nordisk A/S)	tocilizumab (Genentech Inc.)
insulin detemir (Novo Nordisk A/S)	trastuzumab (Genentech Inc.)
insulin glargine (sanofi-aventis U.S. LLC)	ustekinumab (Janssen Biotech Inc.)
insulin glulisine (sanofi-aventis U.S. LLC)	vedolizumab (Takeda Pharmaceuticals America, Inc.)
insulin human (Novo Nordisk A/S)	

1.3. Discussion

1.3.1 Detailed analysis of the filtered protein drug products

Our analysis identified worldwide more than 60 protein drugs with a mandatory filtration during preparation or administration (Table 1). Only for six proteins an explicit filtration prohibition has been found (Table 2). Figure 2 visualizes the ratio of filtered to non-filtered drugs registered in Germany. Interestingly, 4 out of 9 protein drugs, which entered the German market in 2014, are filtered (Govi-Verlag Pharmazeutischer Verlag GmbH; Kadcyla® - Anhang 1 - Zusammenfassung der Merkmale des Arzneimittels, accessed 07/2014; NovoEight® - ANNEX I - Summary of product characteristics accessed 07/2014; Sylvant® (siltuximab) [Package Insert], 06/2014; Vimizim™ (elosulfase alfa) [Product Monograph], 2014). For another product the compatibility with polyestersulfone filters was at least checked (Gebrauchsinformation: Information für Patienten Gazyvaro™ 1.000 mg Konzentrat zur Herstellung einer Infusionslösung, 07/2014). A closer look at Table 1 reveals the following. Filtration of the drugs is applied during preparation in 44.6 % and during administration in 55.4 % of all cases, respectively. Further, the list contains 67.7 % lyophilisates and 32.3 % solutions whereupon intravenous immune globulin drugs are excluded in this ratio, because both dosage forms exist for intravenous immune globulins. For the majority of the listed protein solutions, filters are mainly applied during administration (80 %), whereas in 20 % the filtration step is carried out during preparation. Only abciximab solution is filtered during either preparation or administration.

On the contrary, 55.6 % of the lyophilisates are filtered during preparation. Filtration of lyophilisates seems to be consistent considering the following facts. The lyophilized drug is stored in a glass vial and sealed with a rubber stopper. For reconstitution the liquid component, which has also an impact on particle formation (Zhang et al., 1996), of the final drug product is added to the lyophilisate. To this end, the stopper is punctured with a sharp cannula. Puncturing of the stopper is also necessary for the drug removal. During these penetration processes coring can occur. Coring describes the shedding of rubber particles into the solution due to the needle insertion (Asakura et al., 2001; Campagna et al., 2013; Waller and George, 1986). Particles present a risk for patients as discussed previously. Subsequently, an incorporated filter during the filling of the syringe or the administration via an infusion set shall protect the patient from the exposure to particles. The origin of these particles can be either inherent to the reconstituted drug solution (Chang and Pikal, 2009), the stopper (Asakura et al., 2001; Campagna et al., 2013) or any part of the infusion set (Madsen and Winding, 1996).

Most of the proteins are filtered using a pore size of 0.2 µm or 0.22 µm, which is also commonly used for sterile filtration. Other frequently stated pore sizes are 5 µm and 15 µm. For some proteins like abatacept, belatacept, abciximab and others (see Table 1) the pore

size of the filter has a wide range from 0.2 µm to 5 µm or even larger. There are no explanations given for the large differences in the pore size. Maybe the manufacturers want to make sure that the medically reasonable application is not hindered ad hoc, due to a single logistic problem at the bedside, where a particular filter is just not readily available. “Better do something than nothing” appears to be the slogan. On the other hand, it may reflect the situation that “normally” the product is clean anyway and the filter is not so relevant. In case precipitation occurs and is not detected visually, also a larger pore size might reduce the particle load dramatically. According to the pharmacopoeia parenteral drugs need to be visually inspected to detect particulate matter and container with defects need to be rejected (European Directorate For The Quality Of Medicine, 2011c; The United States Pharmacopeial Convention, 2011a). Consequently, the drug products are 100 % visually inspected after filling by trained operators, semi- or automatic processes and acceptable quality level checks before release (Doessegger et al., 2012). Nevertheless, the chance exists that particles are formed during transportation and storage of the drug product. Besides, particulate matter is one of the top ten reasons for the recall of parenteral drugs (Doessegger et al., 2012). Hence, the visual inspection of the parenteral dosage form before administration by healthcare professionals or the patient is of utmost importance. Instructions for the performance of such a visual inspection can also be frequently found in package inserts.

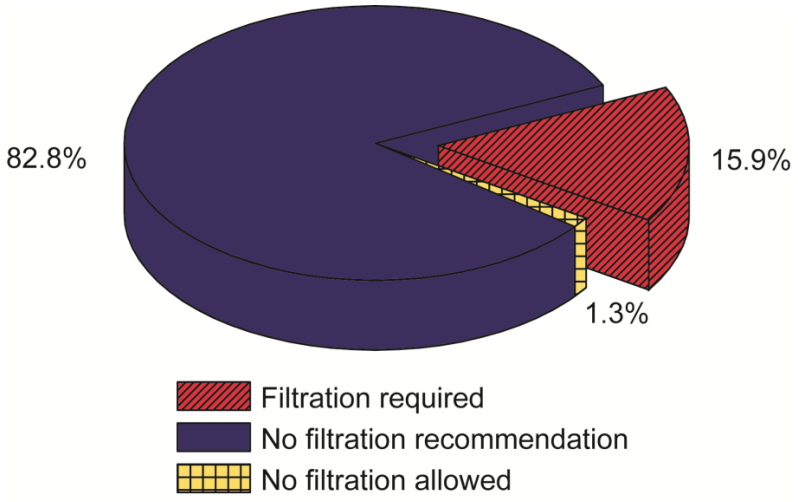

Figure 2: Filtration is already carried out for 15.9 % of all approved protein drugs listed in the Rote Liste® as the pie chart displays (Rote Liste® Service GmbH Frankfurt/Main, 01/2015, 2013).

1.3.2 Consideration of possible negative filtration impacts

1.3.2.1 Filter shedding

Although filters remove particle or bacteria, the following facts need to be considered. Filters themselves can be the source of particles and extractables (Eisenberg, 1974; Huang et al., 2011; Liu et al., 2012). Already in 1974 the pharmaceutical industry was aware about the particle shedding from filter membranes (Eisenberg, 1974). At that time especially the shedding of toxic asbestos filters was a problem and led to an FDA announcement to improve the filtration methods (Eisenberg, 1974). However, particle shedding from filters is still a serious issue as a study by Liu *et al.* shows (Liu et al., 2012). Particle burden of a buffer and protein solution increased after filtration for some filter types (Liu et al., 2012). This also suggests different quality levels by the filters itself. Hence, the quality of the filters has to be harmonized. Until then, the manufacturers need to state clearly which filter should be applied for their product or even better provide the right filter with their product. Particulate matter can also not be easily diminished by preflushing. Further, the toxic impact of these particles is not known (Liu et al., 2012).

1.3.2.2 Extractables deriving from filters

Extractables are another important topic. Especially extractables of filter membranes used for protein filtration, like polyethersulfone or polyvinylidene fluoride, show surface activity. Depending on the membrane type the extractables can have a positive or negative influence on the particle level after agitation. Further, it is particularly important to know about the surface activity of the extractables, if only small volumes are filtered due to possible changes of the surface tension. Flushing the filter before usage is the best option to diminish the amount of extractables (Huang et al., 2011). For clinical reasons a study by Harrison and Healy is highly relevant suggesting a higher effectiveness of filters, if the flow rate is low (Harrison and Healy, 1974).

1.3.2.3 Drug adsorption on filters

Another critical aspect regarding the usage of filters is the possible retention of the drug or other formulation components. Protein fouling is of little importance if the solutions are aggregate free. If aggregates are present they associate with protein monomers (Kanani et al., 2008). Then fouling is able to reduce the flux and increase the membrane resistance, respectively, transmembrane pressure (Güell and Davis, 1996; Kanani et al., 2008). Severe filter clogging making filtration impossible during the investigated volumes and times was not observed (Güell and Davis, 1996; Kanani et al., 2008). The adsorption of the protein on the filter may be not a problem because in many cases the manufacturers do not instruct to use

a specific filter membrane. But it is also possible that protein drug adsorption on filters has not attracted enough interest by many of the manufacturers. Several package inserts just recommended the use of a “low protein binding filter”. This is not very precise, as many different filter membranes are available and not everybody in nursing is familiar with protein binding capacities of filter membranes. Membranes with low protein binding properties are polyethersulfone, polyvinylidene fluoride and cellulose acetate (Mahler et al., 2010). Intensive care units often use nylon or polyethersulfone membranes as a standard, cellulose filters get more and more unimportant (Gasch et al., 2011; Leopold et al., 2013). Specific guidance would therefore be needed as nylon would be a particularly inappropriate choice for protein drug products. The package inserts of two products exclusively mention the adsorption issue. For Bexxar® the manufacturer warns to switch the filter between the infusion of the non- and radiolabelled antibody, because this action will lead to a loss of the drug (Bexxar (tositumomab and iodine I 131 tositumomab) [Package insert], 05/2013). To ensure a complete drug delivery, a flushing of the infusion set including the filter takes place after administration of Yttrium-90 ibritumomab tiuxetan (Fink-Bennett and Thomas, 2003; Zevalin® (ibritumomab tiuxetan) [Package insert], 11/2011). For obinutuzumab the company has investigated incompatibilities between the final drug solution and polyethersulfone filters, even though filtration is not obligatory (Gebrauchsinformation: Information für Patienten Gazyvaro™ 1.000 mg Konzentrat zur Herstellung einer Infusionslösung, 07/2014). Although no incompatibilities are found, the information about the filter is missing in the American package insert (Gazyva® (obinutuzumab) [Package insert], 12/2014; Gebrauchsinformation: Information für Patienten Gazyvaro™ 1.000 mg Konzentrat zur Herstellung einer Infusionslösung, 07/2014). Unfortunately, adsorption studies for filters are lacking, particularly for proteins. A study by Gasch *et al.* in 2011, is the first independent binding study for polyethersulfone filters, although this filter membrane was already introduced in the late 1980ies (Gasch et al., 2011).

However, taking a look at small molecule anticancer drugs, which present a formulation challenge due to their properties, for example their instability in aqueous systems, and the need for solubilizers (Hatefi et al., 2004), shows that the retention of drugs by filters should generally not be neglected. The examination of the adsorption of drugs on filters has started in the late 1970ies (Rusmin et al., 1977). Anticancer drugs were in the focus of the studies, because patients suffering from cancer are prone to infections due to their suppressed immune status (Bethune et al., 2001; De Vroe et al., 1990; Francomb et al., 1994). The most important facts, which can be learned from the investigation of the adsorption behavior of small molecule anticancer drugs onto filters and should be transferred to protein filtration, are shortly summarized.

Drug adsorption to the filter occurs frequently in the initial state of infusion. After a while the defined concentration is reached and the total loss of the drug is not of any clinical concern (De Vroe et al., 1990; Francomb et al., 1994; Rusmin et al., 1977). Although some drugs do not adsorb to different filter types, many cases provide evidence that each combination of a drug and filter needs to be evaluated before clinical administration (Gasch et al., 2011). A change of the filter type can have a severe influence on the concentration recovery as shown for vincristine (Butler et al., 1980; Ennis et al., 1983; Francomb et al., 1994; Pavlik et al., 1983; Pavlik et al., 1982). But also the same membrane type of different suppliers can change the drug loss as demonstrated for bleomycin (De Vroe et al., 1990). Factors, which also need to be considered are drug concentration, pH, different infusion fluids or flow rates (De Vroe et al., 1990). In particular low concentrated drugs (Butler et al., 1980; Rusmin et al., 1977) with a small volume or drugs with a short half-life with low flow rates are endangered to have a significant potency loss (Francomb et al., 1994). To prevent underdosing of vital drugs the Intravenous Nurses Society guidelines state that due to a retention of a drug on the filter membrane, the use of filters might be contraindicated for some drugs, particularly those administered in low doses (Ortolano et al., 2004). The binding of cytotoxic agents to filters might not be a large problem nowadays anymore as a recent study by Bononi *et al.* with paclitaxel suggests (Bononi et al., 2011). They conclude from their experiment that the filter manufactures have meanwhile rendered the membranes successfully to reduce the adsorption of drugs to a minimum (Bononi et al., 2011). However, a study confirming such an improvement for protein filtration is still lacking.

As already pointed out above, the findings from the anticancer drugs are useful, because most of these drug molecules are hardly water soluble and/or hydrophobic. Hence, they stick more easily to the membrane material. Further, these molecules are instable in solution, showing a high tendency to precipitate, which can be triggered by filtration. However, drug adsorption on filters is only critical for low dose proteins like interferons or hormones, but not for monoclonal antibodies or enzymes. Moreover, low dosage protein products comprise a surfactant very often. Subsequently, extraction of filter components is another issue beside adsorption. For low dosage drugs the protein recovery from different membranes of various suppliers should be evaluated. Further, the impacts of the drug concentration, infusion fluid and flow rate also needs to be determined.

1.3.3 Proposals for future filter usage

Considering the facts, summarized above, the authors like to make some proposals. First, to qualify some lead qualities, for example the specific use of polyethersulfone or polyvinylidene fluoride as membrane type for protein drugs which are already filtered. Next, picking up an idea from Liu *et al.* (Liu et al., 2012), products, in this specific case filters, which do not shed

particles themselves need to be identified. On this field more basic and intensive research needs to be performed. The authors also like to encourage establishing a filtration routine for not yet filtered protein drugs. Any decision should be based on a risk analysis considering incidence of particle formation as well as incidence and severeness of potential immunogenicity related clinical consequences. An additional filtration step shall be considered as additional safety measure with the aim to reduce potential immunogenicity. In our point of view a more general usage of filters can be beneficial to all patients receiving protein drug products until particles can eventually be excluded as a potential cause for immunogenicity in the future. Patients who are treated with protein drugs suffer from severe diseases and are already weakened by their disease. A recent study by Ahmadi *et al.*, which shows that particularly aggregates of proteins with little or no clinical immunogenicity have the potential to induce clinical immunogenicity supports our approach (Ahmadi et al., 2015). Further, the authors suggest to perform statistical analysis between same or similar products, which are filtered or not filtered to see, whether a difference in the immunogenicity rate can be found.

1.4. Conclusion

Particles in parenteral preparations are associated with risks for the patient. In addition to injection site reactions or occlusion of capillary blood vessels, a more recent problem emerging is immunogenicity caused by non-native protein species. The authors therefore want to stimulate a discussion on a more widespread usage of in-line filters as a possible solution. This is supported by the fact that already 15.9 % of all protein drug products and a high percentage of newly approved drugs are filtered.

Further, the filtration practice should be revised and harmonized. Three central questions need to be addressed. First, which filter membranes and pore sizes shall be used for protein filtration? Next, does significant protein adsorption occur, especially for low dosed drugs? Third, are filters beneficial or do they contribute to the particle burden? Studies to these topics are lacking, although important. Independent investigations shall be encouraged, but also the manufacturers should provide more details about their filter recommendations. This may finally ensure reasonable filter usage to as many as possible drugs for the reduction of immunogenicity issues and other risks associated with particles.

With the proposed approach using in-line filters we might be able to improve the quality and safety of all protein drug products.

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Chapter 2

Expanding bedside filtration – a powerful tool to protect patients from protein aggregates

Chapter 2 is intended for publication.

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Abstract

Today it is commonly accepted that protein aggregates and protein particles also in particular with silicone oil can induce immune reactions and can therefore endanger the safety and efficacy of a biopharmaceutical drug. A total absence of protein aggregate formation is even with the most stable formulation impossible to achieve. An application of bedside filtration, a filtration during the preparation or administration of the drug product, has the potential to increase the safety of every drug container and could diminish the undesired injection of particulate matter into the patient. In this study the high efficiency of filtration for reducing the amount of protein particles was demonstrated with more than 19 stressed and non-stressed biopharmaceutocal products which covered a broad concentration and molecular weight range. Further, critical aspects regarding the usage of filters such as particle shedding from filters, protein loss as a result of protein adsorption or the hold-up volume of the filter were assessed. Although differences between the filters were observed, no negative impacts were generally caused by the investigated filters. As bedside filtration provides great benefits, its broader application and its establishment are proposed.

Keywords

protein aggregates, immunogenicity, in-line filtration, bedside filtration, protein adsorption, leachables, ejection force, protein denaturation, particle, filter

Abbreviations

CD, Circular Dichroism; DLS, Dynamic Light Scattering; FT-IR, Fourier Transform Infrared Spectroscopy; GC-MS, Gas Chromatography-Mass Spectrometer; HP-SEC, High-Performance Size Exclusion Chromatography; ICP-OES, Inductively Coupled Plasma Optical Emission Spectrometry; LO, Light Obscuration; MFI, Micro-Flow Imaging; NTA, Nanoparticle Tracking Analysis; n.a., not analyzed; PdI, Polydispersity Index; PES, polyethersulfone; PVDF, polyvinylidene difluoride; RP-HPLC, Reversed-Phase High-Performance Liquid Chromatography; Z-Ave, Z-Average.

Supplementary

Additional information is available in the supplementary (Chapter 2 Supplementary). Figures and tables found in the supplementary are referred in the text to “Figure S <Number>” or “Table S <Number>”.

2.1. Introduction

Biopharmaceuticals are complex, sensitive and highly developed products which can generally be considered as relatively safe. Before the final high quality drug product is available on the market, a lot of research is invested in finding the best, most stable formulation. Even with all the current knowledge available it is nearly impossible to absolutely exclude the formation of protein aggregates in these optimized formulations [1-4]. Multiple factors like oxygen, light, shaking or temperature can lead to chemical and physical degradation of the protein resulting in the generation of protein aggregates [3-5].

These non-native protein species are associated with immunogenicity risks. Anti-drug antibodies are only one type of immune reaction. The formation of antibodies against the therapeutic protein after administration is known for nearly all protein drug products [5]. Anti-drug antibodies have been observed e.g. for adalimumab [6], insulin [7], human growth hormone [8], erythropoietin [9], interferon [10, 11], etanercept [12] and many more. Clinical consequences of these unwanted immune responses are often negligible, but can also be severe like anaphylaxis and serum sickness or can even be life threatening, if an endogenous protein like erythropoietin is neutralized [2, 9, 13]. Improvements in the design of human recombinant proteins like sequence modifications or fully humanized proteins have not been able to eliminate the issue of immune responses against non-native protein species [5, 11, 13-16]. Although dose, dosing frequency, route of administration or the immune status of the patient play a major role for the incidence of immunological reactions [5, 13, 17], the safety and efficacy of the drug is endangered by the presence of protein aggregates [5, 15, 17, 18]. Beside protein aggregates, particles consisting of protein adsorbed to silicone oil or glass flakes can also be formed in the final product with both combinations known to activate the immune system [19, 20]. All these protein particles can be formed not only during the manufacturing and filling process, but also afterwards during shipping and storage [3, 5, 16, 21].

Product quality is monitored by the manufacturers by a comprehensive quality assurance such as stability tests, equipment validation or in-process controls. In the end a batch is statistically monitored before it is distributed and afterwards no more product characterization with analytical tools can be performed. The only check before administration will be visual inspection by the medical personnel or by the patient himself as instructed in package inserts [22]. Most of the particles found in such parenteral products have sizes of less than 10 µm [23]. An unaided eye, however, detects particles of a size from 100 µm upwards [17]. Hence, a lot of potentially immunogenic protein particles would go unnoticed and could be injected into the patient.

Being aware of the risks associated with the protein aggregates and the fact that it is impossible to totally exclude protein aggregation measures should be taken. The safety provided during product release should be complemented by an approach to ensure cleanliness for every single container. Therefore, expansion of bedside filtration which is already carried out for several biopharmaceuticals is proposed in this study. Due to the fact that bedside filtration is already used in the field, it should be easy to implement [16, 22]. Bedside filtration has the capability to provide more safety for every single container resulting in a lower exposure of the patients to particulate matter. A survey analysis of more than 300 marketed protein drug products showed an application of bedside filtration during preparation and administration of the drug in already 16 % of all products. For less than 1.5 % of the drugs filtration was explicitly forbidden, but for the overwhelming majority no filtration recommendations exists [22]. However, new approvals of biopharmaceuticals in Germany in the last three years showed that about 45 % of these approvals are bedside filtered. So, a clear tendency towards more filtration is obvious. Our considerations are confirmed by the fact that authorities now request the additional monitoring of particles in the low micrometer range (< 10 µm) [16, 18, 24].

Bedside filtration is mostly carried out with 0.2 µm filters with a low protein binding polyethersulfone membrane [22]. The usage of common 0.2 µm filters in clinical settings ensures a broad and cheap distribution and provides an additional sterile filtration step. Further, a reduction in the occurrence of sepsis, infection and thrombi is observed by the usage of 0.2 µm filters [25, 26].

However, several technical aspects regarding the filter usage need to be addressed. First and foremost, it has to be assessed, if filters possess the capability to remove protein particles effectively. Second, literature reports about some filters shedding particles which could contribute to the overall particle count of protein solutions [27]. This would thwart the entire idea, because particulate matter should be effectively removed by filtration and it should be not exchanged by other impurities from filter components. Third, it has to be ensured that there is no protein loss due to adsorption to the filter membrane or due to protein denaturation caused by shear stress. Next, especially for products with small volumes, the filter hold-up volume, which represents the remaining volume in the filter housing after the filtration process, has to be as low as possible, because biopharmaceuticals are typically expensive and unnecessary overages have to be avoided. Further, it was determined, if soluble leachables are present in the filtered protein solutions [28, 29] and if a filter affects negatively the ejection force.

These important technical aspects are critically investigated in this study and a recommendation regarding the application of bedside filtration in a more routinely way is presented at the end.

2.2. Materials and Methods

2.2.1 Materials

Products, materials and their brand names might be protected due to intellectual property rights although they might not be marked with intellectual property signs.

2.2.1.1 Chemicals

Pure sodium chloride was purchased from Bernd Kraft GmbH (Duisburg, Germany). di-sodium hydrogen phosphate dihydrate p. A., sodium dihydrogen phosphate dehydrate p. A. and L-Arginine base pure Ph.Eur., USP were bought from AppliChem GmbH (Darmstadt, Germany). Sodium acetate, 99 %, anhydrous, p. A. was used from Grüssing GmbH (Filsum, Germany). Trisodium citrate, anhydrous, 99 % was purchased from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). Citric acid monohydrate AnalaR Normapur® derived from VWR BDH Prolabo® (Leuven, Belgium). Polysorbate 80 was from Fluka® Analytical (Buchs, Switzerland). Glycine ReagentPlus® ≥99 (TLC) was bought from Sigma Life Sciences (Taufkirchen, Germany) and mannitol was obtained from Boehringer Ingelheim (Ingelheim, Germany).

2.2.1.2 Proteins

Proteins used in this study are listed in Table 1. All samples were received as expired material, except Beriglobin®, from manufacturers, pharmacies and hospital pharmacies for non-medical, non-commercial use. All products were stored at 2 °C to 8 °C.

Table 1. List of proteins investigated in this study

Information about brand name, manufacturer/supplier, protein international nonproprietary name, protein concentration, product formulation, primary packaging and protein class is provided.

Product Name	Protein Name	Concentration	Formulation	Primary packaging Liquid or Lyophilisate	Protein class
Erypo® 4000 (Janssen-Cilag GmbH)	Epoetin alfa	4000 I.U.	Polysorbate 80, Gly, NaCl, Na_3PO_4, $Na_4P_2O_7$	Glass vial Liquid	cytokine
Erypo® 4000 (Janssen-Cilag GmbH)	Epoetin alfa	4000 I.U.	Polysorbate 80, Gly, NaCl, Na_3PO_4, $Na_4P_2O_7$	Prefilled syringe Liquid	cytokine
Intron A® Injection 1 Mio (Essex Pharma GmbH)	Interferon alfa-2b	1 Mio I.U./mL	Gly, NaH_2PO_4, Na_2HPO_4, HSA	Glass vial Lyophilisate	cytokine
Kineret® 100 mg (Swedish Orphan Biovitrum AB)	Anakinra	100 mg/0.67 mL	Na-citrate, NaCl, NaEDTA, Polysorbate 80, NaOH	Prefilled syringe Liquid	cytokine
NeoRecormon® 2000 IE (Roche)	Epoetin beta	2000 I.U.	Urea, NaCl, Polysorbate 20, NaH_2PO_4, Na_2HPO_4, CaCl, Gly, Leu, Isoleu, Thr, Glu, Phe	Glass vials Lyophilisate	cytokine
NeoRecormon® 2000 IE (Roche)	Epoetin beta	2000 I.U.	Urea, NaCl, Polysorbate 20, NaH_2PO_4, Na_2HPO_4, CaCl, Gly, Leu, Isoleu, Thr, Glu, Phe	Prefilled syringe with 27G 1/2 Liquid	cytokine
NeoRecormon® 10000 IE (Roche)	Epoetin beta	10000 I.U.	Urea, NaCl, Polysorbate 20, NaH_2PO_4, Na_2HPO_4, CaCl, Gly, Leu, Isoleu, Gly, Thr, Glu, Phe	Prefilled syringe with 27G 1/2 Liquid	cytokine
	Erythropoietin	84 µg/mL	NaCl, Polysorbate 80,	Glass container	cytokine

			NaH_2PO_4 x 2 H_2O, Na_2HPO_4 x 2 H_2O, glycine	Liquid	
Rebif® 22 µg Injection solution (Serono)	Interferon beta-1a	22 µg/0.5 mL	Mannitol, HSA, NaOAc, HOAc, NaOH	Prefilled syringe Liquid	cytokine
Roferon®-A (Roche)	Interferon alfa-2a	3 Mio. I.U./0.5 mL	NH_4OAc, NaCl, Benzyl alcohol, Polysorbate 80, HOAc, NaOH	Prefilled syringe Liquid	cytokine
Roferon®-A (Roche)	Interferon alfa-2a	9 Mio. I.U./0.5 mL	NH_4OAc, NaCl, Benzyl alcohol, Polysorbate 80, HOAc, NaOH	Prefilled syringe Liquid	cytokine
Cerezyme® 200 Units (40 U/mL) (Genzyme B.V.)	Imiglucerase	2.5 Units/kg 3.5 Units/kg	Mannitol, Na-citrate, Polysorbate 80, Citric acid or NaOH	Glass vial Lyophilisate	enzyme
Hylase® „Dessau“ 300 I.E. (Pharma Dessau GmbH)	Hyaluronidase	300 I.U.	Gelatine	Glass ampoule Lyophilisate	enzyme
Urokinase 100 000 HS (medac)	Urokinase	2 000 I.E./mL	Na_2HPO_4, NaH_2PO_4, HSA	Glass vial Lyophilisate	enzyme
Urokinase HS medac 500 000 I.E. (medac)	Urokinase	50 000 I.E./mL	Na_2HPO_4, NaH_2PO_4, HSA	Glass vial Lyophilisate	enzyme
	Reteplase	1 mg/mL	Arginine	Glass container (Thawed) Liquid	enzyme
Minirin® parenteral (Ferring Arzneimittel GmbH)	Desmo-pressinacetat	4 µg/1 mL	NaCl, HCl	Glass ampoule Liquid	hormone
	Human Growth Hormone	0.8 mg/mL	Gly, Mannitol, NaH_2PO_4, Na_2HPO_4	Glass container Liquid	hormone
	anti-TNF-α IgG 1	1 mg/mL 40 mg/mL	NaCl, Polysorbate 80, NaH_2PO_4 x 2 H_2O, Na_2HPO_4 x 2 H_2O, Mannitol, Citric acid x H_2O, Sodium citrate	Glass container Prefilled syringe Liquid	antibody
Beriglobin® (CSL Behring GmbH)	Human antibodies against Hepatitis-	160 mg/mL	Gly, NaCl, NaOH or HCl	Prefilled syringe Liquid	antibody

	A-Virus				
Mabthera® 100 mg (Roche)	Rituximab	1 mg/mL 1.5 mg/mL 4 mg/mL	Na-citrate, Polysorbate 80, NaCl, NaOH, HCl	Glass vial Liquid	antibody
Orthoclone® OKT 3 (Janssen-Cilag GmbH)	Muromonab CD3	1 mg/mL	NaH_2PO_4, Na_2HPO_4, NaCl, Polysorbate 80	Glass ampoule Liquid	antibody
Zenapax® 5 mg/mL (Roche)	Daclizumab	0.5 mg/mL	NaCl, Na_2HPO_4, NaH_2PO_4, HCl, NaOH	Glass vial Liquid	antibody
Bioclate™ Antihemophilic Factor (recombinant) 250 (Centeon Pharma GmbH)	Blood coagulation factor VIII	0.08 mg/10 mL	HSA, NaCl, His, Macrogol 3350, $CaCl_2$ x 2 H_2O	Glass vial Lyophilisate with 16G filter needle	antihemophilic drug
Helixate® 250 (Centeon Pharma GmbH)	Octocog alfa	250 I.U.	Gly, NaCl, CaCl, HSA	Glass vial with filter needle Lyophilisate	antihemophilic drug
Albumin (Human) U.S.P. Albutein® 25 % solution (alpha® Therapeutic Corporation)	Human Serum Albumin	12.5 g/50 mL	Na-caprylate, Na-acetyltryptophanate	Glass vial Liquid	plasma protein

Disclaimer:

The particle numbers reported here shall by no means represent the typical particle status of the respective product as they were all expired, except one product and they were analyzed at different points in time after expiration. Using expired materials was intended to challenge the filters with worst case particle numbers which result from normal storage, from not artificial and untypical stress.

2.2.1.3 Filter

Names and supplementing information about the filters applied in this study can be found in Table 2.

Table 2. List of filters used in the study

Brand name, manufacturer and information about the filter membrane, housing, diameter and pore size as well as order number and additional properties of the filters are listed.

Filter Name in Publication	Brand Name	Manufacturer	Filter Membrane / Housing Material	Filter Diameter / Filter Pore Size / Filtration Area	Additional Information / CAT Number
Filter diameter 13 mm - polyethersulfone (PES) & polyvinylidene difluoride (PVDF)					
Pall Acrodisc	Acrodisc® 13 mm Syringe Filter	Pall Corporation (Ann Arbor, MI, USA)	Supor® Membrane (PES) with PP housing	13 mm 0.2 µm 1 cm^2	sterile non-pyrogenic CAT 4602
Millipore PES 13	Millex®-GP	Merck Millipore Ltd. (Tullagreen, Irland)	Fast Flow & Low Protein Binding Millipore Express® PES with PP housing	13 mm 0.2 µm 0.8 cm^2	non-sterile CAT SLGPX13NL
Millipore PVDF 13	Millex®-GV	Merck Millipore Ltd. (Tullagreen, Irland)	Low Protein Binding Durapore® (PVDF) Membrane with PP housing	13 mm 0.2 µm 0.8 cm^2	non-sterile CAT SLGVX13NL
Whatman PVDF 13	Whatman™ Puradisc™ 13 mm Syringe Filter	GE Healthcare UK Limited (Buckinghamshire, UK)	PVDF with PP housing	13 mm 0.2 µm 1.3 cm^2	non-sterile CAT 6779-1302
Filter diameter 25/28 mm - polyethersulfone (PES)					
Pall PharmAssure	PALL PharmAssure 25 mm Syringe Filter with 0.2 µm Supor® Membrane	Pall Medical (Cornwall, UK)	Supor® Membrane (PES) with modified acryl housing	25 mm 0.2 µm 2.8 cm^2	sterile non-pyrogenic CAT HP1002

Pall AEF	Supor® AEF Infusion Filter	Pall Medical (Fribourg, Switzerland)	Supor® Membrane (PES)	not declared 0.2 µm 4.5 cm^2	sterile non-pyrogenic CAT AEF1NTE
Millipore PES	Millex®-MP	Merck Millipore Ltd. (Tullagreen, Irland)	Fast Flow & Low Protein Binding Milipore Express® PES	25 mm 0.22 µm 3.9 cm^2	sterile non-pyrogenic CAT SLMP025SS
Whatman PES	Whatman™ Puradisc™ 25 AS	GE Healthcare UK Limited (Buckinghamshire, UK)	PES with PP housing	25 mm 0.2 µm 4.2 cm^2	sterile non-pyrogenic CAT 6780-2502
Sartorius PES	Minisart® High Flow Syringe Filter	Sartorius AG (Goettingen, Germany)	Hydrophilic PES with methacrylate-butadiene-styrene housing	28 mm 0.2 µm 6.2 cm^2	sterile CAT 16532K
I.V.Star®	Codan I.V.Star® 1.6	CODAN Medizinische Geräte GmbH & Co KG (Lensahn, Germany)	PES with methyl methacrylate-butadiene-styrene housing	20 mm 0.2 µm 1.6 cm^2	sterile non-pyrogenic CAT 76.3365
Filter diameter 25 mm - polyvinylidene difluoride (PVDF)					
Millipore PVDF	Vented Millex®-GV	Merck Millipore Ltd. (Tullagreen, Irland)	Low Protein Binding Durapore® (PVDF) Membrane	25 mm 0.22 µm 4.0 cm^2	sterile non-pyrogenic CAT SLGVV255FF
Whatman PVDF	Whatman™ Puradisc™ 25 mm	GE Healthcare UK Limited (Buckinghamshire, UK)	PVDF with PP housing	25 mm 0.2 µm 4.2 cm^2	non-sterile CAT 6746-2502

PP = polypropylene

2.2.2 Methods

2.2.2.1 Filtration effect

Original products and stressed protein solutions were analyzed for particle count and size with particle measurement systems like light obscuration, Micro-Flow Imaging, dynamic light scattering and nanoparticle tracking analysis. To evaluate the capability of filters to remove protein aggregates, protein solutions were analyzed before and after filtration. Solutions were aspirated in sterile NORM-JECT® Luer Lock syringes (Henke Sass Wolf, Tuttlingen, Germany) and either no filter or a 0.2 µm filter was attached before analysis. NORM-JECT® Luer Lock syringes were flushed with highly purified water before aspiration of the protein solutions. Samples were pooled in case of small volume products. Filters used for a specific protein are listed in the measurement data tables (Tables S4 to S6 and Table 7) or figures (Figure 1 to 5 and Figure S2). Additional information about a specific filter is shown in Table 2. Measurements were carried out at least in triplicates and presented as mean ± standard deviation.

2.2.2.1.1 Stressed protein drug products

Proteins (anti-TNF-α IgG 1, erythropoietin, human growth hormone, Albumin (Human) U.S.P. Albutein® 25 % solution) were exposed to stirring with a stir bar at 300 rpm for 3 h on a Heidolph MR 3001 K (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at room temperature.

Harsh stress conditions were applied to Cerezyme® and Zenapax®: After dilution with an isotonic sodium chloride solution to their final concentration, 8.5 mL diluted protein solution were filled into 15 mL Greiner tubes (Greiner Bio-One GmbH, Frickenhausen, Germany), fixated horizontally on an Eppendorf Mixer 5432 (Eppendorf AG, Hamburg, Germany) and run at 1,450 rpm for 3 h at room temperature. Mabthera® 100 mg, Orthoclone® OKT 3, Erypo® 4000 and NeoRecormon® 2000 IE were stressed by exposing them in their original packaging to end-over-end rotation stress at 45 rpm for 3.5 h at room temperature.

Measurements were performed at least in triplicates and presented as mean ± standard deviation.

2.2.2.1.2 Particle analysis

2.2.2.1.2.1 Nephelometry

The turbidity of the samples was assessed with a Nephla turbidimeter (Dr. Lange, Düsseldorf, Germany). Scattered light from a laser with a wavelength of 860 nm is detected at an angle of 90°. Results are presented in triplicates and in the form of formazine nephelometric units (FNU).

2.2.2.1.2.2 Light Obscuration (LO)

Particles in the sub- and visible particle range were quantified with a SVSS-C device from PAMAS GmbH (Rutesheim, Germany). For the analysis the PAMAS PMA Program V 2.1.2.0 software was used. To ensure the system cleanliness the particle count of highly purified water was determined before each sample. The system was flushed with 0.3 mL or 0.4 mL sample before sample triplicates with a volume of either 0.2 mL or 0.3 mL were analyzed. Three of these runs were carried out for all samples resulting in a total of nine single measurements. The solution was aspirated at a speed of 10 mL/min. The system was calibrated with the usage of Duke Standards™ covering the particle range from 1 µm to 200 µm and Count-Cal™ (5 µm) Particle Size Standards (both Thermo Scientific, Fremont, CA, USA).

2.2.2.1.2.3 Micro-Flow Imaging (MFI)

For the particle count and characterization a Micro-Flow Imaging DPA 4100 device (BrightWELL Technologies Inc., Ottawa, Canada) with a 100 µm flow cell in the operation mode “high magnification” was used. For the analysis the MFI™ Particle Analyzer V6.9.7.2 software was used. Flow cell cleaning was performed with highly purified water and flow cell cleanliness was evaluated between each run. Prior each sample run the flow cell was flushed with 0.5 mL of the appropriate protein solution buffer or with highly purified water to carry out “optimize illumination”. This step guarantees a correct system threshold. After “optimize illumination” the flow cell was flushed with at least 0.25 mL sample, before 0.65 mL sample were analyzed for particle count and characterization. Lower analysis volumes were used for some small volume products. The flow rate of the sample run was set to 0.1 mL/min. Triplicate measurements were carried out. Duke Standards™ (10 µm) and Count-Cal™ (5 µm) Particle Size Standards (both Thermo Scientific, Fremont, CA, USA) were used for the calibration of the MFI system.

2.2.2.1.2.4 Nanoparticle Tracking Analysis (NTA)

To quantify and size nanoparticles, the movement of nanoparticles was recorded for 60 s with a NanoSight LM20 (NanoSight, Amesbury, UK) using the software NTA 2.3. The camera shutter was set to 1,499 for both proteins. The gain was set to 113 for NeoRecormon 2000 IE and 138 for anti-TNF-α IgG 1 40 mg/mL. Highly purified water was used to clean the flow cell. 0.5 mL sample were loaded air bubble free into the cell. Triplicate measurements were carried out. Between each measurement the cell content was replaced by another 0.1 mL sample. A 1 mL Terumo® Syringe without needle was used for cell loading (Terumo (Philippines) Corporation, Laguna, Philippines). No flow was applied during the 60 s of video capture.

2.2.2.1.2.5 Dynamic Light Scattering (DLS)

Dynamic light scattering was performed on a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) to determine the particle size and size distribution in the nanometer range. 400 µL protein solution were analyzed in triplicates at 20 °C. Particle size is given with the Z-Average [Z-Ave] value and the polydispersity index (PdI) represents the particle size distribution.

2.2.2.2 Particle shedding from filters

Filter cleanliness was tested by flushing the filters with ultra pure buffers. These buffers were first filtered (0.2 µm), then centrifuged with at least 4,000 rpm for 20 min. Only the supernatants of these purified solutions were used. Filters of interest were flushed with 2 mL of these buffers before the analysis with light obscuration and Micro-Flow Imaging. The investigated filters were Pall Acrodisc, Millipore PES and PVDF 13, Whatman PVDF 13 (Table 2). The prepared buffers for flushing were a 50 mM acetate buffer pH 3.8 with an ionic strength of 154 mM and 200 mM, a 50 mM phosphate buffer pH 6.8 with an ionic strength of 154 mM and a 50 mM phosphate buffer pH 8 with an ionic strength of 154 mM and 200 mM. Either no or 0.1 % polysorbate 80 was added to the buffers. Highly purified water was used for preparation and rinsing of container systems. Measurements were carried out at least in triplicates and presented as mean ± standard deviation.

2.2.2.3 Protein Adsorption

For the determination of protein loss caused by adsorption to the filter high-performance size exclusion chromatography (HP-SEC) was performed.

The mobile phase consisted of 300 mM sodium chloride and 50 mM phosphate pH 7 in highly purified water. A flow rate of 0.5 mL/min was applied and a TSKgel 3000SWXL

column was used (Tosoh Bioscience GmbH, Stuttgart, Germany). Before analysis samples were centrifuged at 14,000 rpm for 10 min. Depending on the protein concentration various amounts of the protein solution were injected. Each sample was run in triplicates. A Waters 2695 Alliance Separation Module (Waters Corporation, Milford, MA, USA) with a Waters 2487 Dual λ Absorbance Detector for UV absorbance at 280 nm or a Dionex system with an ASI 100 autosampler, a RF 2000 Fluorescence (extinction: 295 nm; emission: 343 nm) and a UVD170U detector (280 nm) (all Thermo Fisher Scientific GmbH, Idstein, Germany) were used. Data analysis was carried out with Chromeleon V6.8. The area under the curve of non-filtered samples was compared to the area under the curve of filtered samples.

2.2.2.4 Hold-up volume

The hold-up volume of Pall Acrodisc filters (n = 9) and seven filters with a diameter of 25 mm or 28 mm (n = 5) (see Table 2), all with a pore size of 0.2 µm, was assessed by filtration of 1 mL or 2 mL highly purified water, respectively. The volume after filtration, the hold-up volume and the hold-up volume after 1 mL air purge were measured.

2.2.2.5 Protein denaturation

To evaluate possible changes in the protein structure caused by filtration four proteins were analyzed before and after filtration with a 0.2 µm and 13 mm or 25 mm filter with far-UV circular dichroism and Fourier transform infrared spectroscopy.

2.2.2.5.1 Fourier Transform Infrared Spectroscopy (FT-IR)

A Bruker Tensor 27 FT-IR device with a Bruker AquaSpec Cell (Brucker Optics, Ettlingen, Germany) was utilized for spectra recording of 40 mg/mL anti-TNF-α IgG 1 solutions. The sensor was constantly cooled before and during measurements with liquid nitrogen and a gaseous nitrogen flow. The spectra were background corrected with the corresponding buffer. The OPUS analysis software was used to obtain the second derivative of the spectra from a wavelength of 1,750 cm^{-1} to 1,450 cm^{-1} and to apply vector normalization.

2.2.2.5.2 Far-UV circular dichroism (CD)

A Jasco J-715 spectropolarimeter (JASCO International Co. Ltd., Tokyo, Japan) was used for capturing far-UV spectra of Erythropoietin, human growth hormone, anti-TNF-α IgG 1 and Beriglobin®. The measurement software J-700 series control driver Vers 1.08.00 and for the analysis the software SpectraManager for Windows 95/NT Spectra Analysis Vers 1.53.07 (Build 1) (both JASCO International Co. Ltd., Tokyo, Japan) were used. Protein solutions with a concentration of 0.150 mg/mL and for Beriglobin® 0.075 mg/mL were filled into a quartz cuvette with a path length of 0.1 cm. Spectra from 250 nm to 195 nm were recorded by a continuous scanning method at 4°C. Four scans were accumulated. Background correction of the corresponding buffer was performed for each protein spectrum. The data is shown in millidegrees of ellipticity as a function of wavelength.

2.2.2.6 Leachable study

For the determination of leachables seven different filters from various suppliers with a diameter of 25 mm or 28 mm and a pore size of 0.2 µm were investigated with reversed-phase high-performance liquid chromatography, inductively coupled plasma optical emission spectrometry and gas chromatography-mass spectrometry. The filters were flushed either with 1 mL highly purified water or with an isotonic sodium chloride solution pH 4 containing 0.1 % polysorbate 80. For the analysis the filtrate of five filters was pooled.

2.2.2.6.1 Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

To detect UV active substances a YMC-Pack C4 (250 x 4.6 mmI.D. S-5µm, 30 nm) column (YMC, Kyoto, Japan) was attached to an UltiMate 3000 system including a UV detector (Thermo Fisher Scientific GmbH, Idstein, Germany) as well as a column oven which was set to 37°C. The UV wavelengths were set to 214, 245, 280 and 330 nm and the extinction and emission wavelengths were at 280 nm and 343 nm, respectively. A linear gradient from 0 % to 100 % within 55 min followed by 5 min 0 % mobile phase B was applied at a flow rate of 0.5 mL/min. Mobile Phase A consisted of 90 % highly purified water, 10 % HPLC grade acetonitrile and 0.1 % trifluoroacetic acid (w/w), whereas mobile phase B was made of 100 % HPLC grade acetonitrile and 0.1 % trifluoroacetic acid (w/w).

2.2.2.6.2 Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

For trace analysis of several ions ICP-OES was applied. The following elements were monitored: Li, B, Na, Mg, Al, Si, P, K, Ca, Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ag, Cd, Sn, Ba, Hg, Pb. For this purpose a Varian Vista RL CCD simultaneous ICP AES Vista RL radial device from Varian (nowadays Agilent Technologies, Santa Clara, CA, USA) was used. 0.1 mL 69 % nitric acid was added to the filtrate to obtain an aqueous 3 % nitric acid solution. Samples were then boiled at 105 °C for 30 min. 3 % standard solutions from Merck KGaA (Darmstadt, Germany) were used for system calibration. Blanks were measured before the samples were analyzed. For each element two to three emission wavelengths were chosen. Each sample was analyzed in triplicates. Instrument settings were the following: performance: 1.2 kW; nebulizer pressure: 180 kPa; pump speed: 17 rpm; plasma flow: 13.5 l/min; auxiliary flow: 1.5 l/min; sample uptake time: 30 s; instrument stabilization time: 45 s; measurement time: 9 s. The maximum percentage variance is 5 % and the minimum correlation coefficient is 0.98.

The filtrates were analyzed by Jaroslava Obel from the analytical division of the Department of Chemistry and Pharmacy at the Ludwig-Maximilians-Universität Munich.

2.2.2.6.3 Gas Chromatography-Mass Spectrometer (GC-MS)

To identify and quantify leachables GC-MS was performed. 1 mL sample filtrate was mixed with either 2 mL of THF-methacrylate or BHT-aldehyde solutions and with 2 mL water for injection. The sample was adsorbed on a PDMS stir-bar for 1 h before thermal desorption and cryogenic focusing of the desorbed agents occurred. The substances were then separated on a GC column (Agilent Technologies, Santa Clara, US) and finally detected with a mass spectrometer (Agilent Technologies, Santa Clara, US).

Provided filtrates were further prepared for GC-MS analysis by Nicole Scherer. GC-MS measurements and analysis of the chromatograms was performed by Nicole Scherer. The evaluation of this data was done by Benjamin Werner.

2.2.2.7 Ejection force

The impact of the filter attachment on the ejection force of the product solution from a syringe was determined using a Texture Analyzer XTplus device (Stable micro Systems, Godalming, UK). Pre-test speed was set to 5 mm/sec, test speed was 6 mm/sec (0.18 mL/min) and post-speed was 10 mm/sec. Marketed products (anti-TNF-α IgG 1 40 mg/mL, Roferon® 3 Mio, Roferon® 9 Mio, NeoRecormon® 2000 IE) were analyzed. 27G ½" needles for attachment were supplied by the manufacturer for Roferon® 3 Mio, Roferon® 9 Mio and NeoRecormon® 2000 IE, whereas the anti-TNF-α IgG 1 product was already filled into

prefilled syringes with a staked-in needle. The product was repacked into glass syringes without a staked-in needle (BD Hypak SCF™, BD, Franklin Lakes, New Jersey, USA) to enable filter and 27G ½" needle (BD Microlance™ 3, Becton, Dickinson and Company Limited, Drogheda, Ireland) attachment. Controls represent the force to eject the protein solution from the glass syringe and the 27G ½" needle. Filters with a pore size of 0.2 µm and a diameter of 13 mm were attached on the luer cone of the syringe. 27G ½" needles were attached to the filter outlet and are later referred to as 27G needles.

For the simulation of injection into subcutaneous tissue, injections were carried out into pig skin. The skin was at least 3 cm thick and the needles were inserted 1 cm into the skin. These experiments were performed with a 120 mg/mL monoclonal antibody solution filled in glass syringes. Either a 27G ½" needle or a filter and a 27G ½" needle were attached before ejection at a speed of 3 mm/s.

2.3. Results

2.3.1 Filter testing by filter manufacturers

Information about the internal filter quality testing was obtained from the four filter manufacturers GE Healthcare, Merck Millipore, Pall GmbH and Sartorius AG. The collected information shows that quality criteria for filters are diverse and not every company was able to provide information on properties like particulates, extractables or product lot release criteria. Regarding biocompatibility all companies run tests according to ISO 10993, two run also 93/42/EEC and USP Class VI Biological tests for plastics. Title 21 Code of Federal Regulations, Section 210.3(b)(6) and 211.72 for non-fiber-releasing filters is carried out by one company. Production and packaging occur under clean room conditions according to several ISO forms or to ISO 9001. Two of the companies have internal limits regarding particulates. In one case the test method according to Annex A.2 of ISO 8536-11 is applied for infusion filters but not for other medical filters. The other company allows less than 50 particles larger than 10 µm/unit. Visual inspections for product lot release are done by two companies. The units should be free of visible fibers and particles under normal plant lighting, respectively, should be free of injection molded particles and loose particles or fibers of sizes larger than 100 µm. One company determines extractables by UV and weighing, although it is not stated which extractables could be expected. Further, the filters are tested for the release of ammonium and heavy metal ions. Another company weighs the amount of non-volatile residues and analyzes them with FT-IR in adaption to Title 21 Code of Federal Regulations parts 176 and 177. However, this test is done for filter cartridges and not for infusion or syringe filters.

2.3.2 Filtration effectiveness

The main aim of bedside filtration is to protect of the patients from any larger protein aggregates and protein particles. Hence, the filters should have the capability to diminish larger protein aggregates effectively. The suitability of several filters was tested by filtration of more than 19 different, highly relevant products. Protein solutions were measured in non-stressed (Tables S4 and S6) and stressed (Table S5) conditions before and after filtration. MFI measurements showed for all cases the capacity of filters to diminish small micrometer particles. The particle counts of four differently stressed products are exemplarily presented in Figure 1. For harshly shaken, reconstituted Cerezyme® 200 Units (3.5 Units/kg) a reduction of the particle count from around 73,000 particles/mL to 2,686 particles/mL for one filter and to values even lower than 800 particles/mL for the other filters was achieved (Figure 1A). This corresponds to reduction factors of up to 894. For Mabthera® (1 mg/mL), which was stressed by end-over-end rotation in the original vial before dilution, the particle count was diminished from 4,535 particles/mL to less than 200 particles/mL, for four out of five filters (Figure 1B). End-over-end rotation stressed NeoRecormon® 2000 IE in prefilled syringes (Figure 1C) and Orthoclone® OKT 3 (1 mg/mL) in 5 mL ampoules had particle reduction factors of above 1,000. For Orthoclone® OKT 3 e.g. the particle burden decreased from 675,394 particles/mL to less than 100 particles/mL (Figure 1D). Note that numbers for the particle sizes 10 µm and 25 µm, which are mandatorily monitored by the pharmacopoeia [30], were either reduced to a minimum of a few particles/mL or were even entirely eliminated. Further numbers of the effective reduction of these two particle sizes with filtration can be found in Figure S2.

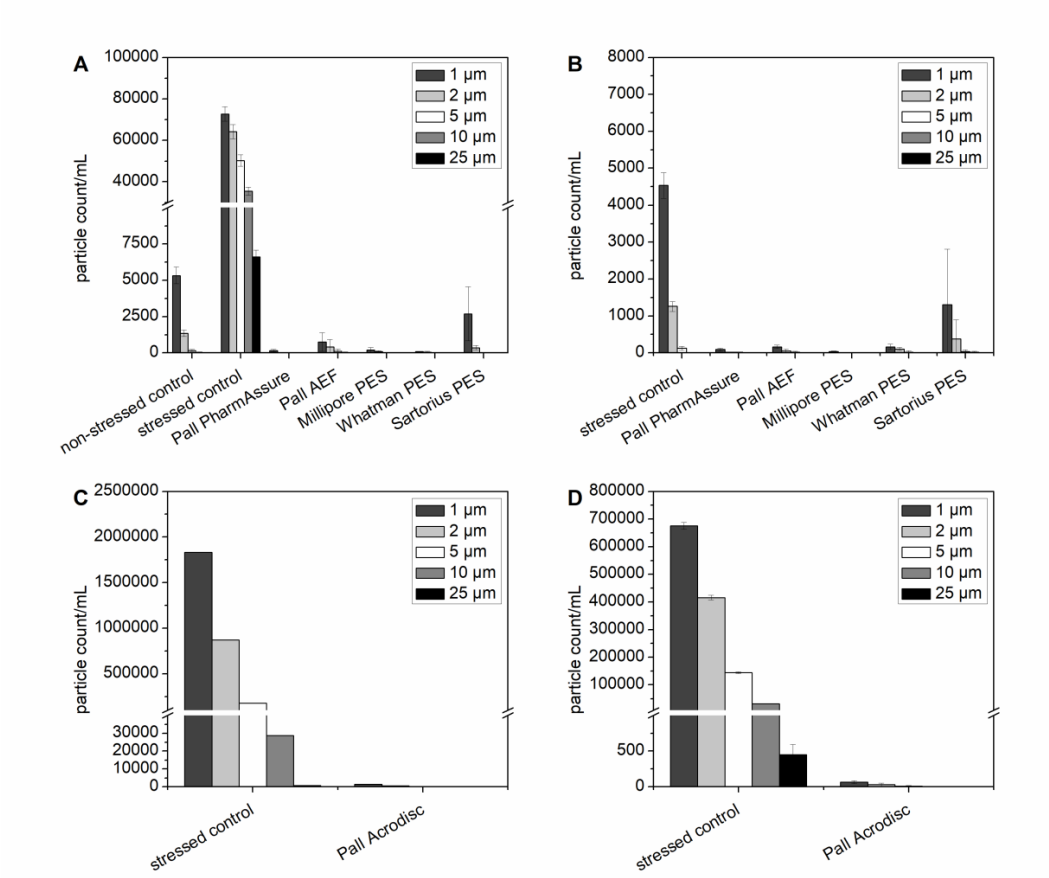

Figure 1. Particle count, as determined with MFI, is shown for shaken Cerezyme® 200 Units (3.5 Units/kg) (**A**) and end-over-end rotation stressed Mabthera® (1 mg/mL) (**B**), NeoRecormon® 2000 IE (**C**) and Orthoclone® OKT 3 (1 mg/mL) (**D**). Non-filtered controls and filtered samples are displayed with the filters applied. The cumulative particle count/mL is shown for particles sizes of 1 µm, 2 µm, 5 µm, 10 µm and 25 µm.

Table S4 and S5 list additional examples for non-stressed and stressed protein drug products displaying the high capability of filters to eliminate larger protein aggregates. Table S4 and S5 include DLS measurement data. In the majority of all cases the hydrodynamic radius of the proteins as well as the PdI remains virtually unaffected by filtration. Yet, in several cases like for Hylase 300®, NeoRecormon® 10000 IE or for stressed anti-TNF-α IgG 1 40 mg/mL, human growth hormone 0.8 mg/mL (Table S4 and S5) or Zenapax® (Figure 3D) a reduction in the hydrodynamic radius after filtration could be observed. This demonstrated that reversible protein aggregates could be destroyed by filtration. In particular in the case of stressed proteins it was shown that the native hydrodynamic radius could be obtained after filtration.

Further, two standard pharmacopoeia methods, LO and nephelometry were additionally carried out for several proteins (Table S6). LO measurements revealed a clear reduction of

the particle burden of the tested protein solutions and consequently confirmed the so far presented MFI results. Nephelometry data showed lower or similar values after filtration indicating the reduction of nanometer sized particles. In Figure 3 the data of a stressed Zenapax solution is exemplarily displayed graphically in order to confirm the observed results.

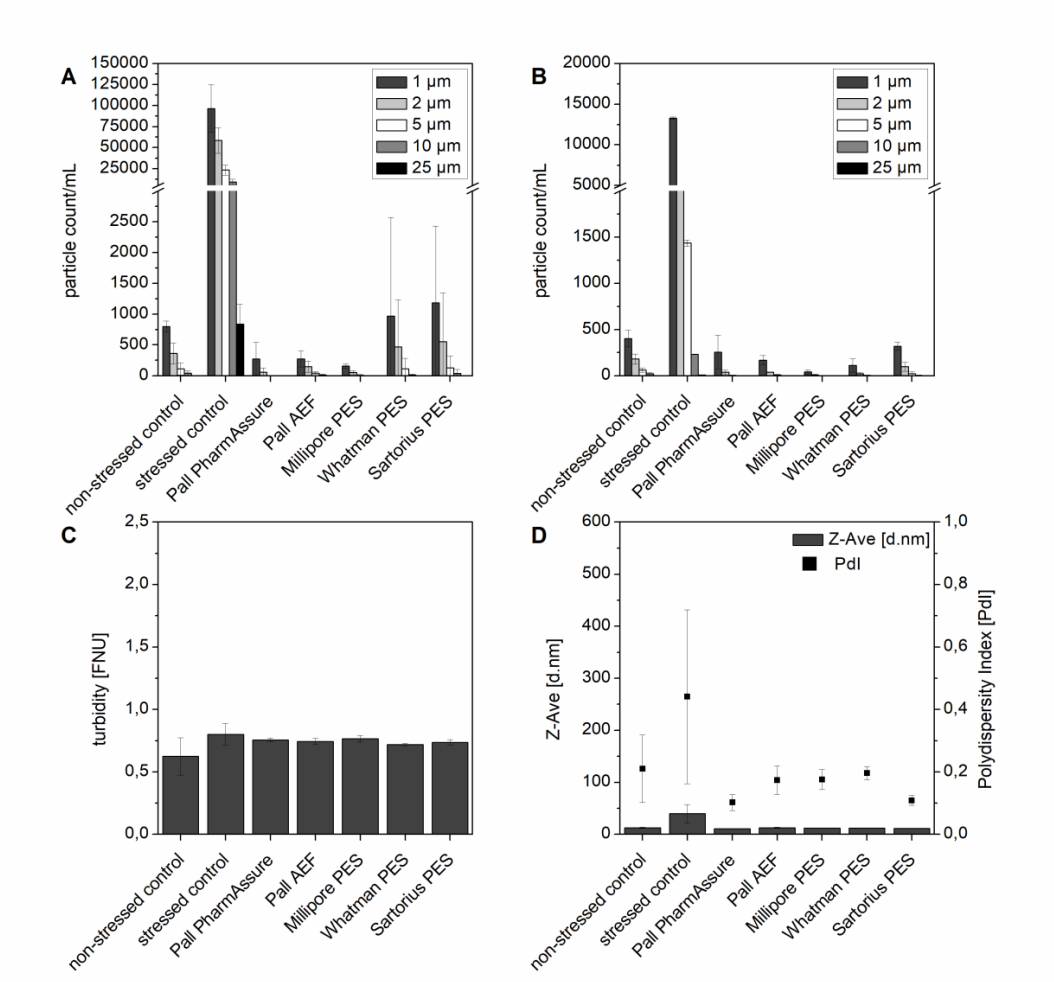

Figure 3. Stressed Zenapax solution was analyzed with MFI (**A**), LO (**B**), nephelometry (**C**) and DLS (**D**). The cumulative particle count/mL is shown for particle sizes of 1 µm, 2 µm, 5 µm, 10 µm and 25 µm. Non-filtered controls and filtered samples are presented.

In order to test the effectiveness of 0.2 µm filters in the nanometer range NTA analysis was performed (Figure 4). The particle count of anti-TNF-α IgG 1 40 mg/mL was around 200 million particles/mL of around 200 nm before filtration and it was reduced by at least 25 % after filtration (Figure 4A). A more than 80 % particle burden reduction was achieved for NeoRecormon® 2000 IE after filtration. Also the particle mean size which was around 324 nm was reduced to around 190 nm after filtration, corresponding well to the filter pore size of 200 nm (Figure 4B). The data illustrated that filtration was also effective in the nanometer range.

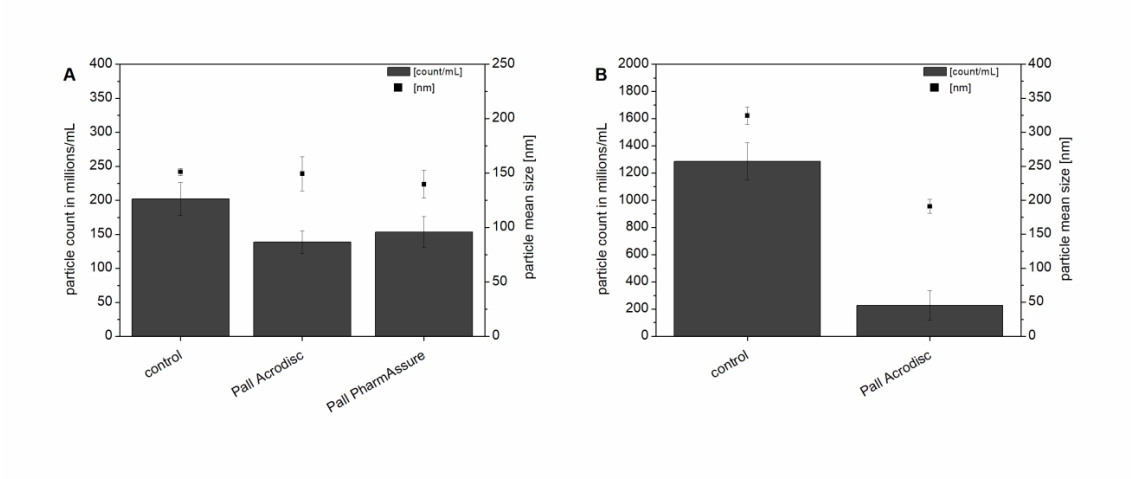

Figure 4. The total particle count in millions/mL and the particle mean size for anti-TNF-α IgG 1 40 mg/mL (**A**) and NeoRecormon® 2000 IE (**B**) solutions are presented before and after filtration, as analyzed by NTA.

Overall, the particle burden of non-stressed and stressed protein solutions can be effectively reduced in the nanometer and particularly in the micrometer range as demonstrated for more than 20 products. All filters were able to diminish the amount of larger protein aggregates in all cases. Regarding their capability to eliminate protein particles most of the investigated filters behaved similar except one filter.

2.3.3 Particle shedding from filters

The particle shedding from filters was determined by filtering purified buffers. In order to identify possible factors leading to shedding from filters, buffers with different pH values and ionic strengths were applied. The presence or absence of surfactants was additionally tested. Apart from few exceptions it was observed that the filtration process led to lower particle counts (Table 3). For some samples the particle burden remained relatively unaffected by filtration which did not surprise, because already very low particle counts were measured in the buffer controls. However, filter shedding can occur in principle. In particular the Whatman PVDF 13 filter led to a higher particle burden in several test solutions after filtration. An explanation for this could be its packaging. The Whatman PVDF 13 filter was delivered in a plain paper box as a non-sterile bulk product, whereas both Millipore filters were sold as non-sterile bulks within a plastic bag and a box as secondary packaging. In contrast, the Pall Acrodisc filter could be obtained as a single, sterile packaged filter. Single, sterile packaging was not available for all filters with a similar filtration area and with the same diameter. An impact of the pH value, the presence of a surfactant, the ionic strength (data not shown) or filter preflushing (data not shown) on the particle load of the solution could not be detected. For a few cases it was shown that the particle burden of the filters varied strongly within the same filter batch as indicated by standard deviations. Overall, particle shedding from filters can be neglected when adequate packaging is applied. As single, sterile packed filters are the target, particle shedding is considered as a controllable, uncritical aspect.

Table 3. Shedding behavior of two PES and two PVDF filters with a pore size of 0.2 µm and a diameter of 13 mm was analyzed using MFI and LO

For each method the total particle count/mL (particles ≥ 1 µm) is stated.

Buffer	Filter Name	MFI ≥ 1 µm/mL	LO ≥ 1 µm/mL
Acetate buffer pH 3.8 ionic strength 154 mM without 0.1 % polysorbate 80	Control	192 ± 201	34 ± 43
	Pall Acrodisc	22 ± 0	3 ± 1
	Millipore PES 13	59 ± 84	16 ± 14
	Millipore PVDF 13	67 ± 22	10 ± 12
	Whatman PVDF 13	229 ± 141	271 ± 46
Acetate buffer pH 3.8 ionic strength 154 mM with 0.1 % polysorbate 80	Control	22 ± 22	24 ± 19
	Pall Acrodisc	81 ± 78	18 ± 6
	Millipore PES 13	44 ± 38	16 ± 6
	Millipore PVDF 13	22 ± 0	8 ± 2
	Whatman PVDF 13	392 ± 201	390 ± 184
Phosphate buffer pH 6.2 ionic strength 154 mM without 0.1 % polysorbate 80	Control	185 ± 194	52 ± 64
	Pall Acrodisc	118 ± 34	17 ± 15
	Millipore PES 13	22 ± 0	23 ± 20
	Millipore PVDF 13	44 ± 22	4 ± 3
	Whatman PVDF 13	44 ± 38	40 ± 24
Phosphate buffer pH 6.2 ionic strength 154 mM with 0.1 % polysorbate 80	Control	192 ± 257	144 ± 221
	Pall Acrodisc	89 ± 38	24 ± 8
	Millipore PES 13	96 ± 90	6 ± 6
	Millipore PVDF 13	89 ± 117	7 ± 11
	Whatman PVDF 13	1,428 ± 955	527 ± 35
Phosphate buffer pH 8.0 ionic strength 154 mM without 0.1 % polysorbate 80	Control	22 ± 0	182 ± 25
	Pall Acrodisc	37 ± 46	20 ± 5
	Millipore PES 13	104 ± 46	15 ± 9
	Millipore PVDF 13	111 ± 97	40 ± 42
	Whatman PVDF 13	118 ± 110	159 ± 37
Phosphate buffer pH 8.0 ionic strength 154 mM with 0.1 % polysorbate 80	Control	81 ± 34	95 ± 60
	Pall Acrodisc	74 ± 71	17 ± 4
	Millipore PES 13	44 ± 22	26 ± 22
	Millipore PVDF 13	111 ± 80	9 ± 3
	Whatman PVDF 13	429 ± 278	236 ± 98

2.3.4 Protein adsorption

Protein drug products have a broad concentration range from a few µg/mL up to concentrations beyond 100 mg/mL. In particular for highly potent drugs in the lower concentration range like cytokines or hormones it is of substantial importance not to lose any protein because of adsorption to the filter membrane or housing. The filters which were used were all defined as low protein binding filters by the manufacturers. The adsorption behavior of several protein drug products and protein solutions, formulated according to products on the market, covering a broad concentration range and different protein classes were filtered with various filters (Table 7). Protein loss due to adsorption could be neglected for the 13 mm Pall Acrodisc filter with a filtration area of 1 cm^2. In the majority of all investigated samples protein adsorption was also not noted for filters with a diameter of 25 mm or 28 mm (Table 7). The best results were achieved with the Pall PharmAssure filter. However, in some cases slight protein losses of around 4 µg for NeoRecormon® 10000 IE and Cerezyme® 200 Units as well as up to 25 µg/mL for human growth hormone were observed.

Overall, protein recovery after filtration was very high in the majority of all cases. Protein adsorption on the filter membrane will be negligible e.g. for high dosed antibodies, but in particular for low concentrated products protein adsorption should be checked as it can occur.

Table 7. Protein adsorption was as determined by HP-SEC

The area under the curve [%] is shown for different proteins before and after filtration with various filters. n.a. = not analyzed.

Product	Before Filtration	Pall Acrodisc	Pall PharmAssure	Pall AEF
anti-TNF-α IgG 1 (1 mg/mL)	100 ± 0.38	100.7 ± 0.35	100.67 ± 0.18	100.87 ± 0.19
Mabthera® 100 mg (1.5 mg/mL)	100 ± 1.25	99.6 ± 1.2	99.62 ± 1.55	98.59 ± 0.78
Mabthera® 100 mg (1.5 mg/mL) - stir stress	100 ± 1.61	98.78 ± 1.29	99.34 ± 2.52	98.36 ± 1.58
Beriglobin®	100 ± 0.95	99.71 ± 0.71	99.84 ± 0.92	n.a.
Human Growth Hormone (0.8 mg/mL)	100 ± 0.95	n.a.	99.72 ± 1.03	100.91 ± 1.33
Human Growth Hormone (0.8 mg/mL) - stir stress	100 ± 2.27	n.a.	102.45 ± 3.83	99.42 ± 1.18
Erythropoietin (84 µg/mL)	100 ± 0.99	100.59 ± 0.59	100.78 ± 1.88	n.a.
Erythropoietin (84 µg/m)L - stir stress	100 ± 0.93	99.12 ± 0.83	99.16 ± 0.81	n.a.
Hylase® „Dessau“ 300 I.E.	100 ± 1.78	99.59 ± 3.47	n.a.	n.a.
Urokinase HS medac 500 000 I.E. (50 000 I.E./mL)	100 ± 0.42	99.29 ± 0.34	n.a.	n.a.
Bioclate™ Antihemophilic Factor (recombinant) 250	100 ± 0.39	99.16 ± 0.39 (supplied filter needle)		
Orthoclone® OKT 3 (1 mg/mL)	100 ± 1.52	100.91 ± 0.93	n.a.	n.a.
NeoRecormon® 10000 IE	100 ± 3.03	95.26 ± 3.15	n.a.	n.a.

Product	Before Filtration	Pall PharmAssure	Pall AEF	Millipore PES	Whatman PES	Sartorius PES	Millipore PVDF	Whatman PVDF
Erythropoietin (84 µg/mL) stir stress	100 ± 3.63	101.24 ± 5.18	98.26 ± 3.12	101.13 ± 2.51	99.34 ± 1.5	98.76 ± 3.36	96.13 ± 3.56	96.17 ± 4.70
Mabthera® 100 mg (4 mg/mL)	100 ± 0.64	100.17 ± 0.31	99.94 ± 0.56	100.27 ± 0.28	99.98 ± 0.36	99.77 ± 0.20	100.44 ± 0.46	100.17 ± 0.65
Cerezyme® 200 Units (2.5 U/kg)	100 ± 1.66	100.07 ± 0.54	99.09 ± 1.35	100.21 ± 1.78	97.09 ± 1.44	99.16 ± 0.90	100.82 ± 2.11	91.03 ± 0.93
Urokinase 100 000 HS (2 000 I.E./mL)	100 ± 0.38	98.73 ± 0.51	99.19 ± 0.67	98.76 ± 0.47	99.25 ± 1.28	n.a.	n.a.	n.a.
Human Growth Hormone (0.8 mg/mL)	100 ± 0.30	101.18 ± 3.48	96.88 ± 2.16	97.12 ± 1.13	97.75 ± 1.86	99.67 ± 0.82	98.82 ± 1.94	96.92 ± 1.69

2.3.5 Hold-up volume

Depending on the filter housing design and the filter size different volumes of protein drug solution remain in the filter. These so called hold-up volumes have to be of small volume in order to assure that the loss of protein drug product is minimized in all cases. For products applied in larger volumes of e.g. ≥ 5 mL a small excess to account for a potential loss in a filter could be considered. For small volume products (≤ 2 mL) the losses would not be acceptable.

Here, eight filters, one with a diameter of 13 mm, six with 25 mm and one with 28 mm (see Table 2) were investigated regarding their hold-up volumes. Generally, rather high hold-up volumes were found. For the smallest filter, the Pall Acrodisc filter with a diameter of 13 mm, approximately 16.3 % of the initial volume remained in the filter after the filtration process (Table 8). For filters with 25 mm or 28 mm diameter the hold-up volumes were in the range of 23 % to 60.5 % of the initial volume.

Table 8. Overview of the initial volume, the volume after filtration, the hold-up volume, the hold-up volume after 1 mL air purge and the declared hold-up volume after 1 mL air purge for eight filters

	Acro-disc	**Pharm-Assure**	**AEF**	**M-PES**	**M-PVDF**	**W-PES**	**W-PVDF**	**S-PES**
Initial Volume [µL]	1,000	2,000	2,000	2,000	2,000	2,000	2,000	2,000
Volume after filtration [µL]	837 ± 25	1,320 ± 30	1,220 ± 30	1,490 ± 30	790 ± 10	1,550 ± 30	1,540 ± 50	1,150 ± 20
Hold-up volume (= remaining volume in the filter) [µL]	163 ± 25	680 ± 30	780 ± 30	510 ± 30	1,210 ± 10	460 ± 30	460 ± 50	850 ± 20
Hold-up volume after 1 mL air purge [µL]	76 ± 32	270 ± 20	370 ± 30	220 ± 10	1,070 ± 10	160 ± 50	270 ± 60	320 ± 50
Hold-up volume after 1 mL air purge as declared by the manufacturer [µL]	≤ 28	50	700	< 100	< 100	not stated	not stated	100 - 150

13 mm diameter filter: Acrodisc = Pall Acrodisc

25 mm diameter filters: PharmAssure = Pall PharmAssure; AEF = Pall AEF; M-PES= Millipore PES; M-PVDF = Millipore PVDF; W-PES = Whatman PES; W-PVDF = Whatman PVDF

28 mm diameter filter: S-PES = Sartorius PES

In order to reduce the hold-up volume filter manufacturers instruct a 1 mL air purge which is however not applicable during drug administration. Even though such an air purge reduced the hold-up volumes of all investigated filters, the loss of solution is still too high for small volume products and did not coincide with the hold-up volumes declared by the manufacturers (Table 8).

2.3.6 Protein denaturation

During the filtration process protein solutions are exposed to a small filter pore size and the filter surface which presents a shear and interfacial stress for proteins. This stress might theoretically lead to protein unfolding, although practically all pharmaceutical protein drug products are sterile filtered at least once, typically at least twice, during the course of their manufacturing. CD and FT-IR measurements were carried out to confirm common knowledge that filtration does not impact the protein structure and to make sure that filtration, which was applied here, did not cause atypical alterations in the secondary structure of the protein. CD spectra were recorded for four model proteins before and after filtration. CD spectra of human growth hormone are exemplarily displayed (Figure 5B). FT-IR spectra were additionally taken for anti-TNF-α IgG 1 (Figure 5A). The results showed that filtration did not cause any changes in the secondary structure of the analyzed proteins.

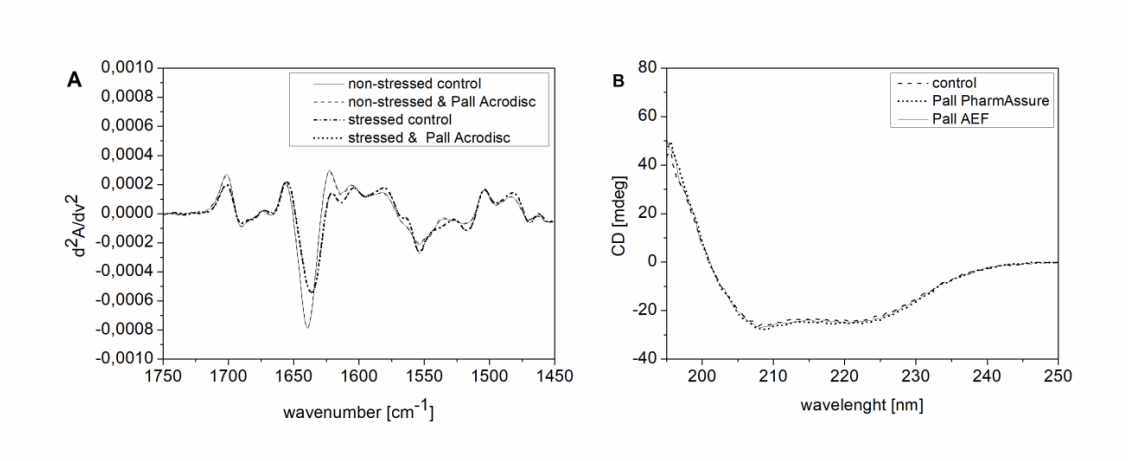

Figure 5. FT-IR spectra of non-stressed and stressed anti-TNF-α IgG 1 40 mg/mL solutions (**A**) as well as the CD spectra of human growth hormone (**B**) are displayed.

2.3.7 Leachables

The existence and quantity of leachables in filtrates was determined by ICP-OES, RP-HPLC and GC-MS after flushing various filters with highly purified water or a 0.9 % sodium chloride solution pH 4 with 0.1 % polysorbate 80.

With ICP-OES more than 20 metal ions were monitored in filtrates, but no toxic heavy metals were detected. Filtrates were analyzed for ultraviolet active leachables using RP-HPLC at four wavelengths. Leachables were only detected in five out of seven filters at 214 nm, 245 nm and 280 nm, but not at 330 nm. While three filters showed each ≤ 5 peaks, two filters had multiple peaks. All these measured impurities were on a low level. Chromatograms of three filters are shown in Figure S6 to display the differences between filters and the low amount of leachables. By analyzing seven filters with GC-MS, more than 60 aromatic and aliphatic leachables were identified in total. As observed with RP-HPLC, differences in the

quantity of leachables were detected. Three filters showed 6 to 12 peaks, one filter 15 peaks and three filters 22 to 28 peaks after filtering the solution which contained the surfactant. A lower number of peaks were visible in the highly purified water filtrates. The concentration of the detected peaks was in general far below 1 ppm, which represents the reporting threshold by the U.S. Food and Drug Administration [31]. The substances neodecanoic acid, dodecanoic acid, 1-dodecanol, 11-methyldodecanol and another not clearly identified one, which could be a polymer chain with several phenyl derivates were in some cases above 1 ppm. 1-dodecanol and dodecanoic acid are frequently found as extractables and their safety hazard is considered as low [32]. Values and risk assessments were not found in the literature for neodecanoic acid and 11-methyldodecanol. However, taking the structural similarity of those two substances into account, it is assumed that their classification is similar to their structural derivates.

2.3.8 Ejection force

The inclusion of a filter into the process of the preparation or administration of the protein drug product should not lead to major handling problems due to high ejection forces caused by the filter resistance. A moderate ejection force with an attached filter, particularly for high dosed and viscous protein formulations is needed. For bedside filtration filters with a pore size of 0.2 µm, a small diameter like 13 mm and a low hold-up volume are required. Although a direct application of filtration for the injection of drugs with volumes below 1 mL is so far practically not possible, due to the high hold-up volumes of the filters, it should be tested, if drug injection at reasonable forces below 20 N is generally practicable.

The ejection force of marketed drug products filled in prefilled syringes was determined at a speed of 0.18 mL/s. anti-TNF-α IgG 1 40 mg/mL filled into prefilled syringes with staked-in 27G needles showed values of around 6.2 N. The same IgG 1 with a 0.2 µm filter (13 mm diameter) and a 27G needle showed values of about 8.7 N (Figure S7). For Roferon® 3 Mio 8.5 N without a filter and 10.4 N with a 0.2 µm filter were necessary (Figure S7). For Roferon® 9 Mio similar values with 9 N and 11.6 N, respectively, were obtained (data not shown). NeoRecormon® 2000 IE had an ejection force of 5.4 N without and 7 N with a 0.2 µm filter (data not shown). The injection of a protein solution into a patient is supposed to require higher forces. A 120 mg/mL concentrated monoclonal antibody was injected at a speed of 0.9 mL/min into pig skin. Easy to inject values of 7.8 N were achieved for the high dosed monoclonal antibody with a 27G needle and of 13.5 N with a 0.2 µm filter and a 27G needle (data not shown). All these measured values are even at high speed well below the critical force of 20 N and are easy to inject.

2.4. Discussion

Bedside filtration could be a straightforward measure to guard patients from potential immunogenicity risks associated with protein aggregates above 0.2 µm in size. Protein aggregation is a broadly accepted factor regarding unwanted immune responses [13] and immune reactions can be reduced by diminishing protein aggregates from the drug product [15]. Bedside filtration is already performed for approximately 16 % of biopharmaceuticals [22].

In this study comprehensive data regarding the efficiency of filtration is presented. Furthermore, critical aspects such as filter cleanliness or protein loss are evaluated. In the end, recommendations for future application of bedside filtration are given.

2.4.1 Filtration efficiency

For drug product solutions from several protein classes with different molecular weights and protein concentrations, it was shown with several filters that protein aggregates ≥ 1 µm can effectively be diminished with filtration. Even enormous particle burden above one million particles (≥ 1 µm)/mL could be reduced to values below a few hundreds of particles/mL. Differently stressed protein samples demonstrated further the capabilities of the investigated filters to diminish the particle count significantly. The total particle count was substantially reduced for all investigated samples, also for products with low particle counts before filtration. Particles ≥ 10 µm or ≥ 25 µm were completely eliminated or remained at numbers well below 100 particles/mL. DLS, nephelometry and NTA measurements further showed filtration effects also in the nanometer range, when using a 0.2 µm filter. The reduction of those small particles is of course not as effective as for the micrometer particles. However, it cannot be the intention of the filtration concept to get rid of protein aggregates below 0.2 µm. Moreover, for the quantification of these small particles HP-SEC studies are more appropriate. Reducing a very high number of protein particles might theoretically lead to lower protein concentrations. However, HP-SEC revealed no differences between non-stressed and stressed samples indicating that the amount of lost protein is too low to be detected. These findings are in agreement with literature [33]. Another study demonstrated for stressed proteins as well that protein loss is in the range of a few µg/mL and might not be measureable in all cases [34].

2.4.2 Filter cleanliness

Concerning the filter cleanliness the number of downstream particles and the concentration of leachables is important. From a previous study [27], we were alert that under certain conditions filter could even contribute to a higher particle load in the filtrate instead of reducing it. It was therefore tested in this study, if filters from various suppliers shed particles and if parameters, such as pH, ionic strength, filter preflushing or presence of a surfactant, would have an impact on the potential shedding behavior. Generally, the detected particle burden was extremely low in the non-filtered controls and in the filtered samples. In the majority of all investigated cases no shedding to such an extent as reported was observed [27]. However, variances between different filters exist. One of the tested filters showed stronger shedding behavior and could not be chosen for the purpose of bedside filtration. This specific filter was not single sterile packed, which explains the particle release from this filter. The shedding behavior was not influenced by pH, ionic strength, presence of a surfactant or filter preflushing. Regarding their shedding behavior the majority of the tested filters could be used for bedside filtration, although most of them are not yet specified by their manufacturers for this purpose but for in vitro, non-clinical use only. Filter companies take already several quality assurance measures in order to provide clean filters. Further, filters are generally manufactured and packaged under clean room conditions according to ISO norms. Internal limits for larger particles exist as well. However, testing for particles in the low micrometer range or for extractables is not routinely performed and should become a standard procedure for medical filters.

Our rather small investigation about leachables in the tested filters revealed overall a low level of impurities by RP-HPLC and GC-MS. No adverse effects of these low concentrated leachables are expected, based on the negative biocompatibility tests of the filters performed by the manufacturers. It was also shown that the amount of detected leachables differed between the filters. To be on the safe side, it would be recommendable that manufacturers evaluate their filters systematically for extractables, which is currently not the case for all medical filters.

2.4.3 Protein loss

Protein can be lost during filtration either due to adsorption of protein to the filter surface or due to the hold-up volume of the filter.

Protein adsorption is a complex process with several factors like buffer, pH, temperature, charge or polarity playing a role [35, 36]. Further, the dilution media, the membrane and even the manufacturing process of the membrane can have an impact on the adsorption of protein to the filter surface [37, 38]. Adsorption of proteins to the filter surface was not observed for the majority of the investigated proteins. However, in some cases lower protein concentrations were measured after filtration. These losses were in the range of around 4 µg/mL for NeoRecormon® 10000 IE and Cerezyme® 200 Units and up to 25 µg/mL for human growth hormone (0.8 mg/mL). Protein adsorption might therefore be of importance for very low concentrated drugs in the µg/mL range, but not for higher concentrated products like monoclonal antibodies [38].

Another reason to lose protein during the application of bedside filtration is the hold-up volume of the filter. A loss of 16.3 % after filtration of 1 mL solution was already measured for the investigated 13 mm diameter filter. The loss was even higher for 25 mm and 28 mm diameter filters. An air purge as instructed by the manufacturer can reduce the hold-up volume. But this is not applicable during drug administration because injected air poses the risk of air embolism. Besides the hold-up volumes are still too high for small volume drug products. It is of utmost importance that filter manufacturers develop filter designs in a way that the hold-up volumes are dramatically reduced for small filters with low filtration areas. A reliable low hold-up volume is in particular necessary for a correct dosing of the drug and to keep potential overfill volumes at a minimum. The hold-up volume is less critical for infusions, because the dosing error is far less and clinically less relevant. Further, infusion sets are often flushed with a saline to avoid drug loss due to the void volume of the system [39].

2.4.4 Protein structure and ejection force

It was the purpose of our studies to confirm that no structural changes of the protein occur during bedside filtration. Our exemplary tests on four proteins showed that filtration in the process of drug preparation or administration did not alter the protein structure, as expected, because sterile filtration is a routine operation unit carried out in the course of aseptic manufacturing of each biotech drug.

In a last step it was further checked, whether the addition of a filter would disturb the proper handling and a smooth drug administration. It was demonstrated with marketed products that easy to inject forces of below 20 N can be achieved with a 0.2 µm and 13 mm diameter filter at high speed [40]. Hence, a direct application of bedside filtration for drugs with volumes less than 1 - 2 mL is possible once filters with low hold-up volumes are on the market.

2.4.5 Expansion of bedside filtration - suggestions on how to proceed

The question which is arising now is how to implement bedside filtration of biopharmaceuticals on a more routine basis as its establishment will take time. Filtration should be first considered for products which already need several handling steps until the injection takes place. In particular lyophilisates need a multiple step process with adding the solvent, dissolution of the powder, inspection after dissolution, aspiration into the administration syringe, change of the needle and dose adjustment before administration. A filter could be easily integrated in such a procedure without much additional effort. This is supported by the fact that 56 % of all lyophilisates with a filtration recommendation by the manufacturer are filtered during drug preparation [22]. Further, an easy implementation of filtration would also be possible for drugs stored in multi-dose or mono-dose vials, because at least the transfer into the administration syringe would be necessary anyway. A routine usage of filters should also be considered in infusion settings. In a later stage the concept of bedside filtration could also be applied to small dosage forms, but redesigned filters are needed as discussed before. For the identification of the most urgent products for the expansion of bedside filtration the prevalence and clinical effects of anti-drug antibodies for a specific product should be considered. Further, the particle burden of the product as well as the stability data of the product provide information whether the product tends to form protein aggregates over its shelf life and should be a candidate for bedside filtration. Safety, not convenience should be the ultimate factor to decide on the preparation and administration routine.

2.5. Conclusion

The presented data show the benefit of bedside filtration for a wide range of protein classes and concentrations. In stressed and non-stressed protein drug products it was demonstrated that filtration is a powerful tool to eliminate larger protein aggregates. Other technical aspects regarding bedside filtration were critically assessed. The results showed no indication against the usage of filters. Filter cleanliness regarding particle shedding from filters was overall good, but variances exist. Several leachables, although on a low level, were detected by RP-HPLC and GC-MS. They have to be further assessed and limited. Protein adsorption on the filter surface is not relevant for high dosed proteins, but should be considered for low µg/mL dosed drugs. The major loss of protein can be traced back to the hold-up volume of the filters. Protein denaturation caused by filtration was not detected and the filter impact on the ejection force is small.

Although the investigated filters performed well, the filter industry has to address two major topics. First, a global quality testing for filters for clinical use should be established. As seen in the study, filter quality might vary regarding filter cleanliness in respect of particulates and extractables within and between batches. It is of utmost importance that a constant filter quality can be assured. Second, filter housings have to be adjusted for the usage of filters for small volume drugs.

In summary, a broader application of bedside filtration is recommended as an immediate and potential measure to improve patient safety.

Disclaimer

The majority of the investigated products derived from the market. Almost all of the analyzed products were expired at the time of analysis. This allows studying an extreme condition. The data is by no means appropriate or intended to describe the actual quality of a particular drug product on the market.

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2.6. Literature

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Chapter 2 Supplementary

Expanding bedside filtration – a powerful tool to protect patients from protein aggregates

Chapter 2 Supplementary is intended for publication.

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Under the scientific guidance and supervision of Prof. Dr. Gerhard Winter, all experiments, except ICP-OES and GC-MS analysis, reported in this chapter have been carried out by Benjamin Werner.

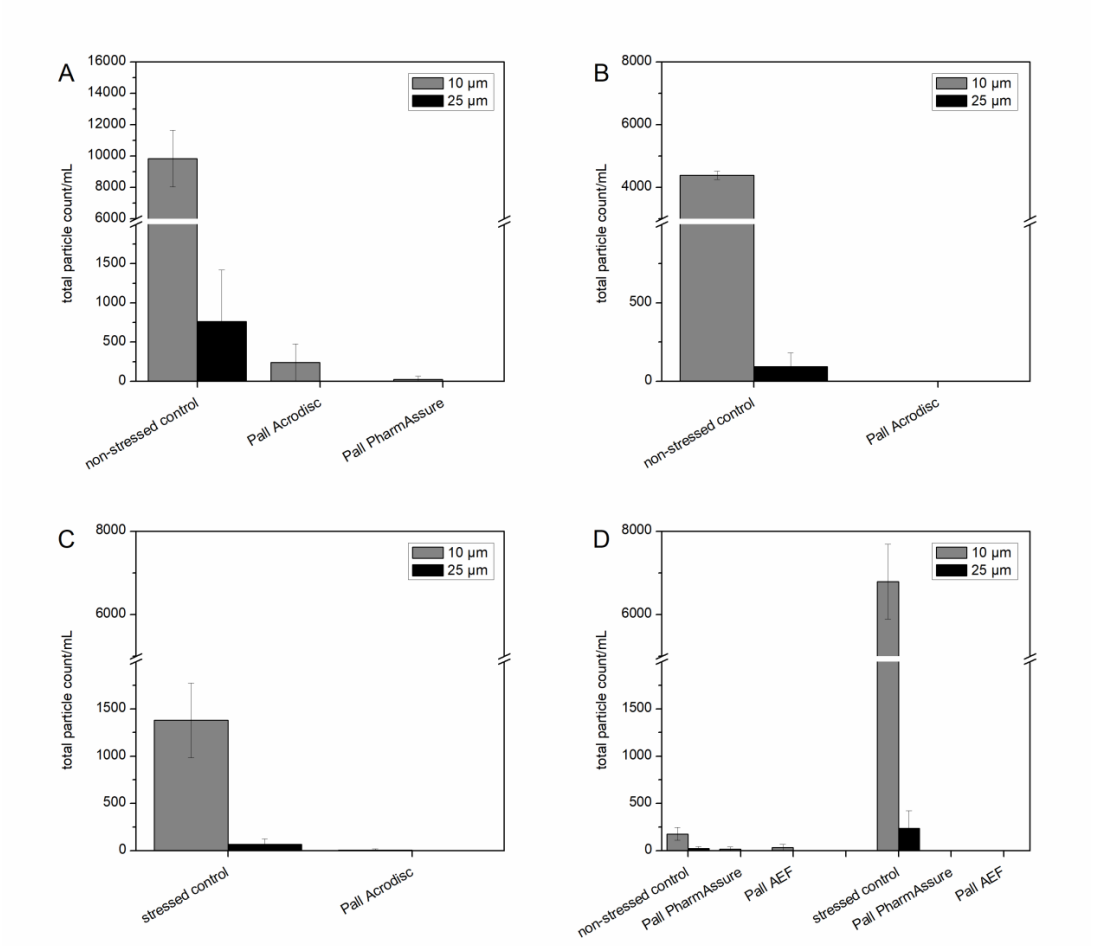

Figure S2. The cumulative particle count/mL for the particle sizes 10 µm and 25 µm, as measured by MFI, is shown for Beriglobin (240 ± 235, respectively, 25 ± 43 particles/mL for 10 µm; 0 particles for 25 µm after filtration) (**A**), Anakinra (0 particles after filtration) (**B**), end-over-end stressed Erypo 4000 (7 ± 13 particles/mL for 10 µm; 0 particles for 25 µm after filtration) (**C**) and for non-stressed (17 ± 24, respectively, 34 ± 34 particles/mL for 10 µm; 0 particles for 25 µm after filtration) and stir stressed (0 particles after filtration) Human Serum Albumin (**D**). Non-filtered controls and filtered samples are presented with the used filters.

Table S4. Marketed drug products and proteins with formulations according to products on the market were analyzed with MFI and DLS before and after filtration with the stated filters. For MFI analysis the cumulative particle count/mL for particles ≥ 1 µm, 2 µm and 5 µm is displayed

Protein	Filter name	MFI ≥ 1 µm/mL	MFI ≥ 2 µm/mL	MFI ≥ 5 µm/mL	DLS Z-Average [d.nm]	DLS Polydispersity Index
anti-TNF-α IgG 1 (1 mg/mL)	Control	4,333 ± 169	1,582 ± 125	198 ± 40	10.97 ± 0.52	0.15 ± 0.05
	Pall PharmAssure	233 ± 68	129 ± 68	0 ± 0	10.80 ± 0.13	0.12 ± 0.02
	Pall AEF	1026 ± 90	304 ± 105	70 ± 41	11.30 ± 0.32	0.17 ± 0.04
anti-TNF-α IgG 1 (40 mg/mL)	Control	330,279 ± 19,038	143,247 ± 5,800	220,807 ± 561	15.45 ± 0.28	0.16 ± 0.03
	Pall Acrodisc	789 ± 319	275 ± 41	80 ± 53	14.68 ± 0.45	0.04 ± 0.01
Kineret® 100 mg	Control	206,992 ± 2,049	110,004 ± 2,490	27,632 ± 2,057	n.a.	n.a.
	Pall Acrodisc	200 ± 40	94 ± 54	12 ± 21	n.a.	n.a.
Beriglobin®	Control	306,683 ± 24,932	158,388 ± 15,198	43,192 ± 5,499	18.45 ± 0.85	0.34 ± 0.03
	Pall Acrodisc	4,333 ± 2,665	3,118 ± 2,149	1,132 ± 905	18.84 ± 1.53	0.37 ± 0.08
	Pall PharmAssure	1,695 ± 638	804 ± 214	210 ± 57	17.97 ± 0.57	0.32 ± 0.02
Bioclate™ Antihemophilic Factor (recombinant) 250	Control	204,117 ± 2,471	70,750 ± 2,424	8,434 ± 542	22.23 ± 3.05	0.70 ± 0.07
	Filter needle	177,943 ± 3,936	63,090 ± 3,117	7,285 ± 682	20.39 ± 1.77	0.78 ± 0.07
Bioclate™ Antihemophilic Factor (recombinant) 250	Control	123,243 ± 3,312	31,392 ± 166	782 ± 189	17.71 ± 0.47	0.67 ± 0.02
	Pall Acrodisc	134 ± 168	61 ± 76	24 ± 42	18 ± 0.12	0.64 ± 0.05
Erythropoietin (84 µg/mL)	Control	841 ± 177	448 ± 46	174 ± 36	7.92 ± 0.49	0.24 ± 0.01
	Pall PharmAssure	355 ± 145	132 ± 80	17 ± 29	8.51 ± 0.21	0.25 ± 0.01
	Pall AEF	502 ± 271	148 ± 107	33 ± 38	8.52 ± 0.19	0.25 ± 0.01
Erypo® 4000	Control	69,060 ± 2,047	19,395 ± 506	1,520 ± 166	n.a.	n.a.
	Pall Acrodisc	542 ± 217	254 ± 125	30 ± 28	n.a.	n.a.
Helixate® 250	Control	1,477,020 ± 219,228	615,949 ± 98,533	92,360 ± 13,901	n.a.	n.a.
	Filter needle	495,301 ± 19,596	264,502 ± 12,137	72,057 ± 4,866	n.a.	n.a.
Human Growth Hormone (0.8 mg/mL)	Control	36,894 ± 1,068	19,699 ± 579	6,702 ± 875	84.89 ± 82.27	0.56 ± 0.34
	Pall PharmAssure	380 ± 174	172 ± 42	60 ± 39	33.87 ± 27.71	0.83 ± 0.20
	Pall AEF	257 ± 111	137 ± 97	34 ± 30	78.41 ± 84.48	0.73 ± 0.21

Table S4 continued

Protein	Filter name	MFI ≥ 1 µm/mL	MFI ≥ 2 µm/mL	MFI ≥ 5 µm/mL	DLS Z-Average [d.nm]	DLS Polydispersity Index
Albumin (Human) U.S.P. Albutein® 25 % solution	Control	4,241 ± 48	2,121 ± 308	558 ± 17	15.72 ± 0.86	0.53 ± 0.00
	Pall PharmAssure	722 ± 72	285 ± 23	50 ± 71	16.68 ± 1.09	0.55 ± 0.00
	Pall AEF	974 ± 118	202 ± 67	78 ± 19	15.91 ± 0.54	0.52 ± 0.01
Hylase® „Dessau“ 300 I.E.	Control	36,145 ± 2,246	13,723 ± 1,630	2,244 ± 409	290.13 ± 34.25	0.89 ± 0.19
	Pall Acrodisc	224 ± 166	112 ± 166	67 ± 89	25.41 ± 1.31	0.97 ± 0.05
Cerezyme® 200 Units (3.5 U/kg)	Control	1,672 ± 83	761 ± 61	324 ± 14	69.81 ± 10.65	0.21 ± 0.05
	Pall Acrodisc	130 ± 78	60 ± 40	17 ± 30	15.55 ± 1.50	0.58 ± 0.15
	IV set control	1,974 ± 349	885± 213	177 ± 16	50.71 ± 8.55	0.28 ± 0.20
	IV set + Acrodisc*	176 ± 133	76 ± 66	34 ± 38	25.32 ± 11.18	0.55 ± 0.38
	IV set + I.V.Star	334 ± 74	163 ± 53	68 ± 29	16.46 ± 0.68	0.68 ± 0.03
Intron A® Injection 1 Mio	Control	83,448 ± 2,866	37,844 ± 1,618	6,156 ± 890	n.a.	n.a.
	Pall Acrodisc	768 ± 1,071	350 ± 521	68 ± 90	n.a.	n.a.
Mabthera® 100 mg (1.5 mg/mL)	Control	2,531 ± 360	937 ± 72	256 ± 70	13.24 ± 3.25	0.13 ± 0.03
	Pall Acrodisc	207 ± 194	137 ± 96	33 ± 27	16.61 ± 4.85	0.14 ± 0.04
	Pall PharmAssure	349 ± 213	101 ± 92	15 ± 29	13.17 ± 2.86	0.15 ± 0.07
	Pall AEF	670 ± 136	197 ± 20	18 ± 32	16.30 ± 4.57	0.15 ± 0.06
Minirin® parenteral (4 µg/1 mL)	Control	8,034 ± 808	2,195 ± 65	256 ± 0	n.a.	n.a.
	Pall Acrodisc	121 ± 119	0 ± 0	0 ± 0	n.a.	n.a.
NeoRecormon® 10000 IE	Control	25,113 ± 4,224	9,372 ± 1,867	1,314 ± 205	188.12 ± 13.95	0.25 ± 0.02
	Pall Acrodisc	313 ± 96	139 ± 55	0 ± 0	137.73 ± 4.60	0.21 ± 0.02
Orthoclone® OKT 3 (1 mg/mL)	Control	249,946 ± 29,704	155,293 ± 10,932	42,034 ± 1,875	11.57 ± 0.10	0.09 ± 0.02
	Pall Acrodisc	43 ± 39	17 ± 29	0 ± 0	11.3 ± 0.1	0.06 ± 0.02
Rebif® 22 µg Injection solution	Control	678,557 ± 313,801	330,744 ± 144,249	75,281 ± 22,819	n.a.	n.a.
	Pall Acrodisc	214 ± 170	102 ± 122	11 ± 20	n.a.	n.a.
Urokinase HS medac 500 000 I.E. (50 000 I.E./mL)	Control	2,390 ± 767	852 ± 57	60 ± 26	9.52 ± 0.44	0.32 ± 0.04
	Pall Acrodisc	177 ± 82	48 ± 14	0 ± 0	10.97 ± 1.73	0.37 ± 0.06

n.a. = not analyzed; * Acrodisc = Pall Acrodisc

Table S5. Marketed drug products and proteins with formulations according to products on the market were exposed to different kinds of stress as stated
Afterwards the products were analyzed with MFI and DLS before and after filtration with the listed filters. For MFI analysis the cumulative particle count/mL for particles ≥ 1 µm, 2 µm and 5 µm is displayed.

Protein	Filter name	MFI ≥ 1 µm/mL	MFI ≥ 2 µm/mL	MFI ≥ 5 µm/mL	DLS Z-Average [d.nm]	DLS Polydispersity Index
anti-TNF-α IgG 1 (40 mg/mL) stir stress	Control	461,215 ± 19,417	163,096 ± 10,238	24,084 ± 610	36.22 ± 1.96	0.39 ± 0.02
	Pall Acrodisc	942 ± 1064	520 ± 652	50 ± 66	17.96 ± 0.45	0.29 ± 0.02
anti-TNF-α IgG 1 (1 mg/mL) stir stress	Control	7,465 ± 568	2,778 ± 305	360 ± 122	11.65 ± 0.53	0.24 ± 0.07
	Pall PharmAssure	102 ± 1	17 ± 29	0 ± 0	12.48 ± 0.61	0.28 ± 0.06
	Pall AEF	513 ± 160	183 ± 80	52 ± 27	12.27 ± 0.89	0.26 ± 0.09
Erythropoietin (84 µg/mL) stir stress	Control	913 ± 238	411 ± 17	173 ± 43	7.81 ± 0.08	0.23 ± 0.00
	Pall PharmAssure	344 ± 42	107 ± 15	25 ± 25	8.87 ± 0.34	0.28 ± 0.04
	Pall AEF	271 ± 89	74 ± 25	16 ± 28	8.48 ± 0.87	0.29 ± 0.07
Erypo® 4000 end-over-end	Control	30,171 ± 1,475	8,963 ± 424	2,820 ± 258	n.a.	n.a.
	Pall Acrodisc	222 ± 365	96 ± 148	22 ± 22	n.a.	n.a.
Human Growth Hormone (0.8 mg/mL) stir stress	Control	68,733 ± 569	30,048 ± 80	7,508 ± 287	311.73 ± 122.5	0.73 ± 0.15
	Pall PharmAssure	635 ± 391	348 ± 249	119 ± 78	75.43 ± 25.44	0.85 ± 0.19
	Pall AEF	606 ± 293	277 ± 89	79 ± 70	74.25 ± 23.40	0.95 ± 0.07
Albumin (Human) U.S.P. Albutein® 25 % solution - stir stress	Control	159,940 ± 12,737	93,632 ± 10,472	30,121 ± 4,392	15.28 ± 0.34	0.52 ± 0.02
	Pall PharmAssure	371 ± 233	112 ± 70	11 ± 19	15.58 ± 1.33	0.53 ± 0.02
	Pall AEF	565 ± 337	194 ± 93	34 ± 48	14.98 ± 0.12	0.53 ± 0.02
Mabthera® 100 mg (1.5 mg/mL) stir stress	Control	9,001 ± 472	2,406 ± 147	232 ± 117	13.23 ± 0.50	0.34 ± 0.05
	Pall Acrodisc	317 ± 320	103 ± 89	17 ± 15	18.95 ± 3.74	0.24 ± 0.05
	Pall PharmAssure	149 ± 57	75 ± 43	0 ± 0	11.01 ± 0.08	0.08 ± 0.03
	Pall AEF	211 ± 90	87 ± 82	35 ± 39	18.35 ± 0.44	0.23 ± 0.01
Mabthera® 100 mg 1 mg/mL shake stress	Control	18,052 ± 885	6,636 ± 491	614 ± 143	n.a.	n.a.
	Pall Acrodisc	1,324 ± 795	333 ± 89	59 ± 34	n.a.	n.a.

n.a. = not analyzed

Table S6. Several marketed drug products and proteins with formulations according to products on the market were analyzed with LO, nephelometry and DLS before and after filtration with the indicated filters

For LO analysis the total particle count/mL (particles ≥ 1 µm) is shown.

Protein	Filter name	LO ≥ 1 µm/mL	Nephelometry [FNU]	DLS Z-Average [d.nm]	DLS Polydispersity Index
anti-TNF-α IgG 1 (1 mg/mL)	Control	2,500 ± 179	0.66 ± 0.07	10.82 ± 0.29	0.14 ± 0.04
	Pall Acrodisc	194 ± 45	0.56 ± 0.08	10.70 ± 0.15	0.12 ± 0.01
Erythropoietin (84 µg/mL)	Control	403 ± 179	0.43 ± 0.03	7.88 ± 0.16	0.22 ± 0.02
	Pall Acrodisc	31 ± 31	0.34 ± 0.09	7.95 ± 0.15	0.22 ± 0.01
Reteplase (1 mg/mL)	Control	15,495 ± 482	0.85 ± 0.02	446.1 ± 179.05	0.18 ± 0.02
	Pall Acrodisc	736 ± 666	0.36 ± 0.02	3.18 ± 6.37	0.32 ± 0.03
Human Growth Hormone (0.8 mg/mL)	control	28,707 ± 3,073	2.26 ± 0.07	86.56 ± 47.06	0.66 ± 0.12
	Pall PharmAssure	14,508 ± 1,505	2.03 ± 0.34	20.76 ± 4.87	0.71 ± 0.09
	Pall AEF	13,415 ± 1,935	1.61 ± 0.45	18.08 ± 12.49	0.58 ± 0.11
	Millipore PES	11,426 ± 2,795	1.47 ± 0.29	17.97 ± 14.62	0.79 ± 0.12
	Whatman PES	14,313 ± 3,155	1.60 ± 0.12	33.25 ± 7.21	0.51 ± 0.12
	Sartorius PES	17,385 ± 2,526	2.04 ± 0.46	56.92 ± 27.29	0.64 ± 0.17
	Millipore PVDF	13,525 ± 3,342	1.2 ± 0.19	35.7 ± 25.47	0.64 ± 0.13
	Whatman PVDF	15,866 ± 2,982	1.64 ± 0.06	16.49 ± 8.39	0.65 ± 0.19
Cerezyme® 200 Units (2.5 U/kg)	control	5,932 ± 481	1.14 ± 0.02	364.73 ± 99.90	0.48 ± 0.04
	Pall PharmAssure	727 ± 237	0.85 ± 0.11	22.75 ± 8.84	0.78 ± 0.21
	Pall AEF	401 ± 140	0.81 ± 0.07	25.49 ± 7.86	0.78 ± 0.13
	Millipore PES	606 ± 82	0.70 ± 0.16	21.84 ± 8.89	0.76 ± 0.21
	Whatman PES	647 ± 195	0.8 ± 0.09	18.25 ± 0.88	0.68 ± 0.03
	Sartorius PES	2,890 ± 686	0.78 ± 0.11	15.69 ± 1.64	0.58 ± 0.11
	Millipore PVDF	887 ± 162	0.86 ± 0.06	22.67 ± 6.96	0.51 ± 0.31
	Whatman PVDF	619 ± 105	0.80 ± 0.05	28.55 ± 2.61	0.86 ± 0.04

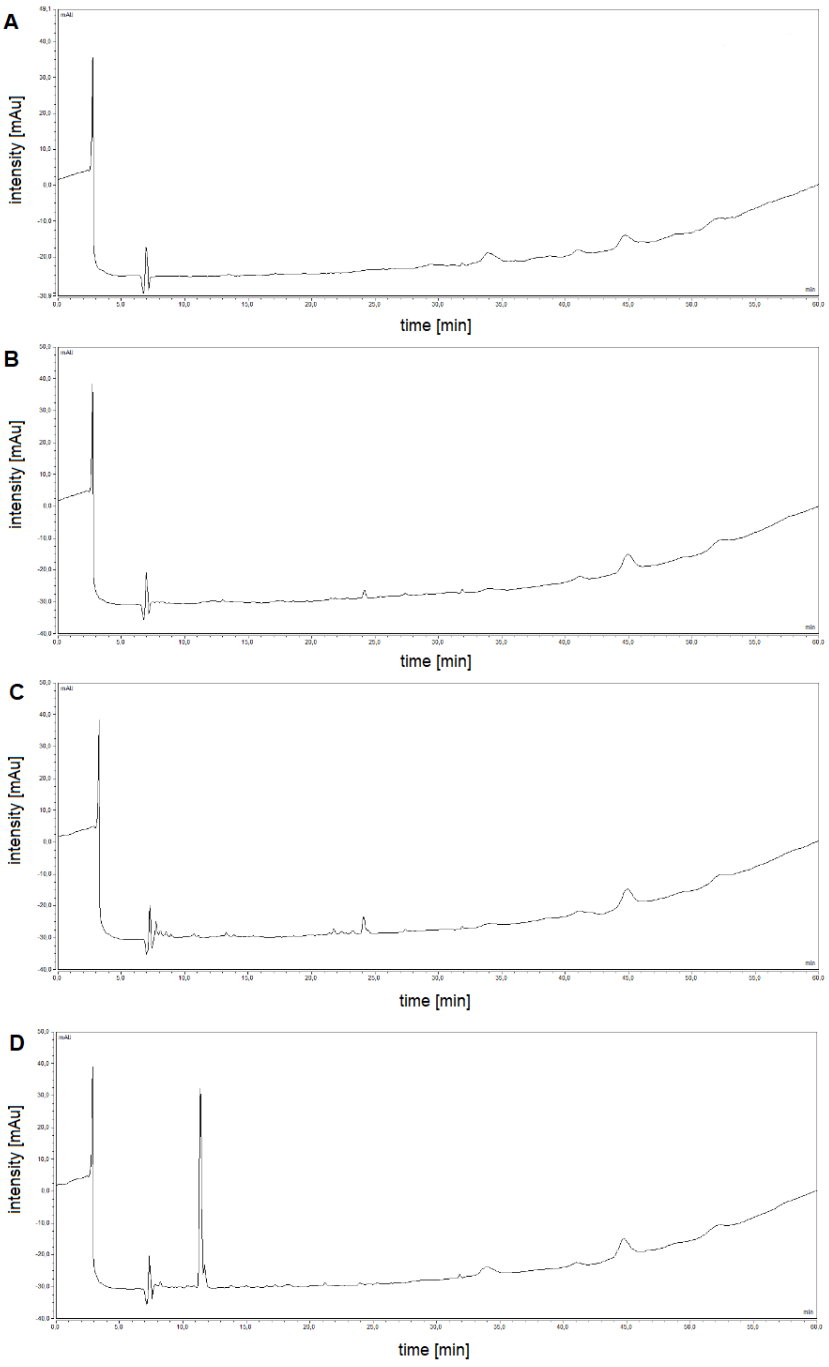

Figure S6. RP-HPLC chromatogramms of an isotonic sodium chloride solution with a pH 4 and 0.1 % polysorbate 80 at 245 nm are presented before filtration (**A**) and after filtration with three different PES filters (**B**) to (**D**).

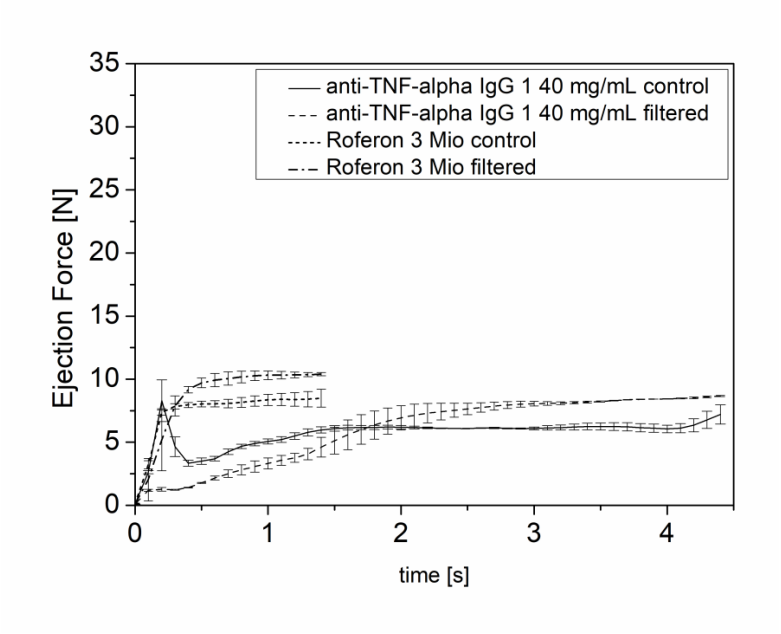

Figure S7. The ejection force at a speed of 0.18 mL/s is measured for an anti-TNF-α IgG 1 40 mg/mL and Roferon® 3 Mio solution without (control) or with (filtered) an attached filter (Pall Acrodisc) and 27G needles.

Chapter 3

Contamination of anti-VEGF drugs for intravitreal injection: How do repackaging and newly developed syringes affect the amount of silicone oil droplets and protein aggregates?

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Abstract

Purpose: The particle counts and the nature of particles of three different antivascular endothelial growth factor agents (VEGF) in different containers in a laboratory setting were compared.

Methods: Original prefilled ranibizumab glass syringes, original vials with aflibercept, and repacked ready-to-use plastic syringes with bevacizumab from a compounding pharmacy and a compounding company (CC) were analyzed. Particle counts and size distributions were quantified by different particle characterization methods (nephelometry, light obscuration, Micro-Flow Imaging, nanotracking analysis, resonant mass measurement). Using high-performance size-exclusion chromatography (HP-SEC), levels of protein drug monomer and soluble aggregates were determined.

Results: Nearly all samples showed similar product quality. Light obscuration and Micro-Flow Imaging showed a 4-fold to 9-fold higher total particle count in compounding company bevacizumab (other samples up to 42,000 particles/mL). Nanotracking analysis revealed highest values for compounding company bevacizumab (6,375 million particles/mL). All containers showed similar amounts of silicone oil microdroplets. Ranibizumab showed lowest particle count of all tested agents with only one monomer peak in HP-SEC. Repackaged bevacizumab from different suppliers showed varying product quality.

Conclusion: All three tested agents are available in similar quality regarding particulate purity and silicone oil microdroplet count. Repackaging can have a major impact on the quality.

Disclaimer

Owing to the limited number of samples and the need to dilute 20 fold for analysis, the statistical value of the data is limited.

Keywords

aflibercept, bevacizumab, contamination, intraocular pressure, protein particles, ranibizumab, silicone oil

3.1. Introduction

Intravitreal antivascular endothelial growth factor (VEGF) agents, including ranibizumab (Lucentis; Genentech, San Francisco, CA), bevacizumab (Avastin; Genentech) and aflibercept (Eylea; Regeneron Pharmaceuticals, Inc, Tarrytown, NY), are used for years to treat several kinds of retinal vascular disorders.[1-3] Short-term and transient intraocular pressure (IOP) rise is well known to occur after intravitreal injection and is easy to explain because of injection of the fluid volume into the vitreous with the different anti-VEGF agents.[4, 5] Long-term or sustained IOP rise has a more difficult mechanism and has been reported clinically in several case reports and case series. Bakri et al[6] first reported sustained IOP rise after ranibizumab injections requiring medical treatment in a small series of four patients and Kahook et al reported similar findings in six patients.[7] Several studies with different number of patients followed reporting prevalence of long-term IOP rise after treatment with ranibizumab, bevacizumab, or aflibercept injections.[7-20]

Recent retrospective analysis of the VIEW 1 and 2 data of 2,457 patients showed significant higher IOP elevation in ranibizumab than in aflibercept injected eyes.[21] This study showed for the first time in a high number of patients a statistically significant IOP elevation difference between these two agents. Notably all studies evaluating IOP elevations after anti-VEGF injections are limited because of the retrospective design of the analysis.

Several mechanisms have been proposed to explain clinically significant IOP elevation after anti-VEGF treatment. Such mechanisms include mechanical trauma to the trabecular meshwork from repeated injection-related IOP spikes and a decrease in aqueous outflow because of VEGF blockade, as potentially mediated by inhibition of nitric oxide synthesis, or inflammation or obstruction by high molecular weight protein aggregates or silicone microdroplets.[22-24] Because of the different size of molecules (bevacizumab has a molecular weight of 149 kDa, aflibercept of 115 kDa, and ranibizumab of 48 kDa), it has been assumed that the agents can accumulate in the trabecular meshwork especially after long-term repeated administration and cause either direct obstruction or indirect change of the outflow facility. Other proposed mechanisms postulate outflow obstruction by protein aggregates and/or silicone oil droplets from the syringes or needles used.[24-26] Liu et al reported both protein aggregates and particles ≥ 1 µm in repackaged bevacizumab obtained from three external compounding pharmacies in the United States.[26]

All three agents are currently prepared and distributed in different containers. Ranibizumab comes in two possible injection containers. First, there is a single-dose vial, which is drawn into a syringe immediately before injection. Second, since 2013, there is a prefilled syringe available in the European Union, which has been approved also by the U.S. Food and Drug Administration (FDA) in 2016. Currently, mainly the prefilled syringe is used in central Europe. [27, 28] Aflibercept is only delivered in a single-dose vial, which is drawn into a syringe

immediately before injection. The use of bevacizumab as intravitreal agent is off-label. Bevacizumab is therefore only available from compounding pharmacies as an agent drawn from a larger vial and then usually filled into multiple syringes, which are stored in a refrigerator at 2°C to 8°C before use.[26] Theoretically, this compounding, refilling, inadvertent freeze and thaw processes carries an increased risk for aggregate and particle generation.

Liu et al quantified levels of subvisible particles and protein aggregates in repackaged bevacizumab syringes obtained from compounding pharmacies, as well as in samples of bevacizumab and ranibizumab vials tested in controlled laboratory experiments.[25] The ready-to-use prefilled ranibizumab was not available at the time of Liu's publication.

Aim of this study was to add further laboratory data on levels of subvisible particles and protein aggregates in a new prefilled ranibizumab syringe product and in the aflibercept product and to compare it to samples of bevacizumab delivered from a compounding pharmacy and from a compounding company.

3.2. Materials and Methods

3.2.1 Materials

The products of interest were bevacizumab 25 mg/mL, repackaged by the central pharmacy of the University Hospital Duesseldorf in 1 mL Braun syringes with a volume of 150 µL (8 units; Avastin Roche 3.75 mg; batch No. B8008H10) (bevacizumab D), bevacizumab 25 mg/mL, repackaged by BA.Herstellung GmbH & Co. KG in a Braun micro-fine insulin syringe U100 with a 30G staked-in needle with a volume of 50 µL (16 units; Avastin Roche 3.75 mg; batch No. 060117APO) (bevacizumab F), original aflibercept 40 mg/mL supplied in a vial and with a 5 µm filter needle (6 units; EYLEA 40 mg/mL injection solution in a vial, Bayer; batch No. 54269C) and original ranibizumab 10 mg/mL in a siliconized glass syringe (6 units; Lucentis 10 mg/mL injection solution in a prefilled syringe; Novartis; batch No. S2050C). All units were packed in shock absorbing containers with cooling packs until arrival at the laboratory of the Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics at the Ludwig-Maximilians-University in Munich. Upon arrival all products were visually inspected for any damages. No damages and no signs of freezing were detected. Samples were stored light protected at 6°C in the middle of a refrigerator to prevent any impact of light or freeze-thawing effects until direct analysis.

3.2.2 Sample Preparation

All steps like extracting the protein solution from the product container, sample dilution, or aliquoting was carried out under a laminar flow hood according to aseptic handling. For the simulation of a real application, all products were prepared according to the manufacturer instructions. An exception of this was that the whole syringe content was used instead of disposing some of the protein solution as instructed to avoid wasting product volume, which was required for analysis. Briefly, aflibercept solution was aspirated from the vial with the enclosed 18G x 1 ½ BD Blunt Fill Needle – Filter (Becton, Dickinson and Company Limited, Franklin Lakes, NJ) into a BD 1 mL syringe with Luer-Lok Tip (Becton, Dickinson and Company Limited). The filter needle was removed. The following procedure was now the same as for the other two products, bevacizumab D and ranibizumab. A 30G ½" BD Microlance 3 (Becton, Dickinson and Company Limited, Drogheda, Ireland) was attached to the syringe and the protein solution was expelled into a 50 mL Greiner tube. The repackaged bevacizumab F was stored in Braun micro-fine insulin syringes U100 with a staked-in needle. In this case, the product was directly expelled into the tube. All aliquots of the same product were pooled in one tube. For analysis, the pooled samples were diluted 1:20 in the appropriate formulation buffer. The buffers were filtered before with a medical polyethersulfone filter with a pore size of 0.2 µm (Pall PharmAssure; Pall GmBH, Dreieich,

Germany) before the addition of the concentrated protein solution. The samples were analyzed within three days after dilution. The presented data show the calculated results for nondiluted samples except for nephelometry where the results of the diluted samples are presented. Particle analysis was performed on calibrated equipment. Duke Standards and Count-Cal Particle Size Standards were used (both Thermo Scientific, Fremont, CA).

3.2.3 Methods

3.2.3.1 Nephelometry

A Nephla turbidimeter (Dr. Lange, Düsseldorf, Germany) was used to determine the turbidity of the samples. At an angle of 90°, scattered light from a laser with a wavelength of 860 nm is detected. Triplicate measurements were performed and presented in the form of formazin nephelometric units (FNU).

3.2.3.2 Light Obscuration

Light obscuration was performed by using a SVSS-C device from PAMAS GmbH and the software PAMAS PMA Program V 2.1.2.0 (Rutesheim, Germany) for the quantification of particles in the subvisible and visible particle range. The system cleanliness was tested before each sample by measuring the particle content of highly purified water. Each sample run was carried out in triplicates of 0.3 mL after a prerun of 0.4 mL. Each sample was analyzed in triplicates resulting in a total of nine single runs. The speed for the aspiration of the solution was set to 10 mL/minute.

3.2.3.3 Micro-Flow Imaging

A Micro-Flow Imaging DPA 4100 device (BrightWELL Technologies Inc, Ottawa, Canada) with a 100 µm flow cell in the operation mode high magnification was used. The software MFI Particle Analyzer V6.9.7.2 was applied for analysis. The flow cell was flushed with highly purified water and flow cell cleanliness was checked between each run. The cell was flushed with 0.5 mL of the corresponding buffer to optimize illumination, which ensures a correct system thresholding, before each analysis. Next, the cell was loaded with 0.3 mL before 0.65 mL of the sample was analyzed at a flow rate of 0.1 mL/minute. Triplicate measurements were performed.

3.2.3.4 Nanotracking Analysis

A NanoSight LM20 (NanoSight, Amesbury, United Kingdom) with the software NTA 2.3 was used for capturing the particle movement in the nanometer range for 60 seconds. The camera shutter and gain were set to 1,497 and 680, respectively. The sample cell was flushed with highly purified water before it was loaded air bubble free with 0.5 mL sample. Between each analysis of the triplicate, 0.1 mL sample was loaded into the cell. Loading was performed with a 1 mL Terumo Syringe without needle (Terumo (Philippines) Corporation, Laguna, Philippines). During video capture, no flow was applied.

3.2.3.5 Resonant Mass Measurement

For the differentiation of protein and silicone oil particles in a particle size range from 300 nm to 5 µm resonant mass measurement with a Hi-Q Micro Sensor was carried out (Affinity Biosensors LLC, Santa Barbara, CA). Before the sample was loaded for 40 seconds, the sensor was flushed with highly purified water. Next, in the automatic limit of detection mode, the limit of detection was determined three times before calculating the mean value. The mean value was then used for the measurement. Analysis was stopped either after the volume reached 150 nL or 10 minutes. The manual stop needed to be performed because of very low particle counts in the samples leading to a wrongly recognized analysis volume. The density of protein particles was set to 1.32 g/mL and for silicone oil to 0.97 g/mL. The software ParticleLab V 1.9 (Affinity Biosensors LLC) was used for analysis. Each product was measured three times.

3.2.3.6 High-Performance Size-Exclusion Chromatography

For the evaluation of the relative amount of soluble protein species, size-exclusion chromatography (HP-SEC) was carried out. A flow of 0.5 mL/minute with a mobile phase consisting of 300 mM sodium chloride and 50 mM phosphate pH 7 in highly purified water was applied on a TSKgel 3000SWXL column (Tosoh Bioscience GmbH, Stuttgart, Germany). Samples were centrifuged before analysis for 10 minutes at 14,000 rpm. Twenty-five microliters of the diluted protein solution were injected. Each product was run six times. Analysis was carried out on a Waters 2695 Alliance Separation Module (Waters Corporation, Milford, MA) with a Waters 2487 Dual λ Absorbance Detector for UV absorbance at 280 nm. Data analysis was carried out with Chromeleon V6.8.

3.3. Results

3.3.1 Nephelometry

The determination of the opalescence of the buffer and protein solutions showed for all samples very low values (Figure 1). The addition of the protein solutions to the corresponding filtered placebo buffers led to a slight increase of the turbidity. One reason for the slightly lower opalescence value for the diluted ranibizumab samples in comparison with the other three products is the lower concentration of ranibizumab by a factor of 2.5 and four to bevacizumab and aflibercept, respectively. Overall the samples were clear and no particles visible.

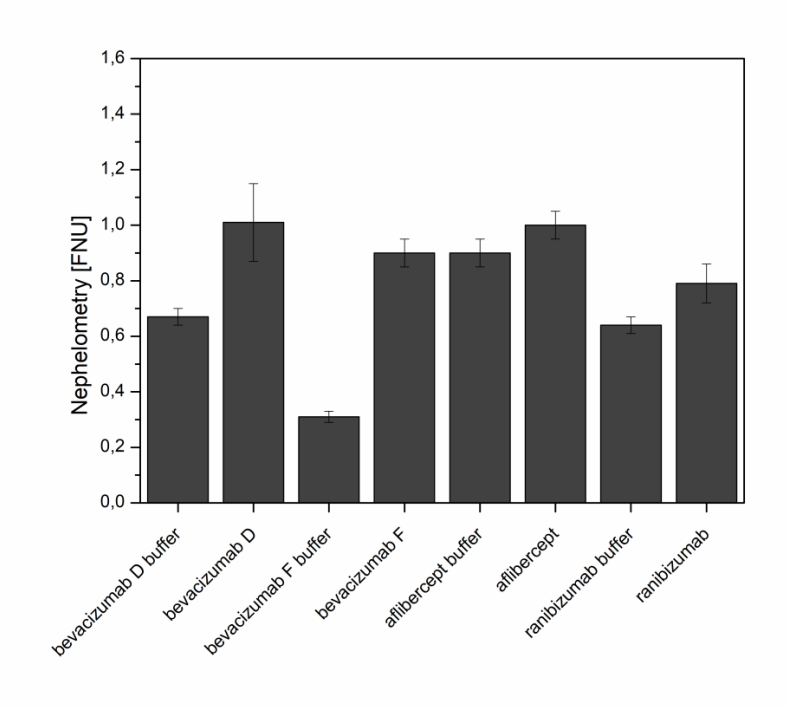

Figure 1. Nephelometry data for buffers and diluted (1:20) protein solutions.

3.3.2 Light Obscuration and Micro-Flow Imaging

The particle burden in the subvisible range was measured with light obscuration and Micro-Flow Imaging (Table 1). The filtered placebo buffer solutions contained only a minor amount of particles (< 1,100 particles [≥ 1 µm]/mL), whereas all protein solutions, except bevacizumab F, showed a (calculated) particle level in a range of around 36,000 to 42,000 particles (≥1 µm)/mL. Bevacizumab F displayed particle counts by a factor of at least four (light obscuration) and nine (Micro-Flow Imaging) higher than the other samples. Ranibizumab had the overall lowest particle count. The difference in the solutions with and without protein clearly demonstrated the presence of many small protein and silicone oil particles in the product solutions. Particles larger than 10 µm as well as 25 µm were on a low level, if existing at all.

Table 1. The Total Particle Count (Particles ≥ 1 µm, Calculated After Measuring Samples With 1:20 Dilution) of Each Sample as Determined by Light Obscuration and Micro-Flow Imaging is listed

	Light Obscuration			**Micro-Flow Imaging**		
	particles/mL ≥			particles/mL ≥		
	1 µm	10 µm	25 µm	1 µm	10 µm	25 µm
Bevacizumab D buffer	546 ± 7	13 ± 6	7 ± 5	54 ± 23	0	0
Bevacizumab D	41,733 ± 9,374	622 ± 212	82 ± 122	42,848 ± 12,097	535 ± 463	0
Bevacizumab F buffer	81 ± 6	3 ± 3	0	498 ± 58	7 ± 13	0
Bevacizumab F	182,919 ± 2,168	2,126 ± 151	37 ± 46	414,308 ± 40,781	2,663 ± 3,107	0
Aflibercept buffer	1049 ± 41	6 ± 4	1 ± 1	255 ± 47	0	0
Aflibercept	36,096 ± 5,356	163 ± 100	7 ± 13	44,299 ± 4,244	271 ± 470	0
Ranibizumab buffer	1,093 ± 95	5 ± 1	0	121 ± 70	0	0
Ranibizumab	37,755 ± 14,715	570 ± 490	82 ± 90	19,815 ± 9,358	267 ± 463	0

The table further contains the particle numbers of interest regarding the USP requirements (particles ≥ 10 µm and particles ≥ 25 µm).

3.3.3 Nanotracking Analysis

Measuring submicrometer particles with nanotracking analysis revealed a particle level of up to 102 million particles/mL for all placebo buffers (Figure 2). In comparison, highly purified water had a particle count of around 12 million particles/mL. In the protein samples, however, hundreds of millions of particles in the low nanometer range were detected. The highest value with 6,375 million particle/mL was obtained for bevacizumab F, followed by aflibercept with 4,082 million particles/mL. The high particle count observed for aflibercept can be attributed to the highest protein concentration. Bevacizumab D showed less than half the number and ranibizumab showed about one fourth of the number of submicroparticles compared with aflibercept. The mean particle size for all drug samples was around 184 nm ± 21 nm. Aflibercept and ranibizumab nanoparticles had a similar mean particle diameter of 163 nm ± 10 nm and 171 nm ± 35 nm, whereas bevacizumab D and bevacizumab F particles showed a broader distribution and a larger mean value of 192 nm ± 62 nm and 209 nm ± 11 nm, respectively.

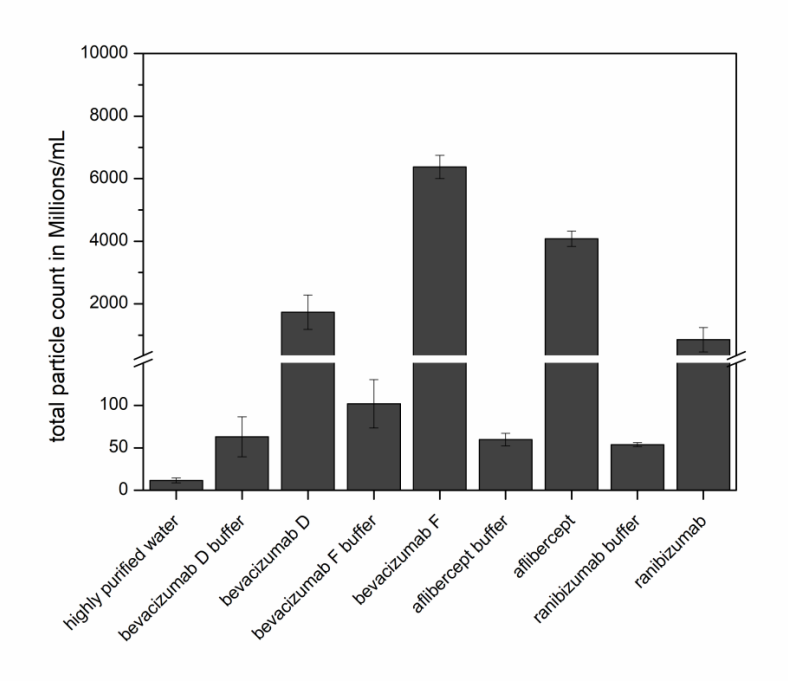

Figure 2. The total (calculated) particle count/ml of all nanometer particles is displayed for each sample as determined by nanotracking analysis.

3.3.4 Resonant Mass Measurement

For the differentiation of silicone oil and protein particles, resonant mass measurement was performed (Figure 3). Overall, the particle burden of all solutions was very low and near the detection limit because of the high cleanliness of the samples. For comparison, highly purified water had a particle burden of around 50,000 particles (≥ 0.3 µm)/ml and a buffer showed values of 400,000 particles (≥ 0.3 µm)/mL. In all cases, silicone oil droplets were detected beside protein particles. The particle numbers were similar for bevacizumab D, bevacizumab F and aflibercept; ranibizumab showed a much smaller overall particle number. For bevacizumab D, bevacizumab F and aflibercept, a relatively small amount of silicone oil in comparison with the amount of protein particles was found. Silicone oil droplets are more dominant for ranibizumab, which is stored in a siliconized glass syringe and has an overall lower protein content.

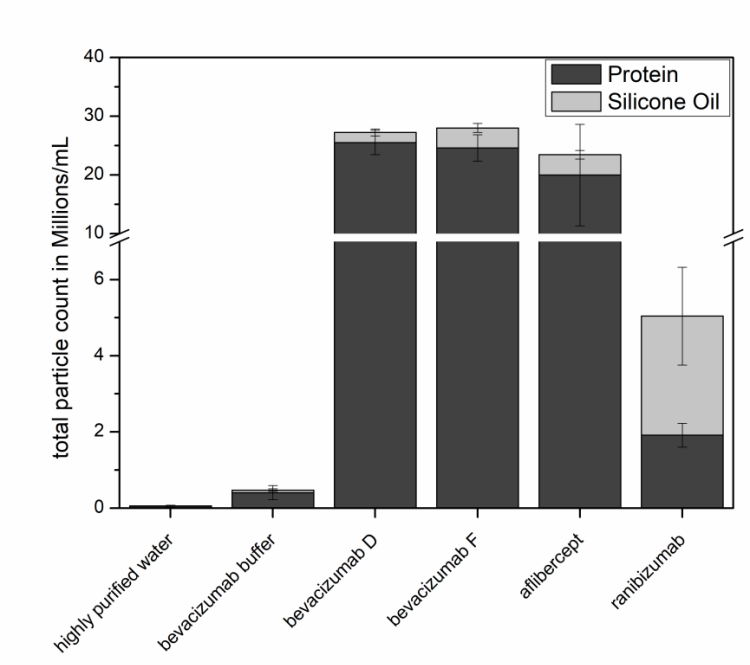

Figure 3. The (calculated) cumulative particle count/mL of silicone oil and protein particles is shown as determined by resonant mass measurement.

3.3.5 High-Performance Size-Exclusion Chromatography

Bevacizumab D and F showed similar shares of soluble protein species as detected with HP-SEC (Figure 4). One peak represented the monomer, whereas the other ones represented dimers and higher molecular weight protein species. Tiny amounts of higher molecular weight protein species made a percentage of around 0.1%, whereas the monomer accounted for around 98.2% and the dimers for roughly 1.7%. Two peaks were found for aflibercept, representing the monomer and higher molecular species with a share of 98.8% and 1.2%, respectively. For ranibizumab, only a monomer peak was detected.

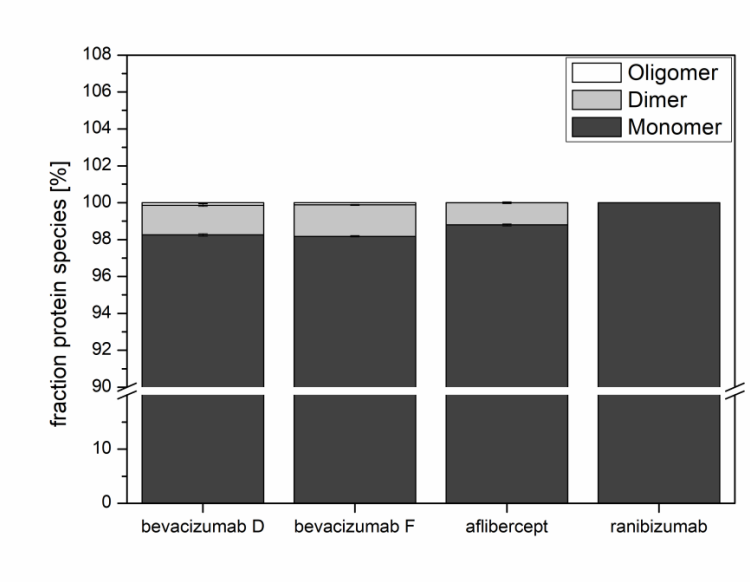

Figure 4. Determination of the relative amount of protein species for different anti-VEGF drugs with HP-SEC.

3.4. Discussion

Intravitreal injection of anti-VEGF drugs can lead to an increased long-term IOP.[4, 5, 7-20] Several mechanisms to explain this phenomenon including the possibility that protein aggregates and silicone oil microdroplets are responsible for the IOP elevation are discussed.[22-24] Protein aggregates can be formed by various pathways, including temperature, light, freeze-thawing or shaking during drug transportation and handling or just by long-term storage.[25, 29] The major source for silicone oil particles is the primary packaging container of the drug substance. Other sources could be silicone oil coated stoppers, the syringe used for drug aspiration or the needles used for withdrawing or injection of the drug. Obstruction of the outflow pathway by particulate matter with IOP elevation is a well-known phenomenon in experimental glaucoma models.[30]

To the best knowledge of the authors, this is the first study directly comparing subvisible particle numbers and type in all three clinically used anti-VEGF drugs in the current application form. In this study, the level and nature of the particles found in anti-VEGF drugs for intravitreal injection were identified and quantified.

The diluted samples showed no sign of visible particles before analysis, which was confirmed by low nephelometry data (Figure 1) and overall moderate-to-low particle counts for all products except bevacizumab F (Table 1). Eventually, the high particle count in bevacizumab F in comparison with the agents could be explained by the primary packaging material. For expelling the protein solution an external 30G needle was attached to the syringe for all agents beside bevacizumab F. Bevacizumab F was supplied with a staked-in needle syringe. In another intravitreal injection study, intravitreal silicone oil droplets were more often detected with staked-in needle syringes than with luer cone syringes with an attached needle. The explanation of the authors was that a 50 µL residual space is present in the needle hub for attached needles. For staked-in needles this space is missing. In this space, silicone oil, which was scratched off the syringe barrel during ejection, can accumulate and is not injected into the eye, whereas it is in the staked-in needle syringe.[31] This hypothesis is supported by observations during preparation of this study. The loss of volume was higher for bevacizumab D; whereas in case of bevacizumab F, the complete small volume of 50 µL could be expelled. Other factors playing a role in the higher particle count of bevacizumab F could be the product itself or handling during storage, repackaging, or transportation. For ranibizumab one should keep in mind that it has a 2.5, respectively, 4 fold lower protein concentration than the other products and the overall lower particle counts can in part be explained by that. Despite moderate-to-low particle counts in three out of four products, it is notable that all investigated products would theoretically not meet the specifications by U.S. Pharmacopeial Convention reference standard (USP) 789 (Table 1).[32] USP 789 deals with the foreign particulate matter in ophthalmic solutions and requires a

particle count below 50 particles/mL ≥ 10 µm, 5 particles/mL ≥ 25 µm and 2 particles/mL ≥ 50 µm,[32] respectively. It has to be mentioned that our measurements were performed according to USP 787, not according to USP 788 (as it is normally required in USP 789).[32-34] USP 787 differs from USP 788 mainly by the analyzed volume. USP 787 is designed for therapeutic protein injections with low volumes and can be performed with 0.2 mL to 0.5 mL, whereas USP 788 would require a volume of 25 mL. The particle number limits are the same for USP 787 and USP 788.[33, 34] The filtered aflibercept shows the best values regarding these requirements, although it would not meet them. Similar findings were already reported by Palmer et al and Yannuzzi et al.[35, 36] They found in repackaged as well as in original bevacizumab particle counts higher than the limits set by the pharmacopoeia.[36] A future solution to reduce particle numbers and to fulfill the requirements by the USP might be to filter the protein solutions before administration with a lower pore size filter (e.g., 0.2 µm).[37] Further, a batch to batch variation cannot be excluded because all our samples derived from one batch. Such a variation is possible as several other reports show.[25, 35, 36, 38] Particle levels do not only vary between different compounders but may even vary between syringes produced from the same original vial content.[25, 26, 36] Analyzed samples from this study were taken from standard clinical settings and therefore reflect real live anti-VEGF samples for injections in patients.

So far only Micro-Flow Imaging data are published to quantify silicone oil and protein particles in ophthalmic preparations.[25] A drawback of this method is that a reliable differentiation is only possible at particle sizes over 5 µm. Because smaller particles might have an effect on the trabecular meshwork, resonant mass measurement was applied. With this technique, also the nanometer range can be analyzed. The analysis revealed millions of nanometer-ranged silicone oil and protein particles (Figure 3). But, overall the samples contain a low amount of silicone oil and the absolute amount is similar for all four products in the study. The total particle number measured with resonant mass measurement is clearly lower for ranibizumab. The contribution of silicone oil microdroplets to the overall particle level in repackaged bevacizumab samples has been shown several times.[25, 38] It was observed that the amount of silicone oil has not changed much over the investigated period of several weeks for nearly all tested samples.[25, 36, 38] Nevertheless, the particle burden in repackaged bevacizumab increased over time,[25, 36] suggesting protein aggregation, beside silicone oil as cause for the particle elevation.[36] Otherwise, bevacizumab seems to be stable for several weeks in repackaged syringes.[25, 36, 38, 39]

Nanotracking analysis measurements revealed a high amount of nanosized particles (Figure 2) and confirmed the overall particle number trends gained by light obscuration and MFI (Table 1).

For the identification of the amount of soluble protein aggregates HP-SEC was carried out

(Figure 4). Overall the data correlate well with the findings for the insoluble particles. In accordance with literature, bevacizumab contains a small percentage of higher molecular weight species. The investigated bevacizumab samples had an amount of roughly 1.8%, whereas others detected a value of 1.56%[38] or even higher.[25, 40] Besides the monomer peak, a protein dimer peak was observed as well for aflibercept in a low concentration of 1.2% of the total protein concentration. The higher molecular species peak was so far not described in literature.[41] The detection of only the ranibizumab monomer peak with HP-SEC was in accordance with literature.[40]

Several authors have postulated mechanisms explaining long-term IOP elevation after repeated anti-VEGF injections.[6, 7, 10, 25] Most mechanisms refer to mechanic or postinflammatory occlusion of the trabecular meshwork outflow pathway. None of these mechanisms have been proved so far, possibly reasons are multifactorial. Direct toxic effects have been examined as well; but even in much higher doses than clinically used, there is no toxic effect of anti-VEGF agents in laboratory setting.[42]

3.5. Conclusion

Summarizing, the product quality of the repackaged bevacizumab D, the aflibercept vial, and the new siliconized glass syringe ranibizumab is overall similar. Repackaged bevacizumab F shows a much higher particulate burden, particularly when the standard method of light obscuration is applied. This might be due to the fact that a staked-in needle syringe was used in contrast to the other products, but also other reasons cannot be excluded. Ranibizumab displays in the majority of the applied analytical methods the highest quality with regard to protein-based submicron particles. However, one should keep in mind that protein concentration in ranibizumab is the lowest one. At least with the new syringe-packaged ranibizumab product, differences to aflibercept like they were hypothesized in the VIEW 1 and 2 study could no longer be correlated with high particle burden in future use. Further, it can be stated that repackaging in general does not lead to inferior quality regarding particulate levels compared with “original” products. But already with only two repackaged bevacizumab products in hand, relevant differences could be detected. With only one batch of repackaged bevacizumab F at disposal, it is impossible to judge the cause for its inferior performance in comparison with the other investigated samples. When it comes to larger, two digit micrometer-sized particles application of a filter (needle) like for aflibercept appears helpful, although such a filter does not reduce smaller particles.

In summary, it is important to make clear that none of the investigated three agents stood out in terms of particulate impurity. We found high numbers of submicron particles with different analytical methods. The relevance of such particles is still unclear, nor do we have specifications, or an industry standard for quantifying them. Therefore caution has to be applied before drawing conclusions. Yet, in the context of the ophthalmic use of protein drug products and the clinical experience with elevated IOP, it appears urgent to put more effort into research correlating these factors.

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Chapter 4

Silicone oil free polymer syringes for the storage of therapeutic proteins

Chapter 4 is intended for publication.

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The presented experiments were conducted by Benjamin Werner. Assistance was provided for the establishment of the ABS assay and during the execution of the LC-MS analyses. ICP-OES measurements were carried out by a contract analytical service. Scientific guidance was provided by Prof. Dr. Christian Schöneich and Prof. Dr. Gerhard Winter.

Abstract

Prefilled syringes are a popular choice for the delivery of biopharmaceuticals. However, glass syringes might not be the optimal primary packaging material for all biopharmaceuticals. There is evidence that the necessary lubricant silicone oil in glass syringes can interact with proteins and can be shed from the surface into the product solution. In the last years, polymer based syringes were developed which are free of silicone oil. Despite the several advantages however, a major shortcoming of these polymer systems is their relatively high gas permeability, which might be a limitation for the storage of oxygen sensitive biopharmaceuticals. So far, no long-term protein stability studies regarding such polymer systems have been published. In this study, two therapeutic proteins were stored in glass syringes and in silicone oil free polymer syringes made out of cyclic olefin polymer. In addition, polymer syringes stored in nitrogen filled aluminum pouches or covered with oxygen-tight labels were included. Similar chemical protein stability was achieved at 4 °C for all different primary packaging materials. However, in contrast to the polymer syringes, high particle counts were observed in the glass syringes. Polymer syringes stored in nitrogen filled aluminum pouches presented a promising alternative for the storage of biopharmaceuticals as they do not expose patients to silicone oil and silicone oil-protein aggregates.

Keywords

cyclic olefin polymer, COP, polymer, silicone oil free, syringes, protein oxidation, protein aggregates, protein particle, therapeutic protein, long-term stability, stability study, oxidation

Abbreviations

ABS, 4-(aminomethyl) benzenesulfonic acid; COP, cyclic olefin polymer; COC, cyclic olefin copolymers; DLS, Dynamic Light Scattering; HP-IEX, High-Performance Ion-Exchange Chromatography; HPLC, High-Performance Liquid Chromatography; HP-SEC, High-Performance Size Exclusion Chromatography; ICP-OES, Inductively Coupled Plasma Optical Emission Spectrometry; LC-MS, Liquid Chromatography-Mass Spectrometry; mAb, monoclonal antibody; MFI, Micro-Flow Imaging; PFS, prefilled syringes; RP-HPLC, Reversed-Phase High-Performance Liquid Chromatography; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.

4.1. Introduction

The number of prefilled syringes (PFS) as primary packaging for biopharmaceuticals has increased in the last years as many biopharmaceuticals are injected subcutaneously and make up for most of the top 10 US best selling drugs [1-3]. As a consequence PFS have a potential to grow by around 90 % up to 6.7 billion units in 2020. The wide spread usage of PFS is based on several benefits like enabling an easy and quick administration. Further, PFS offer an increased safety due to direct labeling and dosing as well as a reduction of needle stick injuries. Other PFS advantages include reduced contamination risks and minimized overfills [1, 2, 4]. However, downsides of commonly used type I borosilicate glass PFS exist as well [1, 2, 5]. Disadvantages of glass are breakage, delamination or pH shifts due to the leaching of alkali metals [2, 5]. Tungsten traces are introduced into the syringe when forming the fluid path with a tungsten pin. Due to high temperatures during syringe manufacturing tungsten oxides react with glass forming tungsten polyanions. Those polyanions are able to induce protein aggregation in certain cases [1, 2, 6-8]. Another major issue is that glass syringes need silicone oil for functionality. Several studies show that silicone oil alone or in combination with stresses like agitation or shaking can lead to protein aggregation [9-14]. Typically 0.2 - 1 mg medical grade silicone oil is used for the lubrication of the syringe barrel by spray-on technology [3, 4]. In the last years attempts were made to minimize the issues associated with silicone oil by optimizing the siliconization processes. Baked-on or cross linked siliconization techniques led to lower silicone oil levels and reduced silicone oil migration into the drug solution [2-4]. However, a silicone oil free glass syringe is not yet on the market. Since siliconization of the syringe barrel is still necessary, syringe manufacturers investigated other materials for silicone oil sensitive biopharmaceuticals. As alternative to glass syringes polymer syringes made of cyclic olefin polymers (COP) or cyclic olefin copolymers (COC) aroused the interest of the main syringe manufacturers [2]. Currently, two companies offer silicone oil free syringe systems based on COP material, other available polymer syringe systems still need silicone oil for lubrication. West Pharmaceutical Services developed a silicone oil free syringe system with Crystal Zenith® from Daikyo Seiko with FluroTec® coated stoppers. Terumo Corporation offers a silicone oil free COP syringe system called PLAJEX™ syringe with i-coating™ coated stoppers [2, 15]. Outside of Japan these polymer syringes are still rarely used, but the interest in these polymer syringes increases [16]. Whilst breakage and delamination of glass syringes are reasons for frequent recalls, these two issues can be neglected with polymer syringes [5, 16, 17]. Further, the polymer syringes show a high transparency, enabling visual inspection, a low impurity and extractable profile, a high moisture barrier, a broad chemical resistance and can be sterilized by steam or e-beam sterilization [17-20]. It is also proven that the polymer is safe and biocompatible and can be used for medical packaging [15]. The tungsten free and

glue free manufacturing process and the absence of silicone oil is more important for the storage of biopharmaceuticals [21, 22]. In comparison to siliconized polymer and glass syringes, lower particle counts in silicone oil free polymer syringes were detected in two studies, in which protein solutions were exposed to shaking for up to one week [15, 23]. However, no report about the development of particle count in protein filled silicone oil free polymer syringes over a longer storage period without artificial stress exists. Moreover, protein adsorption is reported to be lower for the polymer than for glass [20]. Despite these advantages polymer syringes have a major shortcoming. The material itself possesses relatively weak gas barrier properties [15, 24]. This might limit its usage as primary packaging material for oxygen sensitive pharmaceuticals [24, 25]. Various options are available to handle the oxygen permeability of the polymer syringes. First, oxygen absorbers could be placed in a packaging blister to control residual oxygen [16, 26]. Second, polymer layers with high oxygen barrier properties can be incorporated into the COP syringe body resulting in multilayer syringes. This approach will not be feasible in the near future due to high costs and low available quantities, which cannot sufficiently guarantee a wide market distribution. Therefore, the storage of a COP syringe in a gas tight aluminum pouch, which would also offer protection against light, might be an inexpensive alternative. The pouch could be filled with nitrogen as performed in this study. Next, labeling of the syringe is required anyway, hence oxygen-tight labels could be used as another rather cheap option. It should further be considered that oxygen permeation might have such a low impact on the stability of some proteins that none of these modifications are necessary. For the first time, in this study two highly relevant therapeutic proteins were stored in five different syringe systems evaluating the suitability of the polymer syringes for the long-term storage of biopharmaceuticals. Their chemical and physical stability was determined at three different temperatures during a period of up to 48 weeks.

4.2. Materials and Methods

4.2.1 Materials

Sodium chloride pure and glacial acetic acid 100 % were purchased from Bernd Kraft GmbH (Duisburg, Germany). Di-sodium hydrogen phosphate dihydrate p. A., sodium dihydrogen phosphate dehydrate p. A. and L-Arginine base pure Ph.Eur., USP were acquired from AppliChem GmbH (Darmstadt, Germany). Sodium acetate, 99 %, anhydrous, p. A. was used from Grüssing GmbH (Filsum, Germany). Trisodium citrate, anhydrous, 99 % was purchased from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). Citric acid monohydrate AnalaR Normapur® derived from VWR BDH Prolabo® (Leuven, Belgium). Polysorbate 80 was obtained from Fluka® Analytical (Buchs, Switzerland). Glycine ReagentPlus® ≥99 (TLC) was purchased from Sigma Life Sciences (Taufkirchen, Germany) and mannitol was obtained from Boehringer Ingelheim (Ingelheim, Germany). Hydrochloric acid, fuming 37 % and sodium hydroxide 32 % were obtained from Brenntag GmbH (Mühlheim an der Ruhr, Germany). Highly purified water was used for the preparation of buffers and mobile phases or for system cleanliness checks.

The following steps were carried out according to aseptic manufacturing rules and under a laminar air flow system. A monoclonal antibody (mAb) with a concentration of 1 mg/mL was formulated in a buffer containing NaCl (105.5 mM), NaH_2PO_4 x 2 H_2O (5.5 mM), Na_2HPO_4 x 2 H_2O (8.6 mM), sodium citrate (1.2 mM), citric acid x H_2O (6 mM), mannitol (6.6 mM) and 0.1 % polysorbate 80 at a pH of 5.2. The buffer for a highly potent cytokine, which was used in a concentration of 84 µg/mL, had a pH of 7.2 and comprised NaCl (75 mM), NaH_2PO_4 x 2 H_2O (7.5 mM), Na_2HPO_4 x 2 H_2O (12.5 mM), glycine (66.6 mM) and 0.03 % polysorbate 80. Used protein solutions and buffers were 0.2 µm filtered. 1 mL protein solution was filled into siliconized BD Hypak SCF™ (BD, Franklin Lakes, New Jersey, USA) or Gx RTF® (Gerresheimer Bünde GmbH, Bünde, Germany) glass syringes or silicone oil free cyclic olefin polymer (COP) syringes (Daikyo Crystal Zenith® Polymer Ready-to-Use syringes, West Pharmaceutical Services, Inc., Lionville, PA, USA). COP syringes were used as supplied and stored as follows. Version A (COP-Open): no further measures taken; Version B (COP-Pouch): storage of the COP syringes in nitrogen (≥ 90 %) filled aluminum pouches (Drylok 3000, Advantek GmbH, Freiburg, Germany); Version C (COP-Label): labeling of the COP syringes with a high oxygen barrier property label (Ceramix® CPT 001 SiOx-coated PET, Tovenca, Ebmatingen, Switzerland). The syringes were individually stoppered by hand. Hereby, after filling 1 mL, trapped air was eliminated through the fluid path. Through this approach a large headspace could be avoided to allow studying the direct impact of the syringe barrel on protein stability without the additional influence of an air bubble. FluroTec® stoppers (West Pharmaceutical Services, Inc., Lionville, PA, USA) were used for all setups. The setups were stored at 4 °C and 25 °C for 48 weeks and at 40 °C and

at 75 % relative humidity for 24 weeks. For the analysis the syringe content was ejected like during a drug administration and the contents of five syringes were pooled. Four of these pools of each syringe packaging configuration and condition were analyzed with different methods. The results are presented as mean ± standard deviation.

4.2.2 Methods

4.2.2.1 Visible particles

Syringes were inspected for visible particles before liquid ejection in front of a matt black and white plate with a light source as described in the European Pharmacopoeia method.

4.2.2.2 Dynamic Light Scattering (DLS)

DLS was carried out with a Zetasizer APS (Malvern Instruments, Worcestershire, UK) to determine the particle size and size distribution in the nanometer range. 200 µL protein solution were measured in duplicates at 20 °C. For each packaging configuration four pools were analyzed in duplicates resulting in a total of eight measurements per syringe packaging configuration. Z-Average value states the particle size and the particle size distribution is represented by the Polydispersity Index.

4.2.2.3 Micro-Flow Imaging™ (MFI)

To evaluate the particle count a Micro-Flow Imaging DPA 4100 device (BrightWELL Technologies Inc., Ottawa, Canada) with a 100 µm flow cell in the operation mode “high magnification” was used. The MFI™ Particle Analyzer V6.9.7.2 software was applied for the analysis. Highly purified water was used for flow cell cleaning. Between each run the flow cell cleanliness was checked. The “optimize illumination” process, which ensures a correct system threshold, was carried out before each sample run by flushing the flow cell with 0.5 mL of the appropriate protein solution buffer. Prior to the analysis of 0.65 mL sample for particle count and characterization the cell was flushed with 0.35 mL sample. A flow rate of 0.1 mL/min was applied for the sample run. Four pools of each syringe packaging configuration were measured. For the system calibration Duke Standards™ (10 µm) and Count-Cal™ (5 µm) Particle Size Standards (both Thermo Scientific, Fremont, CA, USA) were used.

4.2.2.4 Chromatography Methods

Mobile phases were prepared with highly purified water or were purchased in HPLC grade and 0.2 µm filtered. All samples were centrifuged at 14,000 rpm for 10 min before analysis. Three different high-performance liquid chromatography systems (HPLC) were used. The absorbance of the mAb was monitored at 280 nm using a Waters 2695 Alliance Separation Module (Waters Corporation, Milford, MA, USA) with a Waters 2487 Dual λ Absorbance Detector. A Dionex Summit system with an ASI 100 autosampler, a RF 2000 Fluorescence (extinction: 295 nm; emission: 343 nm for the cytokine) and a UVD170U detector (280 nm for the mAb) (all Thermo Fisher Scientific GmbH, Idstein, Germany) were used for both proteins. A Dionex Ultimate 3000 system with a fluorescence detector with the same wavelengths as stated before was used for the cytokine analysis. Data analysis was carried out with Chromeleon V6.8. Each pool was injected twice resulting in a total of eight runs per syringe setup and condition.

4.2.2.4.1 High-Performance Size Exclusion Chromatography (HP-SEC)

The amount of soluble protein species such as native, fragmented or aggregated protein was determined with HP-SEC.

Cytokine: The mobile phase consisted of 300 mM sodium chloride and 50 mM phosphate pH 7 in highly purified water. A flow rate of 0.5 mL/min was applied and a TSKgel® 3000SWXL column was used (Tosoh Bioscience GmbH, Stuttgart, Germany). Run time was 30 min.

mAb: A buffer with 20 mM phosphate and 150 mM sodium chloride at a pH of 7.5 was used as mobile phase. Separation was performed for 1 h on a Superose™ 6, 10/300 GL column (GE Healthcare, Uppsala, Sweden) with a 0.5 ml/min flow rate.

4.2.2.4.2 High-Performance Ion-Exchange Chromatography (HP-IEX)

Chemical modifications of the proteins were detected by HP-IEX.

Cytokine: A 20 mM Tris buffer pH 7.5 (mobile phase A) and mobile phase A plus 500 mM sodium chloride pH 7.5 (mobile phase B) were used. A constant gradient from 5 % to 40 % B over a time range of 50 min, followed for 2 min at 100 % B and a reduction to 5 % B over 2 min, which was kept for 5 min, was applied with a flow rate of 0.5 mL/min. A YMC BioPro QA column (YMC, Kyoto, Japan) was used.

mAb: A 10 mM phosphate buffer pH 7.5 as mobile phase A and mobile phase A plus 0.5 M sodium chloride pH 5.5 as mobile phase B were used. Using a ProPac™ WCX-10G (BioLC™ Guard, 4 x 50 mm) and a ProPac™ WCX-10 (BioLC™ Analytical, 4 x 250 mm) (both Thermo Fisher Scientific, Rockford, USA) column for separation with a flow rate of 1 mL/min, the

share of B was increased from 6 % to 16 % within 20 min before 100 % was reached within 2 min. 100 % B was kept constant for 4 min before it was reduced again to 6 % within 2 min.

4.2.2.4.3 Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) – Cytokine only

RP-HPLC was used to determine the share of chemically modified cytokine. Mobile Phase A consisted of 90 % highly purified water, 10 % HPLC grade acetonitrile (Acetonitrile, HiPerSolv CHROMANORM® Super gradient for HPLC, VWR BDH Prolabo® Chemicals, Ismaning, Germany) and 0.1 % trifluoroacetic acid (w/w) (Trifluoroacetic acid, reagent grade 99 %, Sigma-Aldrich Chemie Gmbh, Munich, Germany) whereas mobile phase B was made up of 100 % HPLC grade acetonitrile and 0.1 % trifluoroacetic acid (w/w). Separation was achieved by using a YMC-Pack C4 (250 x 4.6 mmI.D. S-5µm, 30 nm) column (YMC, Kyoto, Japan) at a flow rate of 0.5 mL/min with the following flow gradient: from 2 min to 25 min a linear gradient from 0 % to 100 % B, followed by 5 min 100 % B and within 1 min back to 0 % B. The column oven temperature was set to 37 °C.

4.2.2.4.3 Protein A Chromatography – mAb only

mAb oxidation was monitored by Protein A Chromatography. Mobile phase A contained 20 mM phosphate with a pH of 7.4, whereas mobile phase B comprised additional 150 mM sodium chloride at a pH of 2.9. From 3 min to 23 min the share of B was increased from 0 % to 40 %, and then to 100 % B within 5 min and kept constant for 10 min. Column equilibration with A was performed before and after the increase of B. A Poros A column (2.1 mm x 30 mm, Applied Biosystems, Framingham, MA, USA) and a flow rate of 0.5 mL/min were employed for separation.

4.2.2.5 Oxygen quantification

The concentration of soluble oxygen within the stored protein solution in the syringes was monitored using a Microx 4 stand-alone fiber optic oxygen meter (PreSens Precision Sensing GmbH, Regensburg, Germany). The tip cap of the syringe was removed and the sensor was inserted via the syringe bore into the protein solution, before the measurement was started. For each syringe packaging configuration twelve measurements were performed. The device was further used to measure the oxygen content in the nitrogen filled aluminum pouches.

4.2.2.6 Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) – Cytokine only

To analyze traces of tungsten in glass syringes a Varian Vista RL CCD simultaneous ICP AES Vista RL radial device from Varian (nowadays Agilent Technologies, Santa Clara, CA, USA) was applied. 3 mL protein solution were mixed with 0.24 mL 69 % nitric acid and double distilled water was added to a final volume of 8 mL before thermal treatment. System calibration was performed with a tungsten standard from Merck KGaA (Darmstadt, Germany). 2 to 3 emission wavelengths were chosen for each element. Each sample was analyzed in triplicates resulting in a mean value of twelve independent measurements for a single syringe setup. The following instrument settings were applied: performance: 1.2 kW; nebulizer pressure: 185 kPa; pump speed: 17 rpm; plasma flow: 13.5 l/min; auxiliary flow: 1.5 l/min; sample uptake time: 31 s; instrument stabilization time: 46 s; measurement time: 9 s. The maximum percentage variance is 5 % and the minimum correlation coefficient is 0.995.

Samples were measured by Jaroslava Obel from the analytical division of the Department of Chemistry and Pharmacy at the Ludwig-Maximilians-Universität Munich. Method development was done in close collaboration between Jaroslava Obel and Benjamin Werner.

4.2.2.7 Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS was performed to identify protein modifications like molecular oxidation of the two proteins. The formulation buffer of both proteins was exchanged for a 25 mM phosphate buffer pH 7.25 via centrifugation at 14,000 rpm for 12 min using Amicon® ultra-0.5 centrifugal filter devices (Millipore Corp. Bedford, MA, USA). This step was repeated twice. DL-Dithiothreitol (DL-Dithiothreitol (≥98% (HPLC), ≥99.0% (titration), Sigma-Aldrich Corporation, St. Louis, MO, USA) was added resulting in a final concentration of 10 mM in the mAb solution. Before LC-MS analysis the solution was again centrifuged at 14,000 rpm for 5 min. For the deglycosylation of the cytokine the enzyme PNGase F was applied overnight at room temperature and DL-Dithiothreitol (10 mM final concentration) was added directly before the analysis. LC-MS analysis was carried out on an Agilent 1200 series LC system and an Agilent 6520 Quadrupole Time-Of-Flight system (Agilent Technologies, Santa Clara, CA, USA). Analysis was performed as described by Okbazghi et al. [27].

Sample preparation was performed by Benjamin Werner. Together with Ishan Shah (Department Pharmaceutical Chemistry, University of Kansas, KS, USA) the samples were run on the LC-MS device.

4.2.2.8 4-(aminomethyl) benzenesulfonic acid (ABS) assay

For the detection of tyrosine and phenylalanine oxidation an ABS assay was carried out. ABS was synthesized as described by Sharov et al. [28]. The cytokine was concentrated and dialyzed against a 100 mM phosphate buffer pH 8.8 using Amicon® ultra-0.5 centrifugal filter devices (Millipore Corp. Bedford, MA, USA). The concentrated cytokine solution as well as the mAb solution were mixed with $K_3Fe(CN)_6$ (Potassium ferricyanide(III), powder, <10 micron, 99+%, A.C.S. reagent, Sigma-Aldrich Corporation, St. Louis, MO, USA) in a ratio of 1:15 and a final ABS concentration of 10 mM. 100 mM phosphate buffer pH 8.8 was added to ensure reaction conditions. As a positive control protein solutions were stressed with 10 mM 2,2-azobis(2-methylpropionamidine) dihydrochloride (Sigma-Aldrich Corporation, St. Louis, MO, USA) for 3 h at 37 °C. The benzoxazole fluorescence of the ABS-derivatized protein samples was quantified using a SpectraMax Gemini XS plate reader (Molecular Devices, Sunnyvale, CA) and the software SoftMax® Pro 4.8. The compounds were excited at a wavelength of 360 nm and the emission maximum at 520 nm was used for the analysis. Measurements were carried out in triplicate for the mAb solutions, whereas the cytokine samples were measured once.

Rupesh Bommana and Christian Schöneich (both Department Pharmaceutical Chemistry, University of Kansas, KS, USA) helped to establish the assay for the cytokine and the mAb.

4.2.2.9 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To detect protein fragments and aggregates non-reducing, denaturing SDS-PAGE was performed. NuPage® Novex® 4 - 12 % Bis-Tris protein gels, NuPage® MOPS SDS Running Buffer (20x) and Mark12™ Unstained Standard were used for the cytokine. NuPage® Novex® 3 - 8 % Tris-Acetate protein gels, NuPAGE® Tris-Acetate SDS Running Buffer (20x) and HiMark™ Unstained Protein Standard were applied for the mAb. Further, NuPage® LDS Sample buffer (4x), SilverXpress® Silver Staining Kit (all substances, Novex® by Life Technologies, Carlsbad, CA, USA), glacial acetic acid 100 % and methanol (Methanol HPLC grade, Fisher Scientific UK, Loughborough, UK) were used for the analysis. mAb was diluted with LDS Sample Buffer (4x) to a concentration of 37.5 µg/mL, whereas the cytokine had a final concentration of 67.2 µg/mL. Samples were denatured at 90 °C for 5 min. 12 µL of each sample were loaded per lane. Markers were prepared according to manufacturer instructions. Gels were run in a XCell SureLock™ Mini-Cell Electrophoresis System (Novex® by Life Technologies, Carlsbad, CA, USA) with the corresponding running buffers at a constant voltage of 200 V (cytokine) or 150 V (mAb) using a Bio-Rad PowerPac 200 (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were stained with the SilverXpress® Silver Staining Kit according to manufacturer instructions.

4.3. Results

Taking the lower gas barrier properties of COP into consideration, COP syringes with no measures (COP-Open), COP syringes stored in nitrogen filled aluminum pouches (COP-Pouch) and COP syringes with an oxygen-tight label (COP-Label) were compared to two commonly used, siliconized glass syringes (BD Hypak SCF™ and Gx RTF®). The physical and chemical stability of a mAb and a cytokine stored in these five packaging configurations was evaluated by visual inspection, MFI, DLS, HP-SEC, SDS-PAGE, HP-IEX, Protein A Chromatography, RP-HPLC, ABS assay, ICP-OES and LC-MS at 4 °C and 25 °C for 48 weeks and at 40 °C for 24 weeks. The dissolved oxygen concentration of the protein solutions within the syringes was determined as well. The results are presented in the following first for the mAb for the three temperatures 4 °C, 25 °C and 40 °C and then in the same order for the cytokine. The sections have all the following structure: each starts with the results of the physical protein stability, followed by the oxygen quantification and continues with the results of the methods detecting chemical degradation.

The protein solutions within the syringes were inspected for visible particles. In the whole study period no visible particles were detected for both proteins at all storage conditions.

4.3.1 Results of the mAb stored at 4 °C

Subvisible particle counting was performed with MFI. For the mAB solution the glass syringes showed overall higher particle counts compared to the COP syringes. Hereby, BD Hypak SCF™ syringes had a starting value of 6,440 particles/mL, followed by Gx RTF® syringes with 3,224 particles/mL, whereas the COP syringes had a particle burden of 531 particles/mL for the mAb (Figure 1A). At 4°C the particle count increased over storage time for all syringes to values of 7,339 particles/mL for BD Hypak SCF™, 4,330 particles/mL for Gx RTF®, 1,336 particles/mL for COP-Open, 2,087 particles/mL for COP-Pouch and 1,113 particles/mL for COP-Label syringes (Figure 1A). DLS showed no increase in the hydrodynamic radius of the protein (data not shown). The monomer content was slightly reduced after 48 weeks with the highest loss observed in the BD Hypak SCF™ syringes with 3.6 %, whereas only 2.6 % of monomer content were lost in COP syringes (data not shown) as assessed by HP-SEC. SDS-PAGE revealed no differences between the syringe packaging configurations (data not shown). Oxygen quantification, Protein A Chromatography, ABS assay and LC-MS were applied to detect in particular oxidative protein modifications due to a possible impact of the oxygen barrier property of the syringe barrel material. First, the dissolved oxygen content was measured in the protein solution within the syringe before expelling the solution. The oxygen saturation was between 19 % and 20.5 % for all packaging configurations at the beginning (Figure 1B). The amount of

dissolved oxygen remained similar for glass, COP-Open and COP-Label syringes over a period of 48 weeks. In contrast, the amount of dissolved oxygen was reduced to around 14.4 % in the COP-Pouch syringes (Figure 1B). HP-IEX revealed a rise of the amount of non-native species from around 10.9 % to around 15.46 % for all syringe packaging configurations after 48 weeks (Figure 1C). With Protein A Chromatography and the ABS assay no oxidation was observed (Figure 1D and 1E). In contrast to the other methods, which detected no or only low oxidative modifications, LC-MS analysis revealed that already 20 % of the mAb were oxidized at the beginning of the study (Figure 1F). The relative amount of oxidized protein increased steadily to 28.2 % for both glass syringes. All COP syringes showed higher amounts of oxidized protein with 33.7 % for COP-Open and 37.6 % for COP-Label syringes. COP-Pouch syringes had only 31.4 % of oxidized species. Oxidation occurred in the heavy chains and no changes were observed in the light chains. The mean mass difference between the native and oxidized species for the samples stored up to 1 year was 15.77 Da. This indicated that besides a methionine oxidation with a mass difference of + 16 Da a tyrosine oxidation product with a mass of tyrosine + 14 Da was present.

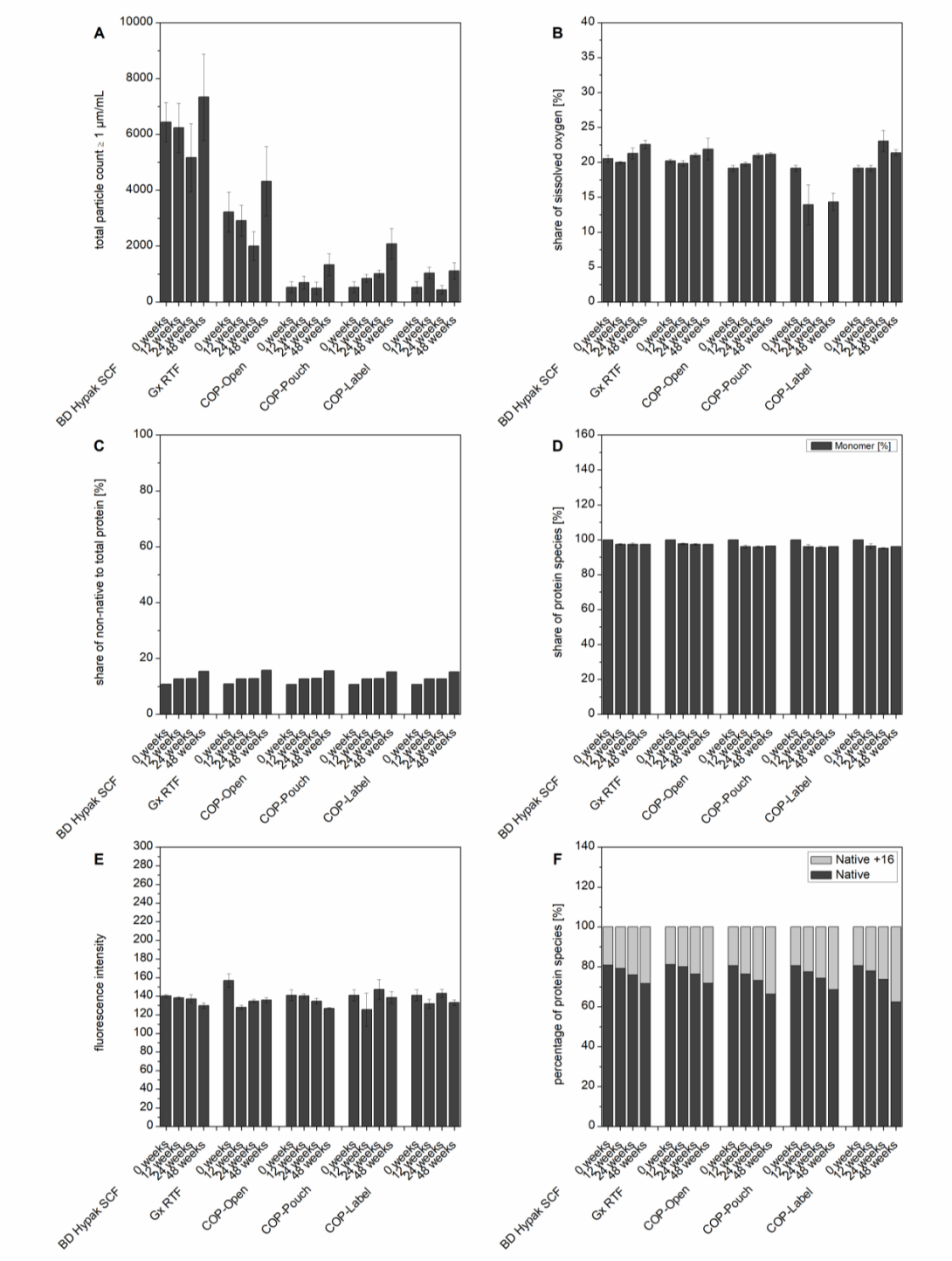

Figure 1: The results of six methods characterizing the mAb solution stored at 4°C for 48 weeks within five different syringe packaging configurations are displayed. The total particle count/mL (particles/mL ≥ 1 µm) was obtained by MFI (**A**). The oxygen saturation of the protein solution within the syringe setups is shown in (**B**)*. Chemical or oxygen specific modifications were determined and quantified with HP-IEX (**C**), Protein A Chromatography (**D**), ABS assay (**E**) and LC-MS (**F**). Oxidation of the mAb (Native + 16) was detected by LC-MS. *The COP-Pouch 24 week value is not shown as it turned out to be an artifact.

4.3.2 Results of the mAb stored at 25 °C

At a storage temperature of 25 °C the subvisible particle count showed in general slightly higher values for the mAb after 48 weeks than at 4 °C. Only the particle count in COP-Label syringes was lower with 899 particles/mL (Figure 2A). No changes were observed with DLS (data not shown). Monomer recovery, as determined with HP-SEC, was lower in glass syringes than in COP syringes (data not shown). Only after 48 weeks fragmentation of the mAb was observed for all syringe systems with approximately 1.5 %. The occurrence of a slight fragmentation was confirmed by SDS-PAGE (data not shown). The measurements of dissolved oxygen content led to similar results as described for 4 °C. COP-Pouch syringes showed an oxygen saturation of around 15.1 %, whereas the other syringe packaging configurations had at least values of 18.4 % (Figure 2B). A 4.2 fold higher amount of non-native protein in comparison to the beginning was detected by HP-IEX for all syringe systems (Figure 2C). Methionine oxidation was detected with Protein A Chromatography (Figure 2D). Hereby, the highest amount of oxidized methionine with around 2 % was measured in the COP-Open and COP-Label syringes. To a lower extent the oxidation was also observed in the other syringe packaging configurations with the COP-Pouch syringe performing nearly as good as the BD Hypak SCF™ (0.32 % vs. 0.16 %). An increase in the fluorescence signal by the ABS assay, indicating a higher level of tyrosine and phenylalanine oxidation was not observed (Figure 2E). Major changes in the amount of oxidized protein became visible applying LC-MS analysis (Figure 2F). Roughly 41 % of the mAb in glass syringes and 48 % of the mAb in COP-Pouch syringes were oxidized in the heavy chain after 48 weeks. For COP-Open and COP-Label syringes 49.4 % of the mAb showed a single oxidation and 34 % of the mAb were even oxidized twice. As observed at 4 °C the native and oxidized species were separated by a mean value of 15.83 Da indicating the presence of oxidized tyrosine + 14 Da. However, after 48 weeks for COP-Open and COP-Label syringes a mass difference of around 17.4 Da was obtained indicating a shift towards an oxidized tyrosine + 18 Da molecule. The mean mass difference between the single and double oxidized mAb was around 1 Da higher than between the native and single oxidized mAb. Oxidized methionine, also detected with Protein A Chromatography, and an oxidized tyrosine + 18 Da derivate were dominating.

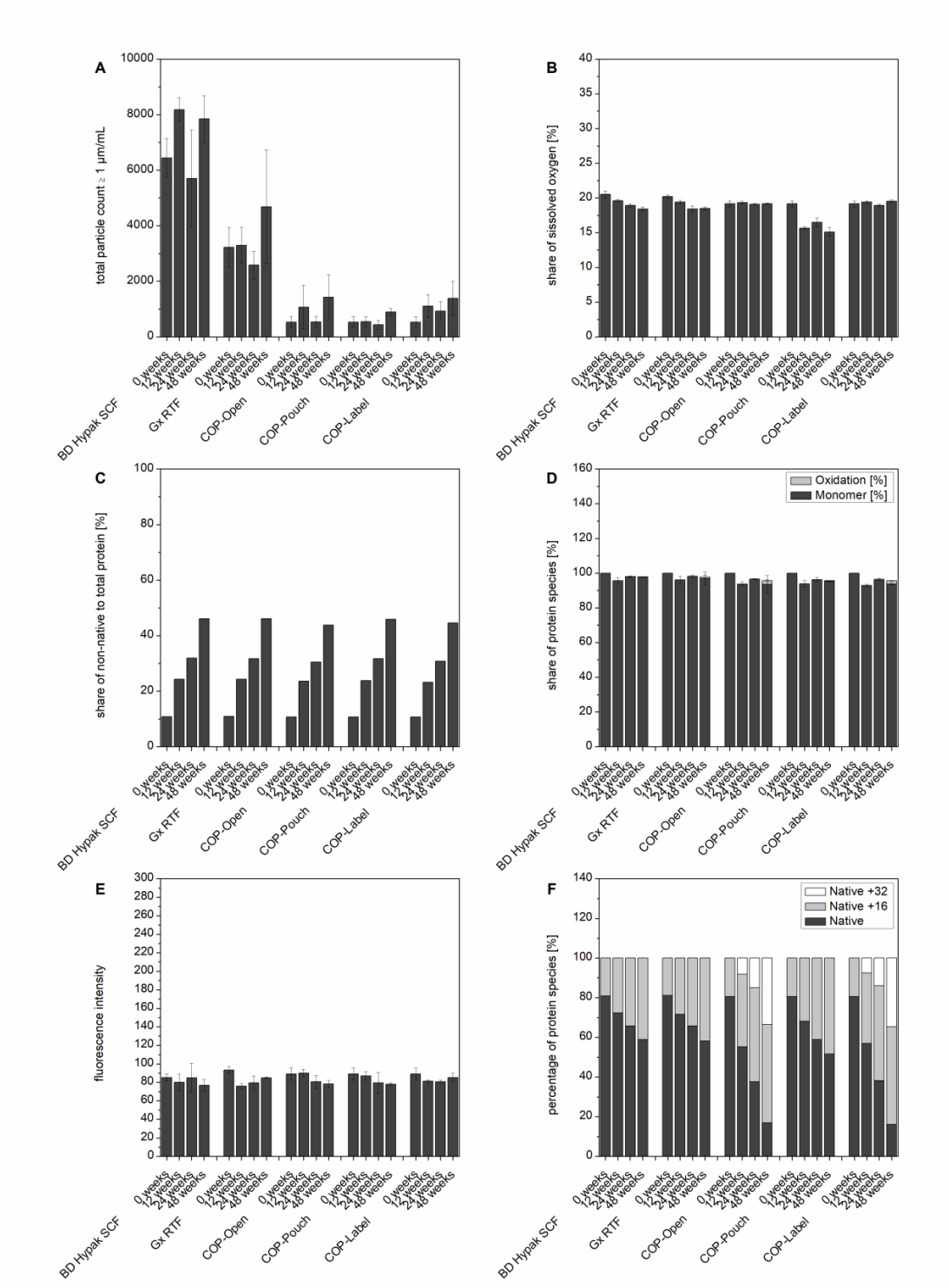

Figure 2: mAb stability at 25 °C for 48 weeks was assessed in five different syringe packaging configurations. MFI measurements revealed the total particle count/mL (particles/mL ≥ 1 µm) (**A**), whilst the determined amount of dissolved oxygen is shown in (**B**). HP-IEX (**C**), Protein A Chromatography (**D**), ABS assay (**E**) or LC-MS (**F**) were applied to evaluate the amount of modified protein species. Single and double oxidations of the mAb (Native + 16 and Native + 32, respectively) were detected by LC-MS.

4.3.3 Results of the mAb stored at 40 °C

Storing the mAb at 40 °C for 24 weeks, as an accelerated stress study, confirmed the results obtained at lower temperatures and longer storage time. In detail, the particle count doubled for BD Hypak SCF™ syringes to 13,069 particles/mL, whereas the particle numbers of the other syringe packaging configurations were similar to those observed at 25 °C after 48 weeks (Figure 3A). DLS showed no changes in the hydrodynamic radius of the protein (data not shown). mAb fragmentation was already detected after 12 weeks with around 3 % in all cases but slight differences between the syringe packaging configurations were visible. After 24 weeks the lowest amount of fragmentation was observed for COP-Pouch syringes (3.86 %), followed by Gx RTF® (3.95 %), BD Hypak SCF™ (4.08 %), COP-Label syringes (4.08 %) and finally COP-Open syringes with 4.38 %. Protein recovery was slightly higher for COP syringes in comparison to glass (data not shown). No differences between the syringe systems were detected by SDS-PAGE (data not shown). Dissolved oxygen level remained similar for all syringe packaging configurations except for COP-Pouch syringes (19.1 % vs. 5.1 %) and COP-Label syringes (19.1 % vs. 15.3 %) (Figure 3B). Chemical modifications of the mAb occurred as identified with HP-IEX (Figure 3C). A 6.34 fold increase of the ratio of native to oxidized mAb was noted for COP-Open syringes. In contrast, COP-Pouch syringes had a ratio of 5.86 %, followed by BD Hypak SCF™ (5.91 %), COP-Label (5.96 %) and Gx RTF® (6.0 %) syringes. After a storage time of 24 weeks methionine oxidation was detectable in all syringe systems (Figure 3D). However, the extent was very low in glass syringes (< 1 %) and in COP-Pouch syringes (1.7 %). In contrast, 9.2 % and 6.6 % of the mAb showed methionine oxidation in COP-Open and COP-Label syringes. The ABS assay also indicated oxidation of tyrosine and phenylalanine over the storage time of 24 weeks (Figure 3E). The data correlated with results obtained by Protein A Chromatography. LC-MS revealed that after 24 weeks the highest amount of native mAb could be found in glass syringes with around 37.5 %, followed by COP-Pouch syringes with 24 % (Figure 3F). COP-Open and COP-Label syringes showed the lowest performance with 90.7 % mAb oxidized. Single oxidation was predominant in all packaging configurations except for COP-Open and COP-Label syringes after 24 weeks of storage. In these two cases the share of double oxidized protein, which occurred in all types of syringes besides single oxidation, made up for 56 %. The mass difference between the native and single oxidized mAb increased with storage time and was principally caused by methionine sulfoxide formation in glass syringes and tyrosine oxidation for the three COP syringe packaging configurations. Whilst in glass and COP-Pouch syringes a reduction of the mass difference between single and double oxidation was observed over time, the values for COP-Open and COP-Label syringes remained nearly constant. All packaging configurations except the Gx RTF® showed values

between 16.6 Da and 16.95 Da for the double oxidation indicating that more methionine than tyrosine oxidized after 24 weeks.

Figure 4 displays mass spectra obtained for the mAb at the different points in time of storage at 40 °C in COP-Open syringes. Besides a varying ratio of mAb to mAb + 16 Da to mAb + 32 Da similar mass spectra were obtained for the other packaging configurations. Mass spectra of mAb stored at 4 °C and 25 °C differed from the shown ones by higher shares of native and single oxidized protein and only for COP-Open and COP-Label syringes double oxidized peaks were observed.

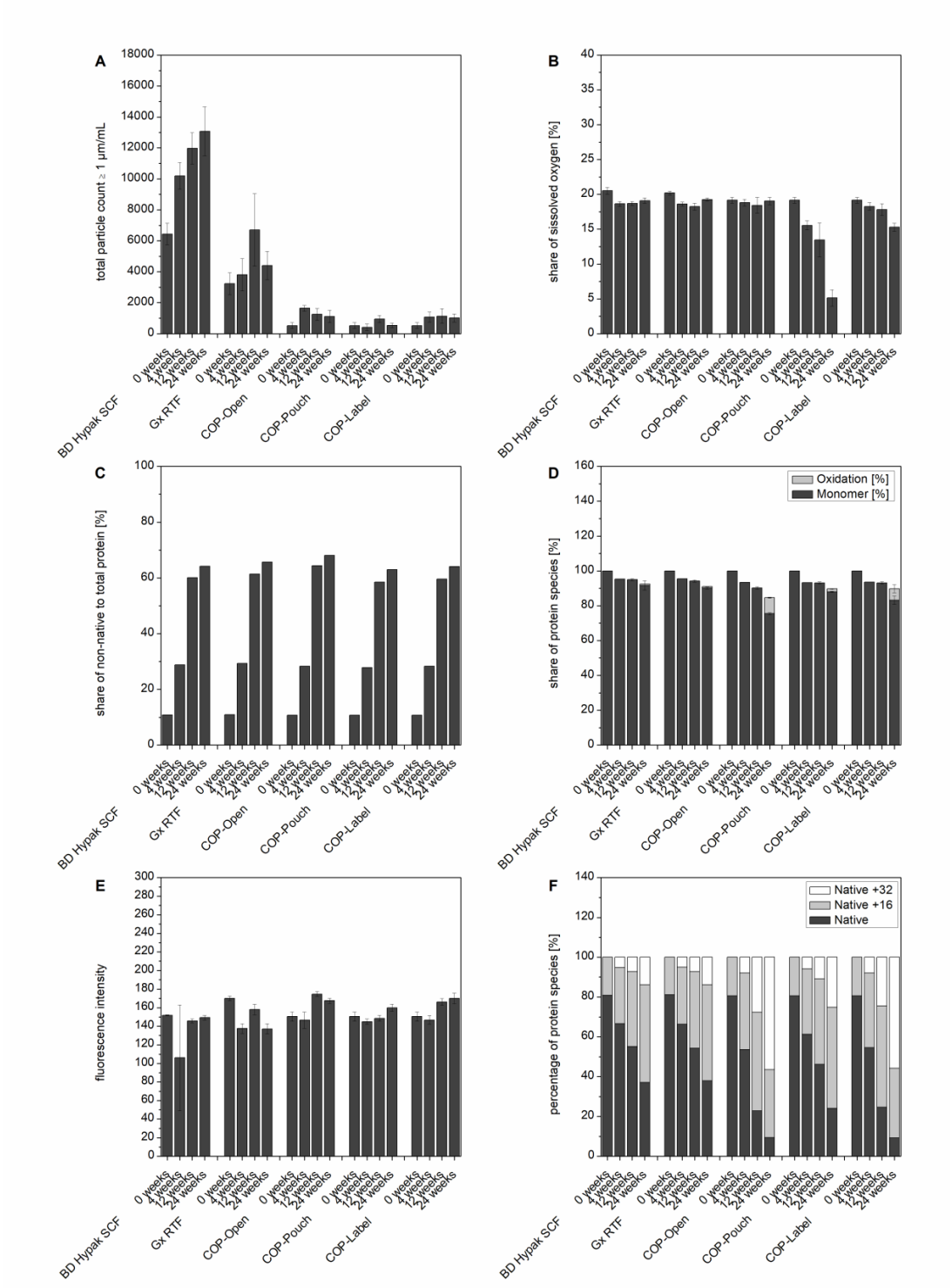

Figure 3: For the mAb stored at 40 °C for 24 weeks in five different syringe packaging configurations the following is shown: the total particle count/mL (particles/mL ≥ 1 µm), the dissolved oxygen content and chemical modifications, as assessed by MFI (**A**), Microx 4 stand-alone fiber optic oxygen meter (**B**), HP-IEX (**C**), Protein A Chromatography (**D**), ABS assay (**E**) and LC-MS (**F**). Single and double oxidations of the mAb (Native + 16 and Native + 32, respectively) were detected by LC-MS.

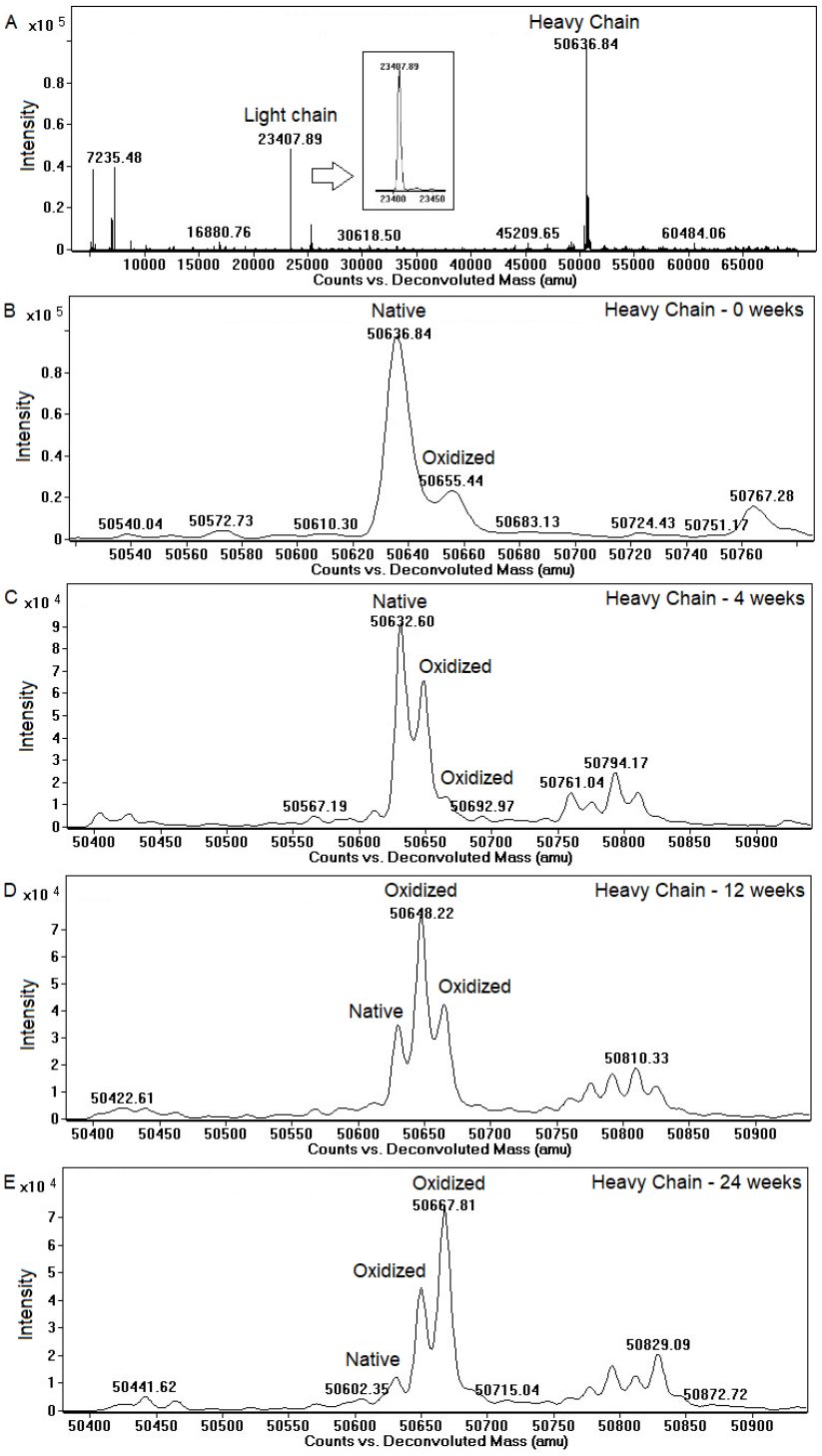

Figure 4: The mass spectra of mAb solution stored in COP-Open syringes at 40 °C over time are displayed. The full mAb mass spectrum with an additional magnification of the light chain (marked with a frame) is presented in (**A**). (**B**) to (**E**) show an enlarged view of the heavy chain at the investigated time points of 0, 4, 12 and 24 weeks. The native and oxidized protein species are indicated.

Table 1 provides an overview of the chemical and physical modifications of the mAb over storage time at the three temperatures.

Table 1: Summary of the mAb changes within the five different packaging configurations over a storage period of up to 48 weeks and at 4 °C, 25 °C and 40 °C as detected with various techniques

	BD Hypak SCF™	Gx RTF®	COP-Open	COP-Pouch	COP-Label
			4 °C		
Visual	O	O	O	O	O
MFI*	+	+	+	+	+
DLS	O	O	O	O	O
HP-SEC	O	O	O	O	O
SDS-PAGE	O	O	O	O	O
HP-IEX	+	+	+	+	+
Protein A Chromatography	O	O	O	O	O
ABS	O	O	O	O	O
LC-MS	O/+	O/+	+	O/+	+
			25 °C		
Visual	O	O	O	O	O
MFI*	+	+	+	+	+
DLS	O	O	O	O	O
HP-SEC	O/+	O/+	O/+	O/+	O/+
SDS-PAGE	+	+	+	+	+
HP-IEX	+/++	+/++	+/++	+/++	+/++
Protein A Chromatography	O/+	O/+	+	O/+	+
ABS	O	O	O	O	O
LC-MS	+	+	++	+	++
			40 °C		
Visual	O	O	O	O	O
MFI*	++	+	+	+	+
DLS	O	O	O	O	O
HP-SEC	+	+	+	+	+
SDS-PAGE	++	++	++	++	++
HP-IEX	++	++	++	++	++
Protein A Chromatography	O/+	O/+	++	+	++
ABS	O	O	+	O/+	+
LC-MS	+/++	+/++	++	++	++

O = no or minor changes; + = changes; ++ = major changes

* = Highest particle count measured in BD Hypak SCF™ > Gx RTF® >> COP (in general)

4.3.4 Results of the cytokine stored at 4 °C

Similar as observed for the mAb the particle burden of the cytokine solution in glass syringes was higher than in COP syringes from the beginning. BD Hypak SCF™ syringes had the highest particle number with 6,776 particles/mL, followed by Gx RTF® syringes with 2,155 particles/mL, whereas in COP syringes 545 particles/mL were detected (Figure 5A). The storage time influenced the number of particles slightly at 4°C. Higher particle numbers where obtained for Gx RTF® (2,666 particles/mL) and for COP-Open syringes (1,444 particles/mL) (Figure 5A). The protein size, as determined with DLS, was not altered (data not shown). Only the monomer was detected by HP-SEC and SDS-PAGE (data not shown). Similar to the described observations for the mAb, the amount of dissolved oxygen was with 14.4 % the lowest in COP-Pouch syringes, whilst the other syringe packaging configurations showed values above 20 % (Figure 5B). After 24 weeks the share of chemical modified protein ranged between 0.5 % and 0.62 % for the cytokine as monitored by HP-IEX (Figure 5C). With RP-HPLC no altered protein was detected (data not shown). The ABS assay also showed no additional oxidation in comparison to the beginning (data not shown). Glycosylated cytokine displayed a high heterogeneity in LC-MS analysis (data not shown). In order to reduce the heterogeneity N-glycans were removed with PNGase F to allow focusing on the O-glycoforms. O-glycoforms with a composition of $NeuAc_1Hex_1HexNAc_1$ and $NeuAc_2Hex_1HexNAc_1$ were obtained [29], where the latter had a higher prevalence. Peaks with a 16 Da higher mass were observed for the major isoform. LC-MS analysis revealed that approximately 25.5 % of the $NeuAc_2Hex_1HexNAc_1$ isoform were already oxidized at the beginning (Figure 5D). The share of oxidized cytokine remained stable for all syringe systems but one. COP-Label syringes displayed an oxidation rate of 40 % after 48 weeks. Approximately 5 % of the main isoform showed an O-acetylation of probably a sialic acid, identified due to a mass increase of 42 Da [29]. Oxidation was also observed for the $NeuAc_1Hex_1HexNAc_1$ molecule. A low percentage of approximately 1.2 % of the main isoform showed dehydration of aspartic acid. To evaluate any oxidation caused by tungsten ICP-OES was carried out for the glass syringes. After 48 weeks a tungsten concentration of 0.06 ± 0.02 µg/mL for BD Hypak SCF™ and of 0.58 ± 0.08 µg/mL for Gx RTF® was measured.

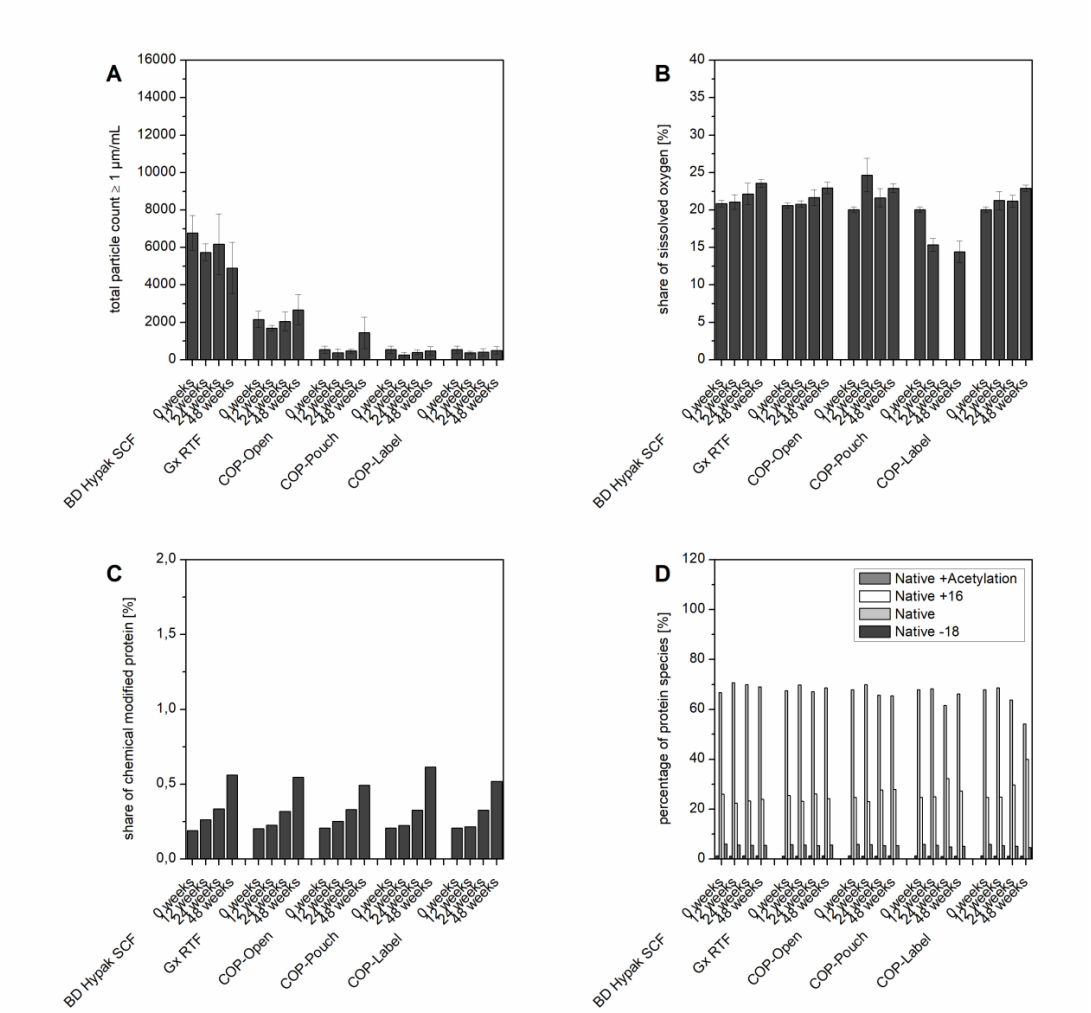

Figure 5: Cytokine stability at 4 °C for 48 weeks in five different syringe packaging configurations was determined. The total particle count/mL (particles/mL ≥ 1 µm) was determined by MFI (**A**). The dissolved oxygen content was measured with a Microx 4 stand-alone fiber optic oxygen meter (**B**)*. The amount of chemically modified protein species was analyzed with HP-IEX (**C**) and LC-MS (**D**). Acetylation (+ 42 Da), oxidation (+ 16 Da) and dehydration (- 18 Da) were detected by LC-MS. *The COP-Pouch 24 week value is not presented, because it turned out as an artifact.

4.3.5 Results of the cytokine stored at 25 °C

At a storage temperature of 25 °C the particle burden of the cytokine solutions were in a similar range at the beginning and at the end of the study (Figure 6A). An increase was only observed for Gx RTF® syringes were the particle number rose from 2,155 particles/mL to 3,854 particles/mL (Figure 6A). DLS and HP-SEC showed no to minor changes during the storage time (data not shown). In HP-SEC aggregate formation with a small fraction of around 0.3 % was detected for all syringe packaging configurations only after a 48 weeks storage time. In contrast, no aggregate formation was observed by SDS-PAGE (data not shown). The same pattern, as described before, was gained by measuring the dissolved oxygen concentration. The lowest value was obtained for COP-Pouch syringes (data not shown). The share of chemical modified protein was around 0.35 % for all packaging conditions after week 24 (Figure 6B). However, after 48 weeks slight differences between glass and polymer syringes were noted by HP-IEX. COP-Open and COP-Label syringes had the highest amount of chemical modified protein with 1.16 % and respectively 1.17 %. The lowest share was detected in BD Hypak SCF™ syringes with 0.96 %, followed by COP-Pouch syringes (1 %) and Gx RTF® syringes (1.14 %). With RP-HPLC the differences were more distinctive than with HP-IEX (Figure 6C). Before week 12 no modifications were obtained. Until week 24 only hydrophilic changes were observed, whereas after 48 weeks both, hydrophilic and hydrophobic changes were detected. RP-HPLC confirmed the results obtained by HP-IEX, as COP-Open and COP-Label syringes had a total share of modified protein species of 1.90 % and 1.81 %, respectively. Glass syringes performed similar (1.48 % BD Hypak SCF™ and 1.46 % Gx RTF®) and COP-Pouch syringes showed the best results with 1.36 % regarding the share of modified protein species. An increase of tyrosine and phenylalanine oxidation was not detected by the ABS assay (data not shown). LC-MS confirmed the results from the other analyses that the amount of oxidized cytokine was the highest in COP-Open and COP-Label syringes (48.0 % and 51.3 %) (Figure 6D). COP-Pouch syringes had a value of 31.2 % of oxidized cytokine. The lowest share of oxidized protein was obtained in glass syringes with approximately 24 %. The amount of acetylated cytokine was slightly reduced from around 6 % at week 0 to a range between 4.8 % and 3.6 % after 48 weeks depending on the amount of oxidized protein. An increase in the dehydration of the aspartic acid in a range from 0.1 % to 0.6 % over 48 weeks was observed. The percentage change refers to the main isoform $NeuAc_2Hex_1HexNAc_1$. The tungsten concentration in BD Hypak SCF™ syringes was 0.06 ± 0 µg/mL and in Gx RTF® syringes 0.59 ± 0.16 µg/mL, similar to the values obtained at 4 °C.

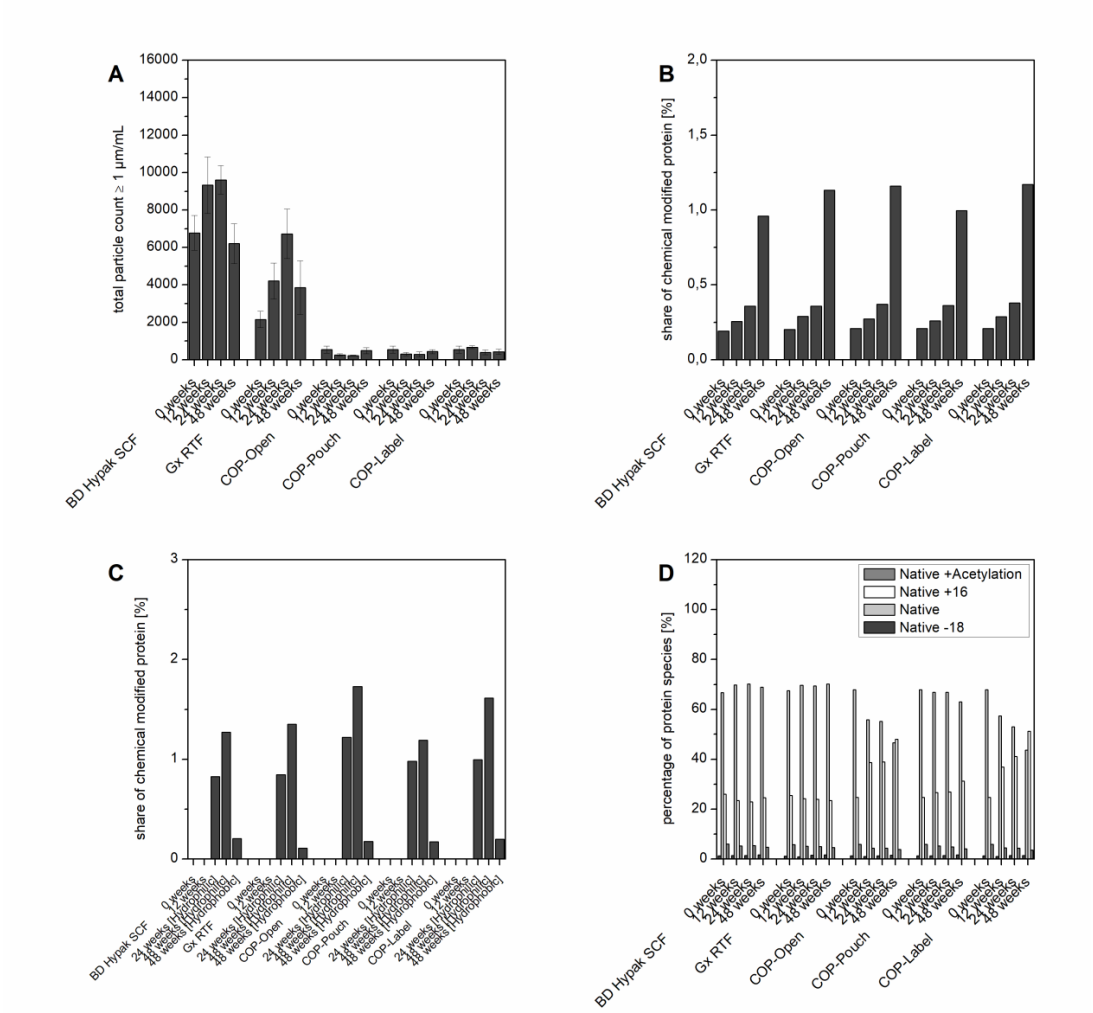

Figure 6: For the cytokine stored at 25 °C for up to 48 weeks in five different packaging configurations data obtained by MFI (**A**), HP-IEX (**B**), RP-HPLC (**C**) and LC-MS (**D**) are shown. Acetylation (+ 42 Da), oxidation (+ 16 Da) and dehydration (- 18 Da) were detected by LC-MS.

4.3.6 Results of the cytokine stored at 40 °C

The storage of the cytokine at 40 °C led to severe modifications of the protein. First, the particle count in COP syringes remained constant over the study period (Figure 7A). On the contrary, the amount of particles in glass syringes significantly increased from 6,776 particles/mL to 11,632 particles/mL in BD Hypak SCF™ syringes and from 2,155 particles/mL to 6,078 particles/mL in Gx RTF® syringes (Figure 7A). Whilst DLS measurements showed little changes over storage time, aggregation was observed from week 12 onwards by HP-SEC (data not shown). At the end the highest share of aggregates in the recovered protein was found for COP-Open syringes (6.4 %). 5.3 % of cytokine

aggregates were formed in Gx RTF® and COP-Label syringes. In BD Hypak SCF™ and COP-Pouch syringes the aggregate level of the recovered protein was at 4.9 %. Besides noting the formation of aggregates, protein loss of up to 45 % of the original protein concentration after 24 weeks was documented as well. In contrast to HP-SEC, aggregation was detected by SDS-PAGE already after 4 weeks (data not shown). Dissolved oxygen concentration was again the lowest in COP-Pouch syringes (10.0 %) (Figure 7B). HP-IEX revealed that the best performances regarding chemical modifications were achieved by COP-Pouch (10.4 %) and COP-Label (11.7 %) syringes (Figure 7C). COP-Open syringes showed the highest share of altered protein with 18.3 %, followed by glass syringes with 15.0 % to 15.5 %. The results were confirmed by RP-HPLC measurements (Figure 7D). Whilst during the first 12 weeks only hydrophilic changes were observed at a level below 5 %, high amounts of hydrophilic and hydrophobic changes were obtained after 24 weeks. In total 28.5 % of the cytokine stored in COP-Open syringes and only 18.1 % of the cytokine stored in COP-Pouch syringes showed chemical modifications. The two glass and the COP-Label syringes were in the range of these values. Although the fluorescence intensity in the ABS assay increased for samples which were stored for 12 or 24 weeks, it was not possible to clearly state, if tyrosine and phenylalanine oxidation occurred because the starting values were too high (Figure 7E). LC-MS provided additional evidence that COP-Pouch syringes were the most suitable packaging condition for the cytokine at 40 °C (Figure 7F). Around 63 % of the main isoform persisted after 24 weeks. The share of oxidized protein remained constant for the whole time at around 25 %, whereas the share of cytokine with a reduced molecular weight of 18 Da increased over time from 1.4 % to 8.9 %. In contrast, COP-Label syringes, performing in HP-IEX and RP-HPLC measurements nearly as good as COP-Pouch syringes, showed with LC-MS similar results as COP-Open syringes. Approximately 43 % of the main isoform and nearly the same amount of oxidized species were detected. Glass syringes preserved around 56 % native cytokine and only a 4 % to 5 % increase of oxidized species was observed. Further dehydration of the cytokine by at least 7.5 % and deacetylation with values of up to 3 % was observed in all packaging configurations.

The cytokine degradation progress over storage time as determined with LC-MS is depicted in Figure 8.

No tungsten was observed in BD Hypak SCF™ syringes, whereas in Gx RTF® syringes a concentration of 0.66 ± 0.22 µg/mL was found.

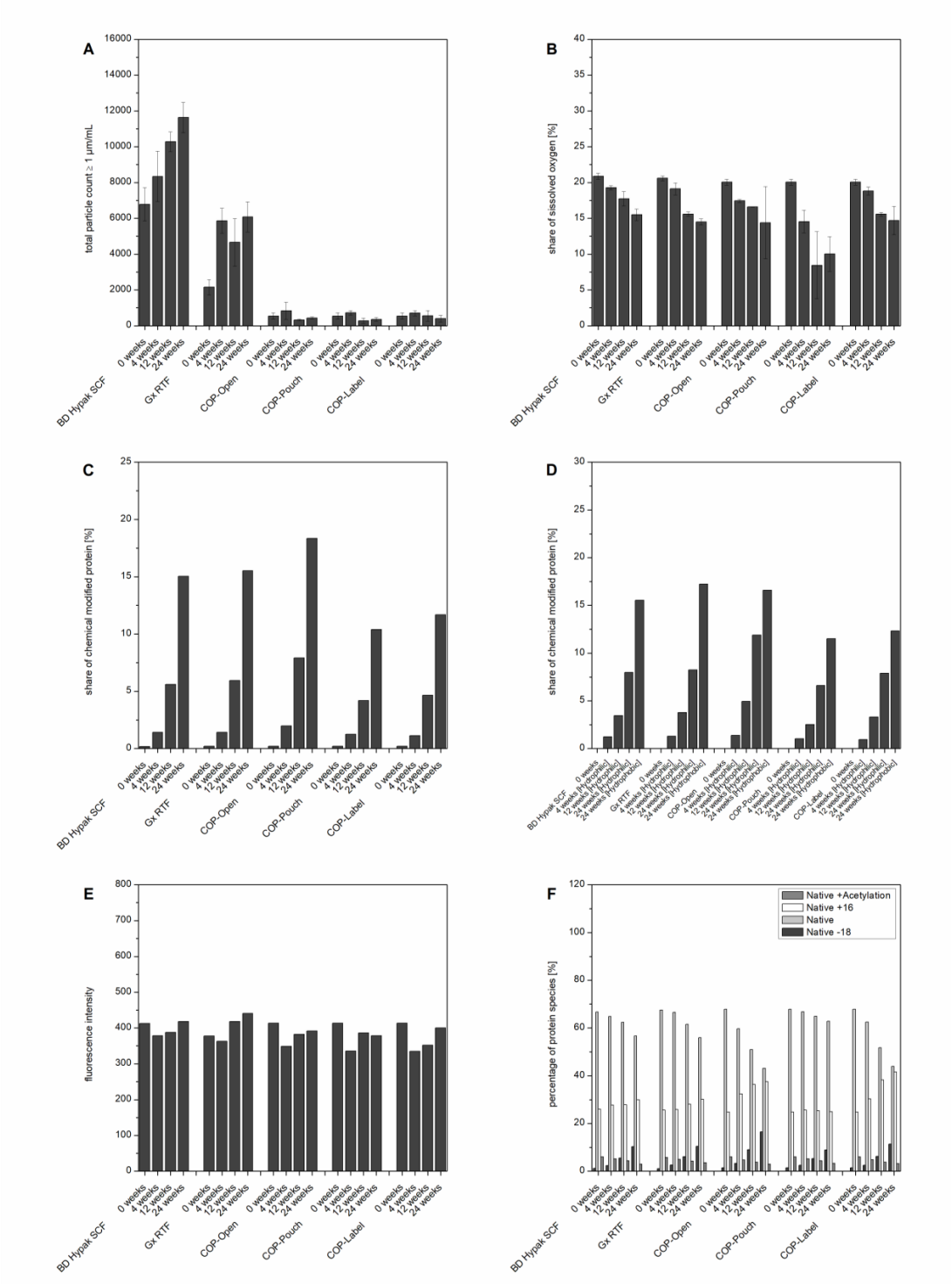

Figure 7: For the cytokine stored at 40 °C for up to 24 weeks in five different packaging configurations the MFI results (total particle count/mL ≥ 1 µm) are shown in (**A**), whilst the amount of dissolved oxygen content is displayed in (**B**). Chemical modifications were determined with HP-IEX (**C**) and RP-HPLC (**D**). Specific oxidation processes were analyzed with the ABS assay (**E**) and LC-MS (**F**). Acetylation (+ 42 Da), oxidation (+ 16 Da) and dehydration (- 18 Da) were detected by LC-MS.

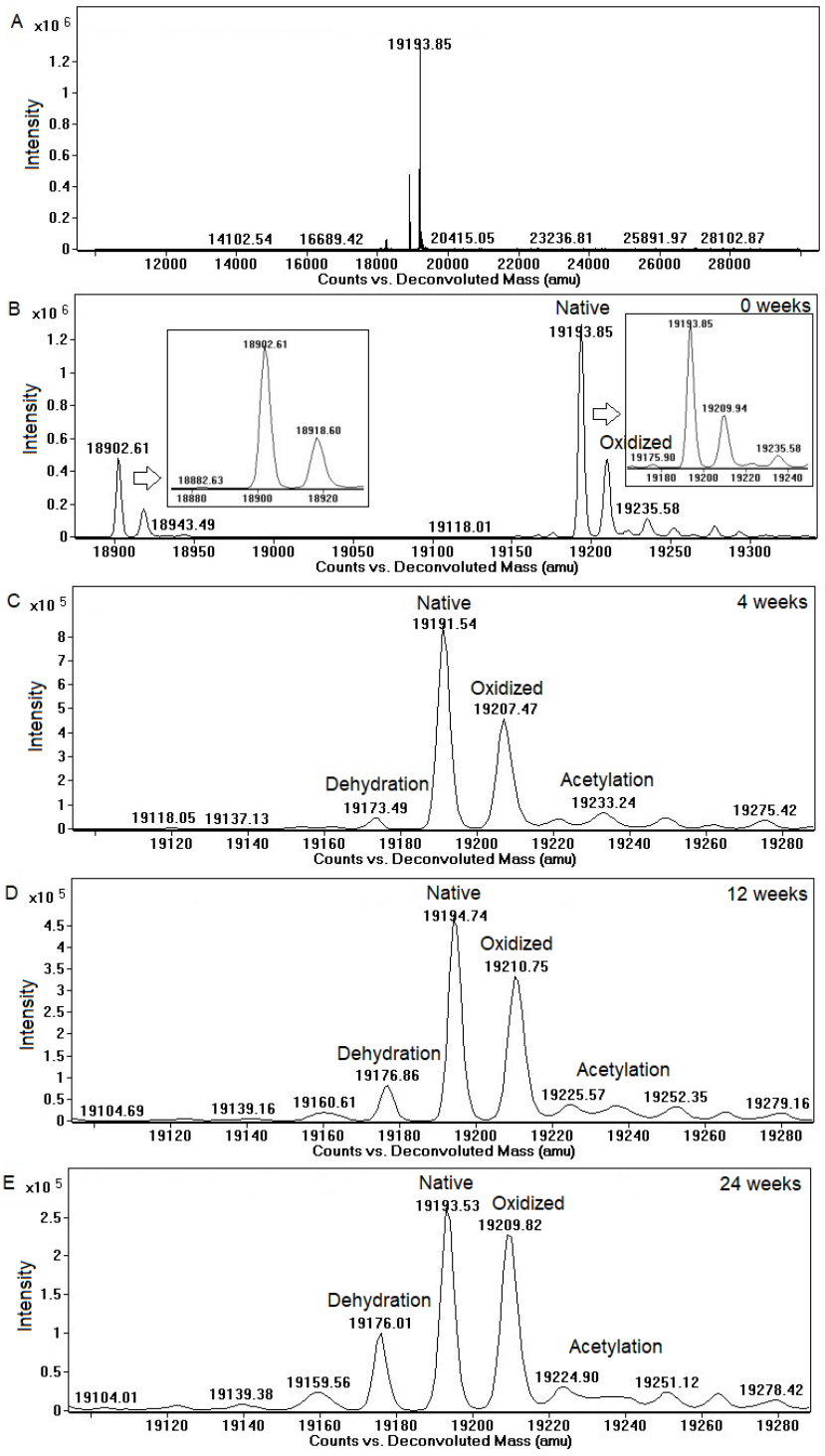

Figure 8: N-deglycosylated cytokine was analyzed with LC-MS. The mass spectrum of the cytokine shows two major isoforms (**A**). These isoforms are enlarged in (B). The molecular changes of the main isoform over storage time at 40 °C in COP-Open syringes are visualized in (**B**) to (**E**). Major peaks are labeled with the cause of the mass difference besides the native protein.

Table 2 summarizes the changes of the cytokine detected with all methods over storage time at all temperatures.

Table 2: Overview of the share of cytokine modifications as detected with various methods over the storage time in the five different packaging configurations

	BD Hypak SCF™	Gx RTF®	COP-Open	COP-Pouch	COP-Label
			4 °C		
Visual	O	O	O	O	O
MFI*	O	O	O	O	O
DLS	O	O	O	O	O
HP-SEC	O	O	O	O	O
SDS-PAGE	O	O	O	O	O
HP-IEX	O/+	O/+	O/+	O/+	O/+
RP-HPLC	O	O	O	O	O
ABS	O	O	O	O	O
LC-MS	O	O	O	O	O/+
			25 °C		
Visual	O	O	O	O	O
MFI*	O/+	+	O	O	O
DLS	O	O	O	O	O
HP-SEC	O/+	O/+	O/+	O/+	O/+
SDS-PAGE	O	O	O	O	O
HP-IEX	+	+	+	+	+
RP-HPLC	O/+	O/+	O/+	O/+	O/+
ABS	O	O	O	O	O
LC-MS	O	O	+/++	O	+/++
			40 °C		
Visual	O	O	O	O	O
MFI*	++	++	O	O	O
DLS	O/+	O/+	O	O	O
HP-SEC	++	++	++	++	++
SDS-PAGE	++	++	++	++	++
HP-IEX	++	++	++	+/++	+/++
RP-HPLC	++	++	++	+/++	+/++
ABS	O/+	O/+	O/+	O/+	O/+
LC-MS	+	+	++	O/+	++

O = no or minor changes; + = changes; ++ = major changes

* = Highest particle count measured in BD Hypak SCF™ > Gx RTF® >> COP (in general)

4.4. Discussion

In this study the suitability of silicone oil free polymer syringes made out of COP for the long-term storage of biopharmaceuticals was evaluated. The impact of the oxygen barrier properties of COP in comparison to glass as syringe barrel material on the physical and chemical stability of two highly relevant therapeutic proteins was assessed. Two secondary packaging concepts to reduce the COP permeability were included in the study as well.
First, the particle concentration within different packaging conditions is discussed. Afterwards, the impact of the packaging material on the amount of dissolved oxygen is commented on. Finally, the protein stability as determined using all methods, in particular with LC-MS, is discussed, starting with the mAb.

Throughout the storage period the particle count showed for all packaging and storage conditions the highest level in glass syringes with values between 3,200 to 13,100 particles/mL, whereas the particle concentration in COP syringes ranged between 200 and 2,100 particles/mL. The elevated particle number in glass syringes could be explained by the presence of silicone oil. The distinctions between the two glass systems are based on the different amounts of silicone oil and siliconization techniques which were applied by the two manufacturers [3]. Overall, our findings are in agreement with other studies investigating silicone oil free syringe systems and syringe systems containing silicone oil [15, 23]. Particles that formed during the storage period of up to 48 weeks can consist of silicone oil microdroplets, protein particles or a combination of both. With the majority of particles having sizes of less than 5 µm, a clear identification of these particles using MFI was not possible. However, at the beginning the differences in the particle counts between glass and COP syringes can be attributed to silicone oil microdroplets.

In all cases an oxygen saturation of around 20 % within the protein solutions was detected at the beginning as expected. In contrast to the other packaging configurations, which remained constant, COP-Pouch syringes showed a maximum value of approximately 15 % oxygen at the end points of all storage conditions. The reason for the reduced value is that COP syringes possess a low oxygen barrier property and oxygen from within the syringe exchanged with nitrogen from the nitrogen filled aluminum pouch. Similar experiences have been made before, e.g. for COP vials [24]. Two other studies took advantage from the COP permeability and used oxygen absorbers within the secondary packaging to control the oxygen level during product storage [16, 26]. Both studies were able to show that the amount of oxidized methionine was reduced in a system consisting of an oxygen absorber and COP syringes [16, 26].

HP-IEX showed for all syringe packaging configurations an increase of chemical modified mAb species by a factor of around 1.4 at 4 °C and around 4.2 at 25 °C. At 40 °C minor differences between the packaging configurations were detectable with a factor of modified protein within the range from 5.85 to 6.34. Whilst differences between the syringe packaging configurations were minor in HP-IEX measurements and changes were not assignable to oxidation processes alone, other techniques not only showed major differences between the systems, but also that these changes were clearly caused by oxidation.

Protein A Chromatography was used to monitor the oxidation of methionine in the Fc region of the mAb, because an oxidation of these methionines resulting in a different binding affinity to Protein A [30, 31]. Methionine oxidation was low at 25 °C (max. 0.85 %) and at most around twice as high at 40 °C for glass syringes and COP-Pouch syringes. Whereas at 25 °C COP-Open and COP-Label syringes performed similar with around 2 % methionine sulfoxide formation, a major difference, not only with regard to the other packaging configurations, but also between COP-Open (6.6 %) and COP-Label (9.2 %) syringes, was noted using Protein A Chromatography at 40 °C. The lower detected amount of methionine sulfoxide formation in COP-Pouch syringes is in good agreement with two reports which use an oxygen absorber within the secondary packaging to prevent methionine oxidation [16, 26].

The data correlated well with LC-MS data, because oxidations were detected in all cases by LC-MS only in the heavy chains of the mAb, as it was observed by Protein A Chromatography, but not in the light chains. Moreover, based on the proposed tyrosine oxidation mechanisms with tyrosine + 14 Da, + 16 Da and + 18 Da products (see Steinmann et al. [32]), it was possible to conclude whether tyrosine or methionine was oxidized the most. Intact mass spectrometry revealed that a combination of tyrosine + 14 Da and methionine oxidation was predominant at 4 °C. Similar results were obtained at 25 °C and 40 °C for glass and COP-Pouch syringes for the single oxidation, whereas tyrosine + 18 Da products were more distinctive for the second oxidation. Tyrosine + 18 Da oxidations were more prevalent for COP-Open and COP-Label syringes for both oxidations in particular at 40 °C. Further, the LC-MS data showed at all temperatures the highest amount of native mAb in the following order: Gx RTF® ~ BD Hypak SCF™ > COP-Pouch > COP-Open ~ COP-Label syringes.

The data obtained by HP-IEX, Protein A Chromatography and LC-MS, showing that COP-Open syringes protected the mAb less from oxidation, was further supported by the ABS assay. After 12 and 24 weeks tyrosine and/or phenylalanine oxidation was detected in COP-Open and COP-Label syringes to a similar extent, whereas for COP-Pouch and Gx RTF® syringes the fluorescence intensity increase occurred to a lower degree or not at all (BD Hypak SCF™). Bommana et al. have shown with LC-MS that a quantitative correlation exists between the amount of oxidized tyrosine and phenylalanine products and methionine

sulfoxide [33]. Based on the results from Bommana et al. it can be stated that there is a strong correlation regarding the mAb oxidation status between the data obtained with ABS and Protein A Chromatography for the syringe systems in the present study.
Overall, the low performance of COP-Open syringes for the long-term storage of the investigated mAb was confirmed by several methods.

Regarding the cytokine only minor changes were observed over the storage period and were comparable for all syringe packaging configurations at 4 °C and 25 °C except for LC-MS. The amount of oxidized cytokine was increased in COP-Open and COP-Label syringes at 25 °C and even at 4 °C for COP-Label syringes. At 25 °C only a slightly higher amount (max. 0.55 %) of chemically modified protein was detected by RP-HPLC for COP-Open and COP-Label syringes in comparison to the other three syringe packaging configurations. Otherwise no major differences have been noticed between the configurations. At 40 °C major changes were observed for all techniques. Strikingly, COP-Pouch syringes performed best in all cases, even better than glass, whereas COP-Open syringes performed worst of all packaging variants in all but one method. Comparable results were obtained from the two glass and COP-Label packaging configurations with BD Hypak SCF™ showing overall slightly better results than Gx RTF® and COP-Label syringes. The reason for the slightly higher amount of chemically modified cytokine in Gx RTF® syringes in comparison to BD Hypak SCF™ syringes might be the presence of low amounts of tungsten. LC-MS showed major changes of the main isoform of the cytokine. The increase of the oxidized species was related to an oxidation of a methionine or a carbohydrate [34]. Deacetylation, a temperature dependent progress [35], of O-acetylated sialic acid was observed with increasing storage time and in particular at high temperature. In accordance with deacetylation, a strong increase of proteins losing a molecular mass of 18 Da was observed at 40 °C. This could be attributed to a dehydration of aspartate [36]. The cytokine amino acid sequence also includes an aspartate-serine motif which promotes dehydration of aspartate [36]. This chemical degradation is again temperature dependent [36, 37]. The findings of the study were consequently in good agreement with literature [36, 37].

Overall, the present study shows that different air bubble free syringe packaging configurations filled with mAb and cytokine solutions displayed at 4 °C a similar suitability for the long-term storage of biopharmaceuticals. We did not investigate the stability of proteins in these syringe packaging configurations with an air headspace for three reasons. First, the main goal was to assess the direct impact of the syringe barrel material and the gas barrier properties of the material. Second, vacuum stoppering resulting only in a tiny air bubble is applied anyway when using FluroTec® coated stoppers in conjunction with silicone oil free

COP syringes [2]. Third, the negative impact of an air bubble within syringes on the protein stability was already demonstrated several times [9, 15, 38]. Based on our own study with vials (Chapter 5), it could be stated that the impact of the primary packaging material on the protein stability was leveled out when a headspace was present (data not shown).

4.5. Conclusion

Two different syringe barrel materials, glass and COP, were compared in their suitability for the long-term storage of biopharmaceuticals. Two siliconized glass syringes and three silicone oil free COP syringe packaging configurations, including labeling or storage in nitrogen filled aluminum pouches, were investigated. At the typical storage temperature for biopharmaceuticals at 4 °C, chemical modifications between the five different syringe packaging configurations were comparable. However, regarding the particle burden significant differences existed. The particle level was significantly lower in COP syringes and did not increase as severely as in glass syringes over the storage period. The data further showed that silicone oil free COP syringes stored in nitrogen filled aluminum pouches could be used as a straightforward and efficient alternative for siliconized glass syringes for the long-term storage of biopharmaceuticals. Without such secondary packaging concepts the oxidation of sensitive proteins would restrict the use of COP syringes. The usage of silicone oil free COP syringes should be considered in the future to avoid the exposure of patients to measurable levels of silicone oil particles and their associated harmful consequences.

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Chapter 5

New Plastic Vials – Does the container material affect protein stability over storage time?

Chapter 5 is intended for publication.

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Abstract

Glass vials are commonly used as primary packaging material for injectable drugs. A well-known issue with glass vials is the occurrence of glass delamination in particular in liquid formulations. Despite several different approaches to get hold of the problem, it is so far not possible to entirely exclude the formation of glass flakes over time. In plastic vials this phenomenon cannot occur. Different plastic vials are available on the market. However, the suitability of plastic vials for the long-term storage of biopharmaceuticals has not been investigated in detail so far. In this study, the stability of two for therapeutic use highly relevant biopharmaceuticals was assessed in monolayer and multilayer plastic vials in comparison to glass vials. The container material had an impact on the protein stability even at 4 °C. Glass vials were able to preserve the product quality best. For the plastic materials multilayer vials should be preferred over monolayer vials.

Keywords

COP, cyclic olefin polymer, CZ-resin, Multishell®, vial, plastic, protein stability, oxidation, protein particle, protein aggregates

Abbreviations

ABS, 4-(aminomethyl) benzenesulfonic acid; COP, cyclic olefin polymer; DLS, Dynamic Light Scattering; HP-IEX, High-Performance Ion-Exchange Chromatography; HPLC, High-Performance Liquid Chromatography; HP-SEC, High-Performance Size Exclusion Chromatography; LC-MS, Liquid Chromatography-Mass Spectrometry; mAb, monoclonal antibody; MFI, Micro-Flow Imaging; RP-HPLC, Reversed-Phase High-Performance Liquid Chromatography; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.

5.1. Introduction

Vials are commonly used containers as primary packaging systems for liquid and lyophilized injectable drugs [1-4]. In the last decades the material of choice for these vials or other containers such as ampoules or prefilled syringes has been borosilicate glass type I [1, 3-8]. Glass offers several advantages like chemical durability, transparency, ease of sterilization and low production costs [2, 3]. The chemical resistance of glass is influenced by the share of alkali oxides, the manufacturing process, sterilization and the interaction with the drug formulation [2, 3, 5, 6, 9-12]. As pH values, buffer components or salts can have a strong impact on the glass durability, drug container compatibility tests are necessary in particular with liquid drugs [4-6, 10-13]. All these factors have also an impact on visible particle formation for products stored in glass vials [4, 6-11]. The presence of particles in injectable drugs is considered as a health hazard and belongs to the top 10 reasons for recalls [2, 3, 5, 6, 8-12]. These particles are associated with adverse effects like acting as an adjuvant on the immunogenicity of biopharmaceuticals or causing a capillary obstruction resulting in embolism [11, 12, 14-16]. Regarding the side effects of particles and the high costs of a recall, this issue attracted the attention of authorities and the pharmaceutical industry [3, 11, 12].

So far two major particle groups which are caused by the vial material have been identified. They either consist of aluminum or silica. This can be explained by the typical composition of type I borosilicate glass, which contains around 81 % silicon dioxide, 13 % boric oxide, 4 % sodium oxide and 2 % aluminum oxide [1]. The elution of aluminum depends on the type of buffer, but not all buffers are able to form aluminum precipitates. In particular phosphate buffers have the capability to elute and interact with aluminum to form white particles at neutral pH [4, 7, 8]. Silica particles are generated through a process called delamination. In glass vials delamination frequently occurs at the vial bottom or shoulder as these regions are exposed to higher temperatures during their manufacturing process and are therefore enriched in silica with a simultaneous accumulation of alkali oxides at the glass surface [1, 2, 5, 6, 12, 13]. This altered glass composition is later prone to undesired glass flake formation [1, 13]. Ammonium sulfate treatment can reduce the amount of alkali ions [1, 13]. However, it might provoke a different effect as its extraction of cations alters the glass structure and therefore triggers the formation of glass flakes [5, 13]. Reducing the conversion temperature during the vial manufacturing process seems to be a more promising approach to diminish the delamination frequency [1]. In general delamination is a slow process and occurs predominantly at higher temperatures [1, 6, 10, 11, 13]. In order to circumvent the time dependent appearance of glass flakes, the shelf life of some products has to be reduced. This in turn, already contributed to acute supply shortages of certain medicines [12]. To mitigate these bottlenecks, the U.S. Food and Drug Administration allowed the temporary

usage of filters for a handful of life-saving drugs in the past. However, in these cases it had to be demonstrated that the filter did not alter the drug and that the filtration was effectively eliminating the particles [17].

Plastic vials might be an alternative for glass vials in particular for liquid formulations with high pH values, buffers like phosphate and chelating agents like citrate [6, 9, 12, 13]. Delamination is not an issue for plastic vials made out of cyclic olefin polymers (COP). Compared to glass, the material properties of these polymer resins show a higher breaking and chemical resistance in a broad pH range preventing unwanted pH shifts [5, 6, 18-21]. Visual inspection is possible due the glass-like clarity of the material [18-20, 22]. Moreover, these resins possess low levels of impurities and extractables and a high moisture barrier and they can be sterilized by various sterilization techniques such as steam or e-beam [18-20, 22-26]. Due to the hydrophobicity of the material, electrostatic interactions between drug and container could be reduced [19]. Besides, protein adsorption is lower in polymer containers for certain proteins compared to glass vials [23, 27]. COP can be considered as biocompatible and safe for its usage as primary packaging material for drug products [18-20]. However, a major disadvantage of these polymers over glass is their permeability for gases like oxygen [28, 29]. This shortcoming might exclude COP based materials as primary packaging containers for oxygen sensitive biopharmaceuticals [23, 29, 30]. A solution to increase the gas barrier properties of COP is the incorporation of a layer with lower oxygen permeability rates, like polyamide, between two COP layers [31, 32]. This approach is found in Multishell® vials from Gerresheimer Bünde GmbH (Bünde, Germany) [33]. The Multishell® vials use the layer composition COP/polyamide/COP [33]. Up to date, literature on the performance of COP monolayer and COP/polyamide/COP multilayer plastic vials for the long-term storage of drugs, in particular of biopharmaceuticals, is scarce [19]. Another approach to obtain COP vials with a higher gas barrier is to coat the interior vial wall with a layer of silicon dioxide [34].

In this study two plastic vial types were compared with commonly used glass vials and were evaluated for the first time for their suitability to store biopharmaceuticals. To this end, two for therapeutic use highly relevant proteins were filled into three vial types and the chemical and physical stability of the proteins was assessed after storage at three different temperatures for up to 48 weeks.

5.2. Materials and Methods

5.2.1 Materials

Di-sodium hydrogen phosphate dihydrate p. A., sodium dihydrogen phosphate dehydrate p. A. and L-Arginine base pure Ph.Eur., USP were purchased from AppliChem GmbH (Darmstadt, Germany). Sodium acetate, 99 %, anhydrous, p. A. was obtained from Grüssing GmbH (Filsum, Germany). Trisodium citrate, anhydrous, 99 % derived from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). Citric acid monohydrate AnalaR Normapur® was purchased from VWR BDH Prolabo® (Leuven, Belgium). Sodium chloride pure and glacial acetic acid 100 % were ordered from Bernd Kraft GmbH (Duisburg, Germany). Polysorbate 80 was purchased from Fluka® Analytical (Buchs, Switzerland). Glycine ReagentPlus® ≥99 (TLC) was obtained from Sigma Life Sciences (Taufkirchen, Germany) and mannitol derived from Boehringer Ingelheim (Ingelheim, Germany). DL-Dithiothreitol (≥98% (HPLC), ≥99.0% (titration), 2,2-azobis(2-methylpropionamidine) dihydrochloride and potassium ferricyanide(III), powder, <10 micron, 99+%, A.C.S. reagent derived from Sigma-Aldrich Corporation, St. Louis, MO, USA. Hydrochloric acid, fuming 37 % and sodium hydroxide 32 % were purchased from Brenntag GmbH (Mühlheim an der Ruhr, Germany). For the preparation of buffers and mobile phases or for system cleanliness checks highly purified water was applied.

The preparation of protein solutions and the filling and closing of vials was performed under a laminar air flow system and in compliance with aseptic manufacturing rules. Two protein solutions, one with an IgG 1 monoclonal antibody (mAb) with a concentration of 1 mg/mL and the other one with a low dosed cytokine (84 µg/mL) were used in this study. The buffer composition of the mAb was NaCl (105.5 mM), NaH_2PO_4 x 2 H_2O (5.5 mM), Na_2HPO_4 x 2 H_2O (8.6 mM), sodium citrate (1.2 mM), citric acid x H_2O (6 mM), mannitol (6.6 mM) and 0.1 % polysorbate 80. The cytokine was formulated in NaCl (75 mM), NaH_2PO_4 x 2 H_2O (7.5 mM), Na_2HPO_4 x 2 H_2O (12.5 mM), glycine (66.6 mM) and 0.03 % polysorbate 80. The mAb formulation had a pH value of 5.2, whereas the cytokine solution had a pH of 7.2. All protein and buffer solutions were 0.2 µm filtered before usage.

The 2R vials of interest, glass vials from Schott AG (Mainz, Germany), cyclic olefin polymer vials from West Pharmaceutical Services, Inc. (Lionville, PA, USA) and Multishell® vials from Gerresheimer Bünde GmbH (Bünde, Germany), had different sizes and diameters. Hence, to ensure a similar headspace to solution ratio the vials had different fill volumes. Glass vials were filled with 2.0 mL, COP vials with 1.17 mL and Multishell® vials with 2.2 mL protein solution. All vials were closed with FluroTec® coated stoppers and sealed with Flip-Off® seals (both West Pharmaceutical Services, Inc., Lionville, PA, USA). Protein stability in these vials was assessed at 4 °C and 25 °C for 48 weeks and at 40 °C and at 75 % relative humidity for 24 weeks. For the analysis the content of several vials from each vial type was pooled. In

particular, the solutions of two glass vials, four COP vials and three Multishell® vials were mixed. At the end, four pools of each vial material and condition existed. Every pool was measured with the following methods. The results are presented as mean ± standard deviation.

5.2.2 Methods

5.2.2.1 Visible particles

Examination for visible particles in the vials was performed in front of a matt black and white plate with a light source as described in the European Pharmacopoeia method.

5.2.2.2 Dynamic Light Scattering (DLS)

The particle size and size distribution in the nanometer range was determined using a Zetasizer APS device (Malvern Instruments, Worcestershire, UK). Four pools of each of the three vial materials were measured in duplicates at 20 °C resulting in a total of eight measurements. The particle size is given by the Z-average values and the particle size distribution is depicted by the polydispersity index.

5.2.2.3 Micro-Flow Imaging™ (MFI)

Micrometer particles were analyzed and quantified using a 100 µm flow cell in the “high magnification” operation mode on a Micro-Flow Imaging DPA 4100 device (BrightWELL Technologies Inc., Ottawa, Canada). For the analysis the software MFI™ Particle Analyzer V6.9.7.2 was used. Flow cell cleaning and cleanliness check were performed with highly purified water. Before each run the command “optimize illumination” was carried out in order to ensure a correct threshold of the system. During this process the flow cell was flushed with 0.5 mL of the corresponding protein solution buffer. In total 1 mL sample was used for the analysis. 0.35 mL were used to flush the flow cell before 0.65 mL of the sample were analyzed. Analysis was performed at a flow rate of 0.1 mL/min. For each vial material and condition four pools were measured. Duke Standards™ (10 µm) and Count-Cal™ (5 µm) Particle Size Standards (both Thermo Scientific, Fremont, CA, USA) were applied for the system calibration.

5.2.2.4 Chromatography Methods

Before chromatography analysis was carried out all samples were centrifuged for 10 min at 14,000 rpm. The mobile phases were filtered with a 0.2 µm filter and prepared in HPLC grade medium or with highly purified water. High-performance liquid chromatography (HPLC)

was performed on systems from Waters Corporation (Milford, MA, USA) or Thermo Fisher Scientific GmbH (Idstein, Germany) using the software Chromeleon V6.8 (Thermo Fisher Scientific GmbH, Idstein, Germany). A Waters 2695 Alliance Separation Module with a Waters 2487 Dual λ Absorbance Detector at 280 nm was applied for monitoring the absorbance of the mAb. For mAb and cytokine analysis a Dionex Summit system with a UVD170U detector and a RF 2000 Fluorescence detector were applied. The mAb was measured with the UVD170U detector at 280 nm. The RF 2000 Fluorescence detector was operated for the cytokine extinction at 295 nm and for its emission at 343 nm. Moreover, a Dionex Ultimate 3000 system was employed to analyze the cytokine with a fluorescence detector (extinction: 295 nm; emission 343 nm). In total eight runs of each vial material and condition were analyzed as each pool was injected twice.

5.2.2.4.1 High-Performance Size Exclusion Chromatography (HP-SEC)

Soluble monomer, fragment or aggregate species of the proteins were quantified with HP-SEC. The cytokine species were separated for 30 min with a flow rate of 0.5 mL/min on a TSKgel® 3000SWXL column (Tosoh Bioscience GmbH, Stuttgart, Germany). The mobile phase contained 300 mM sodium chloride and 50 mM phosphate pH 7 in highly purified water. For the separation of the mAb a mobile phase comprising 20 mM phosphate and 150 mM sodium chloride at a pH of 7.5 and a Superose™ 6, 10/300 GL column (GE Healthcare, Uppsala, Sweden) were applied. Run time was 1 h with a flow rate of 0.5 mL/min.

5.2.2.4.2 High-Performance Ion-Exchange Chromatography (HP-IEX)

HP-IEX was used to detect chemical modifications of the proteins. Cytokine analysis was carried out on a YMC BioPro QA column (YMC, Kyoto, Japan) with a flow rate of 0.5 mL/min. The mobile phases consisted of 20 mM Tris buffer pH 7.5 (mobile phase A) and of 20 mM Tris and 500 mM sodium chloride pH 7.5 (mobile phase B). Over a period of 50 min a constant gradient from 5 % to 40 % of B was applied. For the next 2 min 100 % B and then back to 5 % B within 2 min were programmed, before the gradient was kept constant for 5 min at a 95:5 ratio of A to B. The mAb was analyzed at a flow rate of 1 mL/min on a ProPac™ WCX-10G column (BioLC™ Guard, 4 x 50 mm) and a ProPac™ WCX-10 column (BioLC™ Analytical, 4 x 250 mm) (both Thermo Fisher Scientific, Rockford, USA). The gradient consisted of mobile phase A (10 mM phosphate buffer pH 7.5) and mobile phase B (mobile phase A plus 0.5 M sodium chloride pH 5.5). At the beginning the amount of A was reduced from 94 % to 84 % within 20 min before 100 % B were reached within 2 min. 100 % B remained for 4 min before the share of A increased again to 94 % within 2 min.

5.2.2.4.3 Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

Chemical modifications of the cytokine were analyzed with RP-HPLC. Mobile Phase A was made up of 90 % highly purified water, 10 % HPLC grade acetonitrile (Acetonitrile, HiPerSolv CHROMANORM® Super gradient for HPLC, VWR BDH Prolabo® Chemicals, Ismaning, Germany) and 0.1 % trifluoroacetic acid (w/w) (Trifluoroacetic acid, reagent grade 99 %, Sigma-Aldrich Chemie GmbH, Munich, Germany). Mobile phase B comprised 100 % HPLC grade acetonitrile and 0.1 % trifluoroacetic acid (w/w). A YMC-Pack C4 (250 x 4.6 mml.D. S-5µm, 30 nm) column (YMC, Kyoto, Japan) was operated with a gradient at a temperature of 37 °C and a flow rate of 0.5 mL/min for separation. The gradient was programmed as followed: the amount of B increased linearly from 0 % to 100 % during the run time from 2 min to 25 min. 100 % B was kept constant for 5 min, before the share of B dropped to 0 % within 1 min.

5.2.2.4.4 Protein A Chromatography

Protein A Chromatography was used to monitor the methionine oxidation of the mAb. Separation was carried out on a Poros A column (2.1 mm x 30 mm, Applied Biosystems, Framingham, MA, USA). A flow rate of 0.5 mL/min with a gradient was applied. The gradient started after 3 min with a rise of B (20 mM phosphate and 150 mM sodium chloride at a pH of 2.9) to 40 % from 100 % A (20 mM phosphate with a pH of 7.4) within 20 min. In the next 5 min the share of B further increased to 100 % and remained constant for 10 min before A was increased to 100 % for 2 min.

5.2.2.5 Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS was carried out to detect molecular changes in the proteins. Amicon® ultra-0.5 centrifugal filter devices (Millipore Corp. Bedford, MA, USA) were applied to dialyze the proteins into a 25 mM phosphate buffer pH 7.25. The buffer was exchanged for the 25 mM phosphate buffer twice. DL-Dithiothreitol was added to the mAb samples in a final concentration of 10 mM. Next, mAb samples were centrifuged before analysis. Cytokine was exposed overnight at room temperature to PNGase F for deglycosylation. As for the mAb solution DL-Dithiothreitol was added in a final concentration of 10 mM directly before analysis. Cytokine samples were centrifuged as well. The devices Agilent 1200 series LC system and Agilent 6520 Quadrupole Time-Of-Flight system (Agilent Technologies, Santa Clara, CA, USA) were applied for the analysis. The method was performed according to Okbazghi et al. [35].

Benjamin Werner prepared the samples and run the samples together with Ishan Shah (Department Pharmaceutical Chemistry, University of Kansas, KS, USA) on the LC-MS device.

5.2.2.6 4-(aminomethyl) benzenesulfonic acid (ABS) assay

An ABS assay was applied to determine the occurrence of tyrosine and phenylalanine oxidation. To concentrate and dialyze the cytokine into a 100 mM phosphate buffer with a pH of 8.8 Amicon® ultra-0.5 centrifugal filter devices (Millipore Corp. Bedford, MA, USA) were used. In a ratio of 1:15 the concentrated cytokine solution and the mAb solution were each mixed with $K_3Fe(CN)_6$ and ABS, which had a final concentration of 10 mM. Basic reaction conditions were achieved by adding 100 mM phosphate buffer pH 8.8. Protein solutions were further exposed to 10 mM 2,2-azobis(2-methylpropionamidine) dihydrochloride for 3 h at 37 °C to serve as positive controls. With an excitation wavelength of 360 nm and an emission maximum at 520 nm the fluorescence intensity of the ABS-derivatized protein samples was measured using a SpectraMax Gemini XS plate reader (Molecular Devices, Sunnyvale, CA) and the software SoftMax® Pro 4.8. As cytokine samples had to be concentrated only single measurements were possible, whereas the mAb samples were analyzed in triplicates.

Assistance in the establishment of the ABS assay was received from Rupesh Bommana and Christian Schöneich (both Department Pharmaceutical Chemistry, University of Kansas, KS, USA).

5.2.2.7 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A non-reducing, denaturing SDS-PAGE was carried out to detect protein fragments or aggregates. For the cytokine NuPage® Novex® 4 - 12 % Bis-Tris protein gels, NuPage® MOPS SDS Running Buffer (20x) and Mark12™ Unstained Standard were applied. Whereas NuPage® Novex® 3 - 8 % Tris-Acetate protein gels, NuPAGE® Tris-Acetate SDS Running Buffer (20x) and HiMark™ Unstained Protein Standard were used for the mAb. For the analysis the following substances were further used: NuPage® LDS Sample buffer (4x), SilverXpress® Silver Staining Kit (all substances, Novex® by Life Technologies, Carlsbad, CA, USA), glacial acetic acid 100 % and methanol (Methanol HPLC grade, Fisher Scientific UK, Loughborough, UK). LDS Sample Buffer (4x) was used to dilute the mAb to a concentration of 37.5 µg/mL. The cytokine was diluted to a concentration of 67.2 µg/mL with the LDS Sample Buffer (4x). Denaturing occurred at 90 °C for 5 min. In each lane 12 µL sample were loaded. Markers were prepared as recommended by the manufacturer. A XCell SureLock™ Mini-Cell Electrophoresis System (Novex® by Life Technologies, Carlsbad, CA, USA) was applied to run the gels in the appropriate running buffers at a constant voltage of 200 V (cytokine) or 150 V (mAb) using a Bio-Rad PowerPac 200 (Bio-Rad Laboratories, Hercules,

CA, USA). Staining of the proteins occurred with the SilverXpress® Silver Staining Kit and accordingly to manufacturer specifications.

5.2.2.8 Oxygen quantification

Glass and plastic vials were closed with FluroTec® coated stoppers and sealed with Flip-Off® seals under nitrogen atmosphere. The vials were stored in oxygen (≥ 75 %) filled aluminum pouches (Drylok 3000, Advantek GmbH, Freiburg, Germany) at 4 °C and 40 °C for 24 weeks. Oxygen concentration in the aluminum pouch and in the vials was measured with a Microx 4 stand-alone fiber optic oxygen meter (PreSens Precision Sensing GmbH, Regensburg, Germany). For each vial material and condition twelve measurements were taken.

5.3. Results

The usage of monolayer and multilayer plastic vials for the long-term storage of biopharmaceuticals has not been described so far. In this study, the physical and chemical stability of a mAb and a cytokine stored in COP monolayer (COP) and COP/polyamide/COP multilayer (Multishell®) vials were compared to commonly used glass vials. Protein stability was assessed by visual inspection, MFI, DLS, HP-SEC, SDS-PAGE, HP-IEX, Protein A Chromatography, RP-HPLC, ABS assay, and LC-MS at 4 °C and 25 °C for 48 weeks and at 40 °C for 24 weeks. The results of the different methods, first for the mAb at the three temperatures 4 °C, 25 ° and 40 °C and then for the cytokine were presented in the order as listed above.

Before further analysis a visible inspection of the vials was performed. No visible particles were detected in any of the vials for both proteins independent of the storage period and condition. The particle sizes 10 µm and 25 µm, according to the specifications of the pharmacopoeia were monitored as well for both proteins, besides the total particle count (particles ≥ 1 µm). The number of particles with sizes of 10 µm and 25 µm was in the range of 0 to 50 particles/mL for the mAb and the cytokine at all storage conditions.

5.3.1 Results of the mAb stored at 4 °C

The particle burden of the vials at the starting point for the mAb was 1,050 particles/mL in glass vials, 908 particles/mL in COP vials and 604 particles/mL in Multishell® vials as determined by MFI. Over the storage period of 48 weeks at 4 °C the particle number nearly doubled to 2,010 particles/mL in glass vials and slightly increased to 1,077 particles/mL in COP vials, whereas in Multishell® vials the particle count was 551 particles/mL (Figure 1A). No changes in the hydrodynamic radius were detected by DLS (data not shown). With HP-SEC only monomer was detected (Figure 1B) and no additional bands were observed by SDS-PAGE (data not shown). HP-IEX displayed for all vial types an increase of chemically modified protein by a factor of around 1.4 (Figure 1C). Protein A Chromatography (Figure 1D) and ABS assay (Figure 1E), both with the capability to detect oxidation processes, showed no changes in the amount of oxidized species. However, LC-MS revealed that oxidation occurred over time (Figure 1F). At the beginning of the study the mAb was already oxidized by 18 %. Depending on the vial type the oxidation of the mAb increased differently within 48 weeks. mAb stored in glass vials showed with 29.6 % the lowest amount of oxidized protein, whereas the plastic vials performed equally regarding the share of oxidized mAb with 37.8 % in the COP vial and 37.1 % in the Multishell® vial. Further, only the heavy chains of the mAb were oxidized as analyzed by LC-MS. In general, during

the storage period the oxidation process led to a mean mass difference between the native and oxidized mAb of 15.72 Da. Based on results by Steinmann et al., who found that tyrosine oxidation products with + 14 Da, + 16 Da and + 18 Da do exist [36], it can be concluded that the observed mass difference of 15.72 Da indicates that tyrosine oxidation with + 14 Da occurred besides methionine and/or tyrosine + 16 Da oxidation.

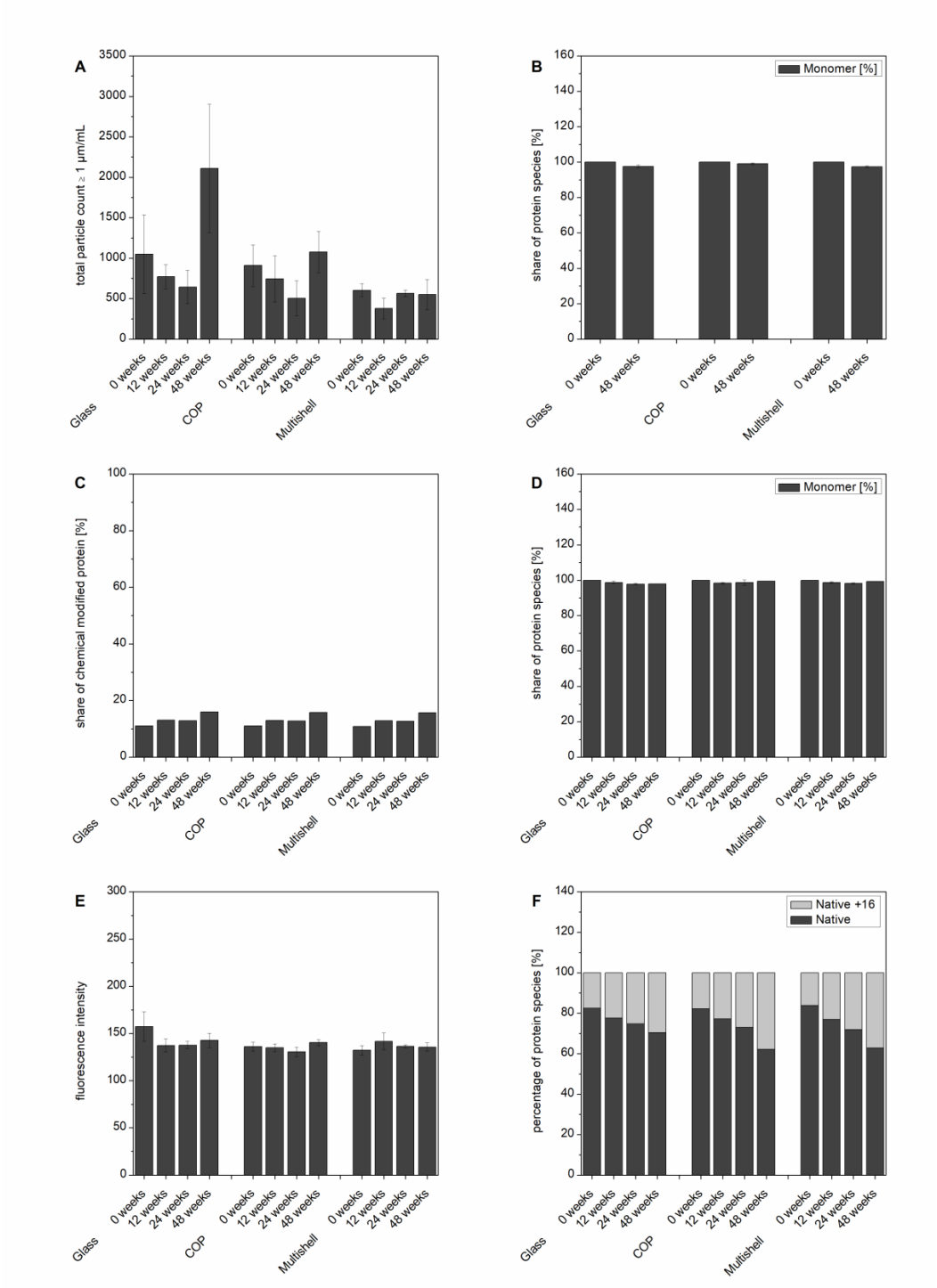

Figure 1: mAb stability in glass, COP and Multishell® vials stored at 4 °C for up to 48 weeks was assessed. MFI was used to determine the total particle count/mL (particles/mL ≥ 1 µm) (**A**). The amount of soluble mAb monomer was obtained by HP-SEC (**B**). Chemical modifications were analyzed and quantified by HP-IEX (**C**), whereas Protein A Chromatography (**D**), ABS assay (**E**) and LC-MS (**F**) showed the occurrence of oxidation (Native + 16 in LC-MS).

5.3.2 Results of the mAb stored at 25 °C

After 48 weeks at 25 °C the amount of subvisible particles in glass vials was 915 particles/mL, while increases to 1,425 particles/mL in COP vials and 1,343 particles/mL in Multishell® vials were observed (Figure 2A). DLS showed no changes in the hydrodynamic radius of the mAb (data not shown). However, a mAb fragmentation of around 1.6 % was detected after 48 weeks by HP-SEC (Figure 2B). This fragmentation after 48 weeks was also confirmed by SDS-PAGE (data not shown). HP-IEX showed increased chemical changes of the mAb in all vial types by a factor of around 4.2 (Figure 2C). After 48 weeks methionine oxidation was detected by Protein A Chromatography (Figure 2D). While methionine oxidation was the strongest in COP vials with 1.97 %, followed by Multishell® vials with 1.74 %, the amount of oxidized methionine in glass vials was 0.39 %. With the ABS assay no changes were observed in all cases except in the samples stored in Multishell® vials after 48 weeks (Figure 2E). Here, an increase of 12.2 % of the fluorescence intensity was observed. The detected protein modifications were further confirmed by LC-MS (Figure 2F). Already after 12 weeks the mAb showed two oxidations in the heavy chain whereas no oxidation was seen in the light chain. For the mAb sample stored in glass vials for 24 weeks a shoulder for a double oxidation was visible. However, the share of this shoulder could not be determined with the software and resulted in slightly too high values for the remaining native species, based on the data obtained for the glass vial sample at 4 °C and 40 °C after 12 weeks. The amount of single and double oxidized protein species increased further during storage time. In the end after 48 weeks, more than 84 % of the mAb were oxidized in the plastic vials, whereas the share of native mAb remained highest in glass vials with 43 %. The mass difference between native and single oxidized mAb after 12 weeks was below 16 Da and it was increased in plastic vials to values above 17 Da in the following weeks. This change did not occur in glass vials where the mass difference between the native and single oxidized protein species remained below 16 Da. The second oxidation (around + 32 Da) showed in the beginning a mass difference of 17.6 Da (glass) to 17.96 Da (Multishell®) and decreased to 16 Da after 48 weeks for plastic vials, whereas in glass vials the mass difference was 17 Da. The results indicate that in the beginning the single oxidation was a combination of tyrosine + 14 Da and tyrosine + 16 Da as well as methionine oxidation (+ 16 Da) and later a combination of methionine oxidation and tyrosine + 18 Da oxidation. In contrast, the second oxidation was mainly of a tyrosine (+ 18 Da) and then shifts towards a mixture of tyrosine and methionine oxidation over storage time. The LC-MS results match with the Protein A Chromatography results.

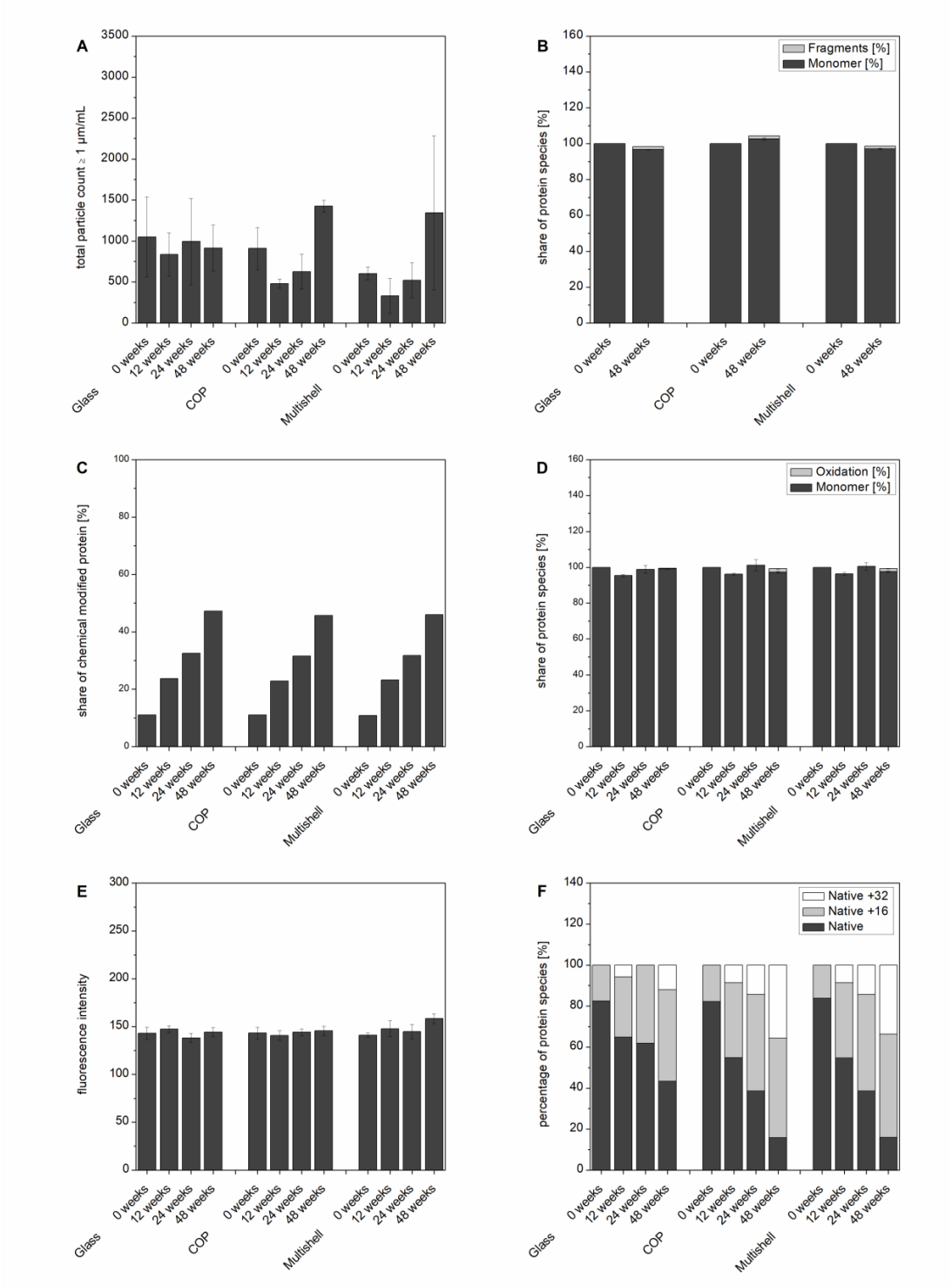

Figure 2: Data of six methods characterizing the mAb solution which was stored at 25 °C for up to 48 weeks in three different vial types is shown. The total particle count/mL (particles/mL ≥ 1 µm) was analyzed by MFI (**A**), whereas the soluble protein content was measured with HP-SEC (**B**). HP-IEX (**C**), Protein A Chromatography (**D**), ABS assay (**E**) and LC-MS (**F**) were applied to detect chemical protein alterations. Molecular weight was changed by one (Native + 16 Da) or two (Native + 32 Da) oxidations as determined by LC-MS.

5.3.3 Results of the mAb stored at 40 °C

At 40 °C the particle count in glass vials was 610 particles/mL after 24 weeks and showed values of 1,675 particles/mL in COP vials and 1,207 particles/mL in Multishell® vials (Figure 3A). Equally as detected at lower temperatures DLS showed no changes (data not shown). Fragmentation was observed from week 12 onwards with a share of 3.07 % in glass vials and up to 3.47 % in COP vials as determined by HP-SEC. The amount of fragments increased to 4.18 % in glass vials, 5.04 % in COP vials and 4.47 % in Multishell® vials after 24 weeks (Figure 3B). SDS-PAGE data confirmed the HP-SEC findings (data not shown). HP-IEX showed chemical modifications of the mAb after a storage period of 24 weeks. 64.37 % respectively 64.62 % of the mAb stored in glass and Multishell® vials were altered, while the mAb solution stored in COP vials showed values of 69.55 % (Figure 3C). Methionine oxidation after 24 weeks was the lowest in glass vials with 1.63 %, followed by Multishell® vials with 6.80 % and COP vials with 8.97 % as determined by Protein A Chromatography (Figure 3D). In a time dependent manner the ABS assay showed an increase of the fluorescence intensity for all vial types indicating an oxidation of tyrosine and phenylalanine (Figure 3E). LC-MS confirmed the so far described results (Figure 3F). In plastic vials more than 90 % of the mAb were oxidized, whereas 26.7 % remained as native species in glass vials after 24 weeks. Double oxidations, detectable as early as 4 weeks after the beginning of the study, accounted after 24 weeks for more than 50 % of the oxidation modifications in plastic vials, while in glass vials single oxidation with 50.9 % was dominating. Over the storage period the mass differences between native and single oxidized mAb rose, indicating that tyrosine oxidations were dominating, which correlated with the observed increase of the fluorescence intensity of the ABS assay. For the double oxidation values of around 17.1 Da were obtained for plastic vials, whereas for glass vials the mass difference was reduced from 17.4 Da to 16.7 Da. The molecular changes of the mAb, which was stored in COP vials at 40 °C, are displayed in Figure 4 as detected with LC-MS.

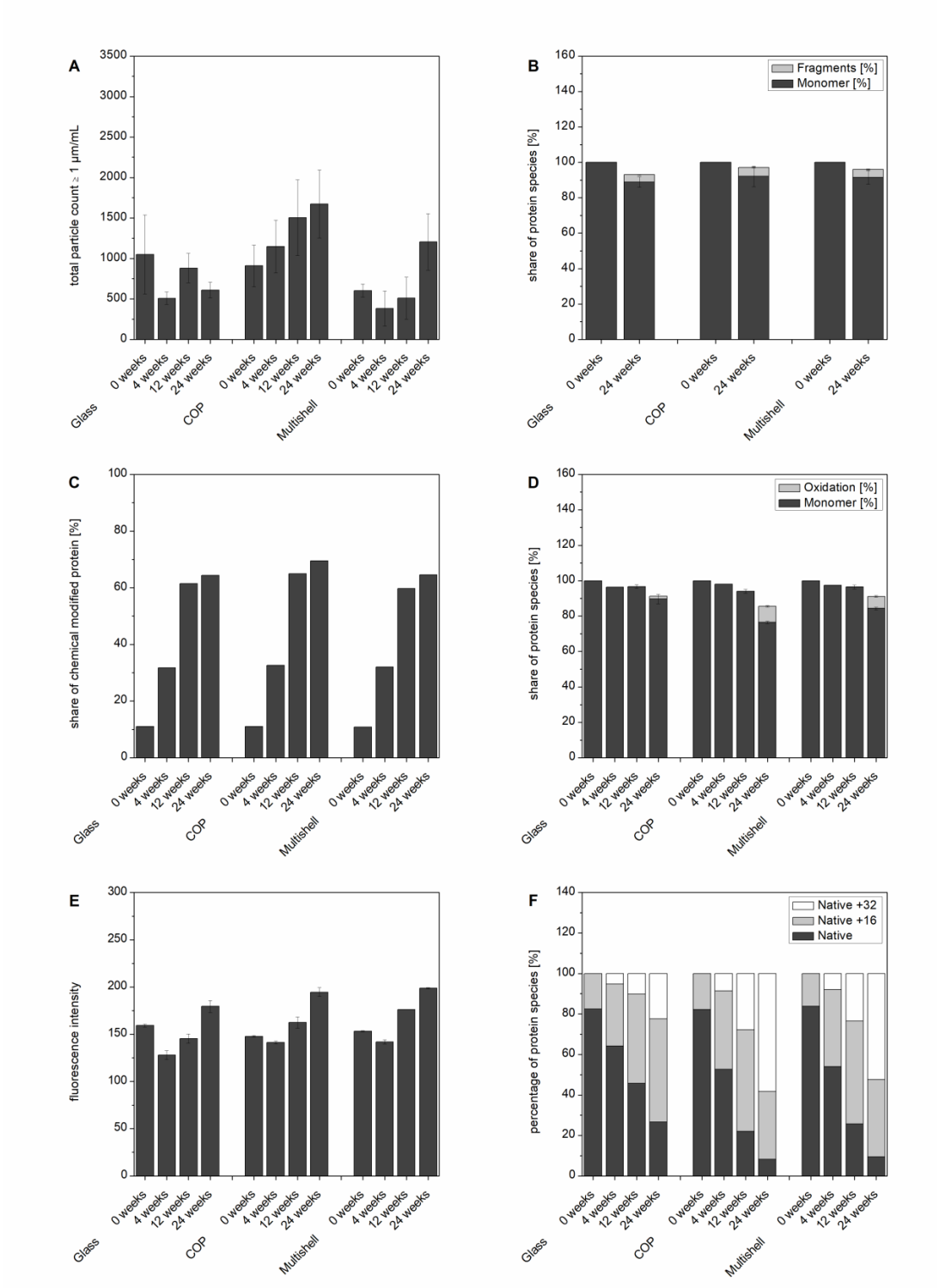

Figure 3: Physical and chemical stability of the mAb stored at 40 °C for up to 24 weeks in the three vial types was monitored by MFI (**A**), HP-SEC (**B**). HP-IEX (**C**), Protein A Chromatography (**D**), ABS assay (**E**) and LC-MS (**F**). A mass increase of + 16 Da and + 32 Da was observed by LC-MS.

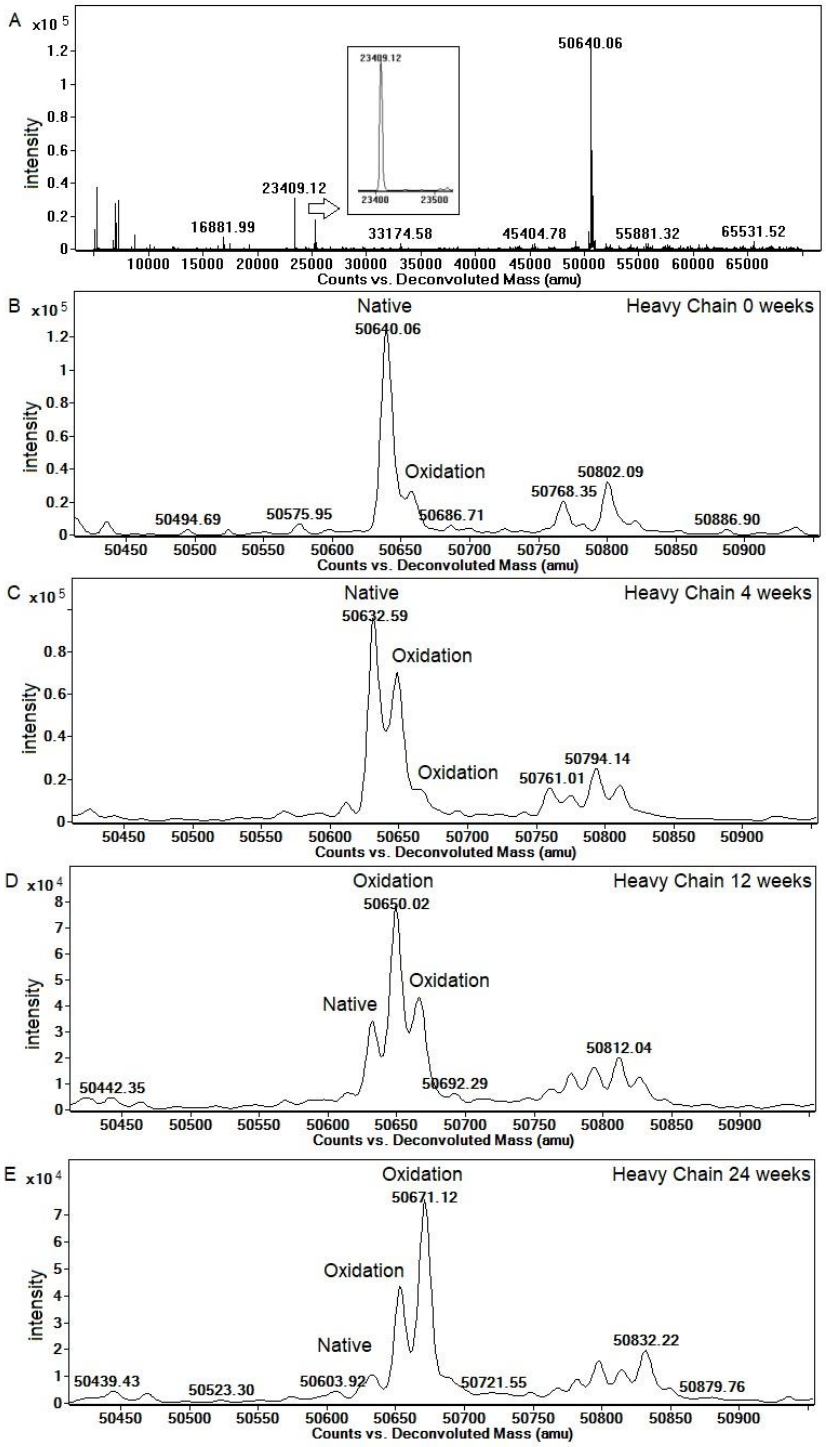

Figure 4: Mass spectrum of the mAb stored in COP vials at 40 °C after 0 weeks is shown in (**A**). The light chain is shown for clarity in a greater magnification (highlighted with the frame) in (A) as well. Magnifications of the heavy chain at different points of analysis (0, 4, 12 and 24 weeks) show the oxidation of the native mAb (**B** to **E**). The peak of the native heavy chain of the mAb and its oxidized versions are labeled.

The particle numbers in the three vial materials were overall on a low level and differed only by a few hundred particles/mL. Changes to a similar extent were observed for all vial materials at the three temperatures regarding the physical protein stability. However, while at 4 °C chemical alterations detected by HP-IEX, Protein A Chromatography and ABS were low and differences between the vial materials were minor, LC-MS showed a higher amount of native mAb in glass vials. At 25 °C and 40 °C similar results were obtained for plastic vials, whereas glass vials conserved the native mAb best.

Table 1 provides a summary of the alterations in mAb stability at the different storage conditions and vial materials.

Table 1: Overview of the changes regarding the chemical and physical stability of the mAb, which was stored at 4 °C and 25 °C over the storage period of up to 48 weeks and at 40 °C for up to 24 weeks in different vial types

	Glass Vial	COP Vial	Multishell® Vial
4 °C			
Visual	O	O	O
MFI	+	O/+	O
DLS	O	O	O
HP-SEC	O	O	O
SDS-PAGE	O	O	O
HP-IEX	O/+	O/+	O/+
Protein A Chromatography	O	O	O
ABS	O	O	O
LC-MS	O/+	+	+
25 °C			
Visual	O	O	O
MFI	O	+	+
DLS	O	O	O
HP-SEC	O/+	O/+	O/+
SDS-PAGE	O/+	O/+	O/+
HP-IEX	+/++	+/++	+/++
Protein A Chromatography	O	O/+	O/+
ABS	O	O	O/+
LC-MS	+	+/++	+/++
40 °C			
Visual	O	O	O
MFI	O	+	+
DLS	O	O	O
HP-SEC	+	+	+
SDS-PAGE	+	+	+
HP-IEX	++	++	++
Protein A Chromatography	+	++	++
ABS	+	++	++
LC-MS	+/++	++	++

O = no or minor changes; + = changes; ++ = major changes.

5.3.4 Results of the cytokine stored at 4 °C

The particle level of the cytokine was very low at the starting point with 50 particles/mL in glass vials, 72 particles/mL in COP vials and 124 particles/mL in Multishell® vials. Over 48 weeks at 4 °C the particle burden increased to 1,316 particles/mL in glass vials, 395 particles/mL in COP vials and 1,365 particles/mL in Multishell® vials (Figure 5A). The hydrodynamic radius of the cytokine remained similar to the start value (data not shown). According to HP-SEC (Figure 5B) and SDS-PAGE only the monomer was present (data not shown). HP-IEX showed for all vial types a share of 0.54 % chemically modified protein (Figure 5C), but no alterations were detected by RP-HPLC after 48 weeks (data not shown). Lower fluorescence intensity for cytokine stored in glass vials compared to plastic vials was obtained in the ABS assay at week 0 (Figure 5D). Therefore, it is not sure, if the increase of the fluorescence intensity was based on tyrosine and phenylalanine oxidation in glass vials. The fluorescence intensity was not elevated for plastic vials after 48 weeks. LC-MS with N-glycan deglycosylated cytokine was carried out to quantify oxidation. After N-glycan deglycosylation only O-glycosylated isoforms of the cytokine remained. The major isoform had the composition of $NeuAc_2Hex_1HexNAc_1$, whereas the other isoform was $NeuAc_1Hex_1HexNAc_1$ [37]. $NeuAc_2Hex_1HexNAc_1$ showed peaks with + 16 Da and approximately 26 % of this predominant isoform were already oxidized at the beginning of the study. Over the storage period the amount of oxidized protein increased in plastic vials and remained stable in glass vials after 48 weeks. Further, around 5.47 % of $NeuAc_2Hex_1HexNAc_1$ were O-acetylated as identified with a mass increase of 42 Da [37]. This share was reduced to the lowest value of 4.49 % found for COP vials. Dehydration of aspartic acid was detected in 1.2 % of the main isoform (Figure 5E). The $NeuAc_1Hex_1HexNAc_1$ isomer was also altered by oxidation.

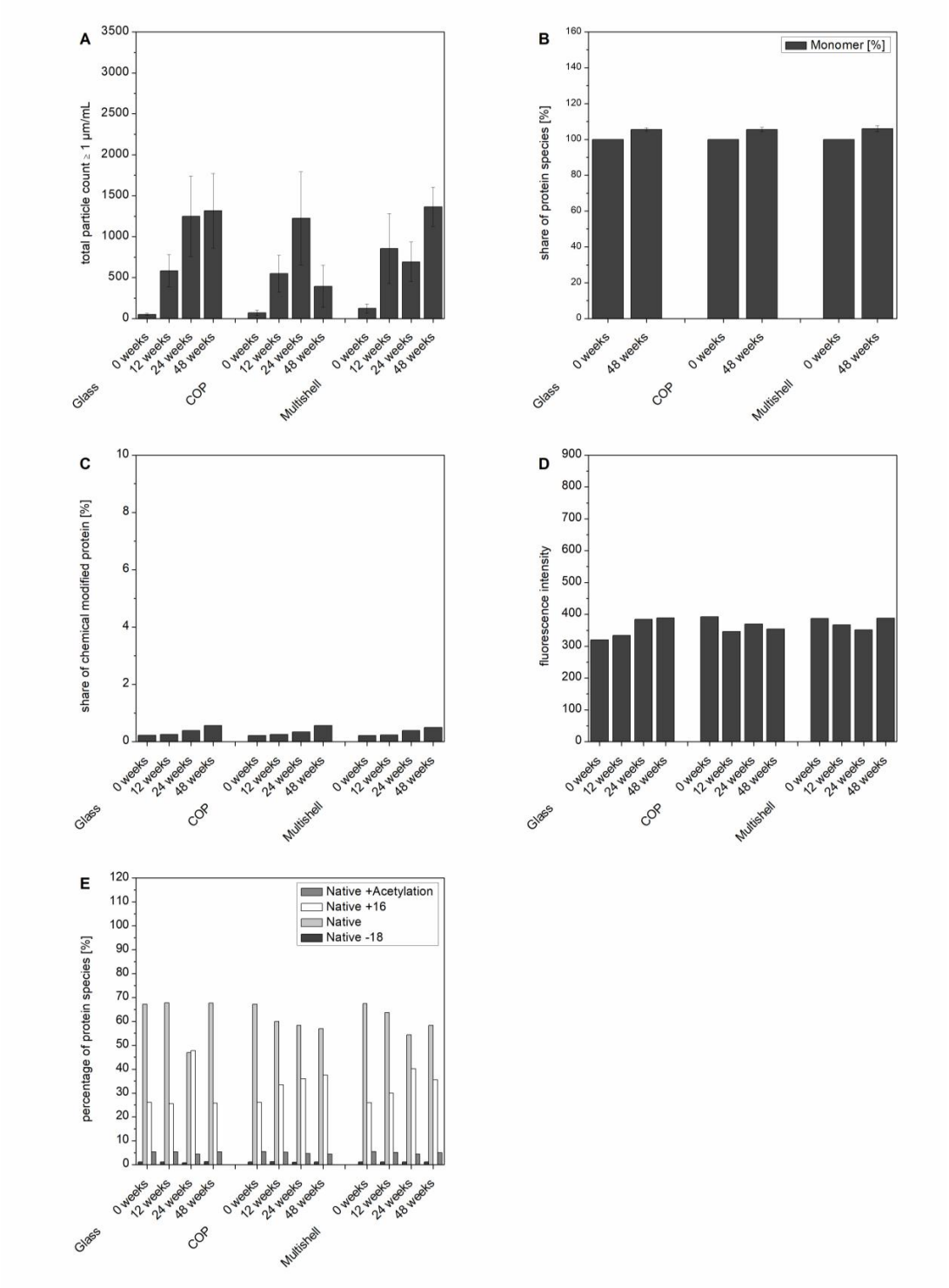

Figure 5: Cytokine stability in glass, COP and Multishell® vials was determined at 4 °C for up to 48 weeks. The total particle count/mL (particles/mL ≥ 1 µm) was determined with MFI (**A**), the soluble protein species was analyzed with HP-SEC (**B**), the amount of chemically modified protein species was assessed by HP-IEX (**C**). Oxidation processes were observed using the ABS assay (**D**) and LC-MS (**E**), which detected acetylation (+ 42 Da), oxidation (+ 16 Da) and dehydration (- 18 Da).

5.3.5 Results of the cytokine stored at 25 °C

At 25 °C the particle number of the cytokine solutions increased in all vial types. After 48 weeks Multishell® vials had the lowest count with 663 particles/mL followed by glass vials with 1,299 particles/mL and COP vials with 1,490 particles/mL (Figure 6A). The hydrodynamic radius of the cytokine remained similar over time (data not shown). Aggregate formation was detected by HP-SEC to a low extent of approximately 0.3 % in all three vial types after 48 weeks (Figure 6B), but not by SDS-PAGE (data not shown). HP-IEX revealed that around 1.49 % of the protein was chemically altered in all three vial types at the end point (Figure 6C). RP-HPLC detected no changes until week 24 (Figure 6D). In week 24 hydrophilic changes of 0.93 % were noted which increased until week 48 to 2.07 % in glass vials, 2.24 % in COP vials and 2.45 % in Multishell® vials. In addition hydrophobic alterations in the range of 0.27 % to 0.59 % for glass and COP vials were observed after 48 weeks. The ABS assay showed for all vial types an increase of the fluorescence intensity over time (Figure 6E). High start values of the plastic vials lead to a smaller increase of fluorescence intensity than for glass vials. Based on HP-IEX and RP-HPLC chromatography and LC-MS results, however, it could be stated that the fluorescence increase could be attributed to oxidation. LC-MS revealed for all vials a higher level of oxidation (Figure 6F). The share of oxidized species rose from approximately 26 % to 36.5 % in glass vials, to 42.1 % in COP vials and to 41.4 % in Multishell® vials. Further, the share of acetylated cytokine was reduced from around 5.5 % to 3.8 % in glass vials and to around 4.1 % in plastic vials. The amount of dehydrated cytokine increased from 1.2 % to a range of 1.6 % to 1.8 % in all vial types.

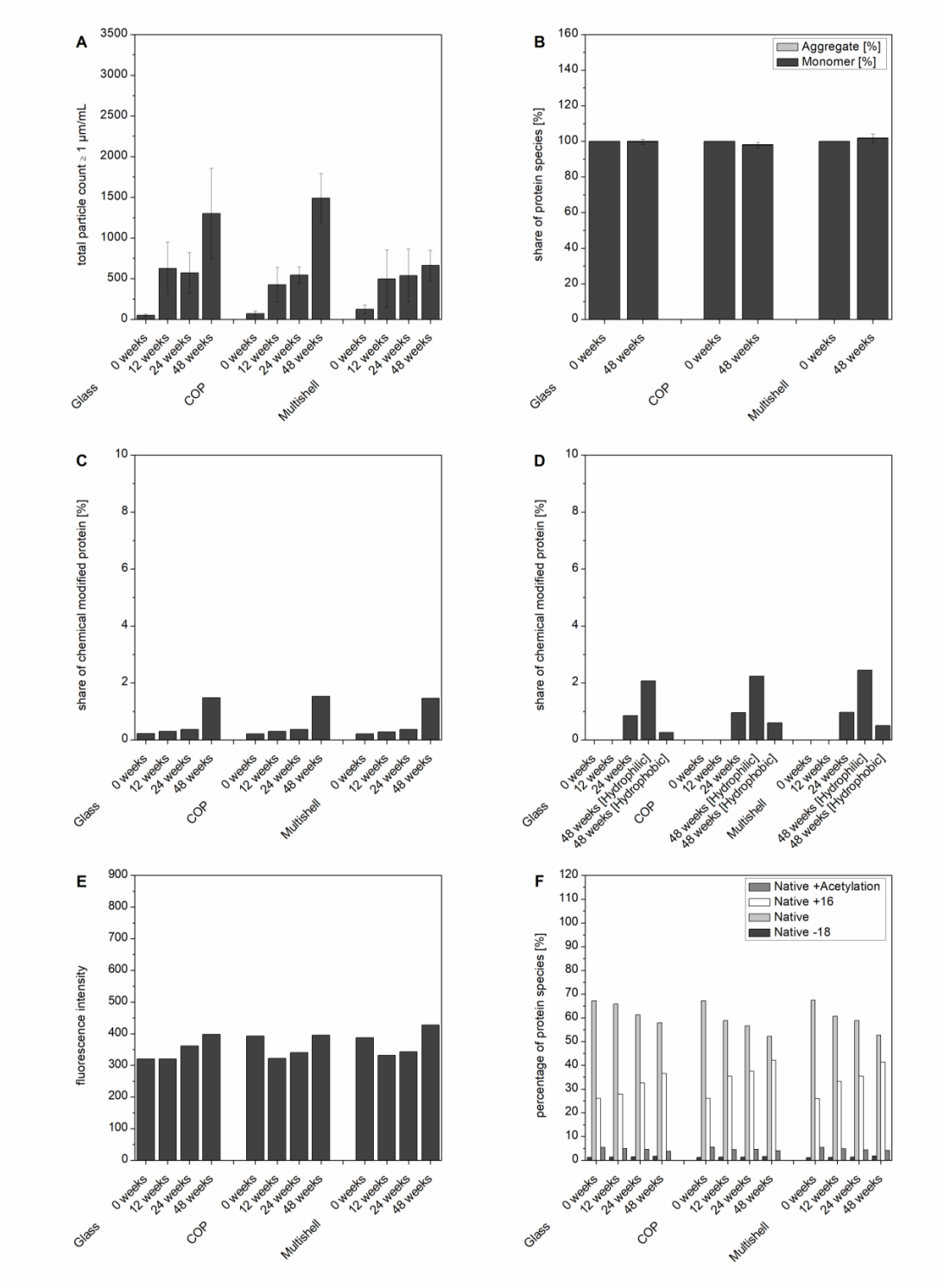

Figure 6: Physical and chemical stability of the cytokine was evaluated at 25 °C for up to 48 weeks in glass, COP and Multishell® vials. The total particle count/mL (particles/mL ≥ 1 µm) was determined with MFI (**A**) and the amounts of native and aggregated protein species were assessed by HP-SEC (**B**). Chemical modifications were detected with HP-IEX (**C**), RP-HPLC (**D**) and ABS (**E**). LC-MS (**F**) revealed acetylation (+ 42 Da), oxidation (+ 16 Da) and dehydration (- 18 Da) processes.

5.3.6 Results of the cytokine stored at 40 °C

At 40 °C the particle counts of the cytokine solutions were below 1,000 particles/mL for all vials after 24 weeks. The lowest particle burden was detected in COP vials with 599 particles/mL and in Multishell® vials with 815 particles/mL. The highest particle concentration was measured in glass vials with 972 particles/mL (Figure 7A). DLS showed a similar hydrodynamic radius for the cytokine after 24 weeks (data not shown). HP-SEC revealed the formation of soluble aggregates after 12 weeks. At least 10 % of the protein was aggregated after 12 weeks in glass and Multishell® vials, whereas in COP vials an amount of 16.6 % of aggregates was found. Protein loss of more than 45 % was observed after 24 weeks. Aggregates were still detected and accounted for 3.7 % in glass vials, 7.4 % in COP vials and 4.4 % in Multishell® vials of the recovered protein (Figure 7B). SDS-PAGE showed in contrast to HP-SEC aggregate formation already after 4 weeks (data not shown). Moreover, the aggregate bands intensified over time. These findings were supported by HP-IEX and RP-HPLC results. HP-IEX showed after 24 weeks that 22.9 % of the cytokine were altered in COP vials and 13.7 % in glass vials and 14.8 % in Multishell® vials (Figure 7C). The same tendency was obtained by RP-HPLC (Figure 7D). In the first 12 weeks only hydrophilic changes and after 24 weeks also hydrophobic alterations were observed. In total 27.6 % of the cytokine were altered in glass vials, 25.9 % of the cytokine were changed in Multishell® vials and 34.4 % of the cytokine were modified in COP vials. The ABS assay showed that tyrosine and/or phenylalanine were oxidized over the storage period with the strongest increase in the fluorescence intensity in glass vials (Figure 7E). The ABS data correlated with LC-MS data (Figure 7F). Glass vials showed the highest amount of oxidized cytokine after 24 weeks with 58.6 %, followed by Multishell® vials with 45.8 % and COP vials with 44.6 %. Further, COP vials showed the highest amount of dehydrated cytokine with 18.0 %. A share of 11.42 % dehydrated protein was measured in Multishell® vials and 9.2 % of dehydrated cytokine were detected in glass vials. As observed at lower temperatures, the amount of acetylated cytokine vanished in glass vials and dropped to 4.6 % in COP vials and to 3.4 % in Multishell® vials. In conclusion, around 32 % of native cytokine were found in glass and COP vials and 39.4 % of native cytokine were found in Multishell® vials. In Figure 8 the chemical modifications of the cytokine at molecular level are displayed as determined by LC-MS. Figure 8 is representative for the other vial types and storage conditions. The intensity of the alterations may vary though.

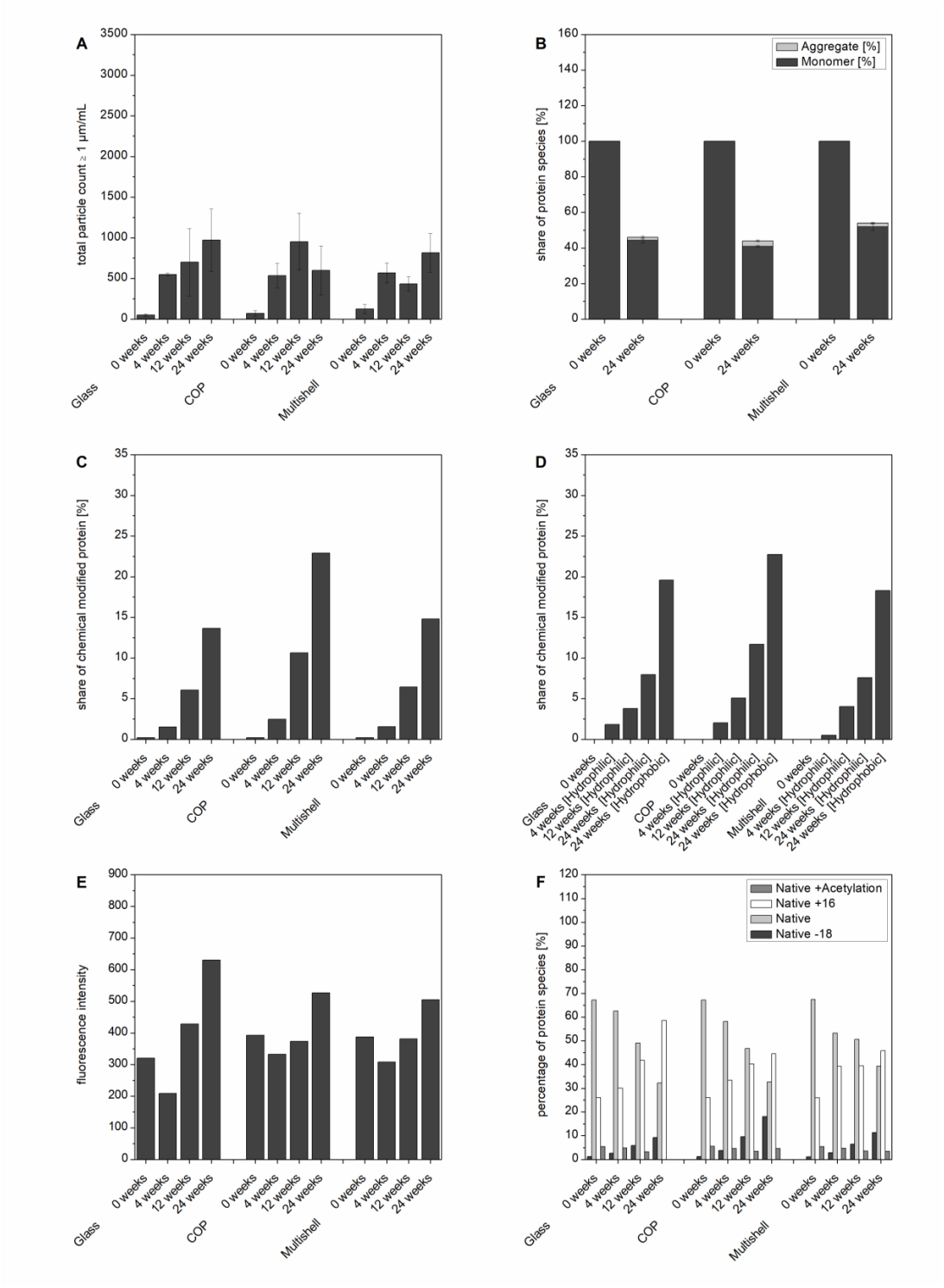

Figure 7: The cytokine was stored at 40 °C for up to 24 weeks in glass, COP and Multishell® vials and the results of six methods are displayed. The total particle count/mL (particles/mL ≥ 1 µm) was determined by MFI (**A**). Soluble cytokine species were analyzed with HP-SEC (**B**) and chemical alterations were quantified with HP-IEX (**C**) and RP-HPLC (**D**). The ABS assay (**E**) and LC-MS (**F**) analysis detected oxidative alterations. Along with oxidation (+ 16 Da) acetylation (+ 42 Da) and dehydration (- 18 Da) were observed by LC-MS.

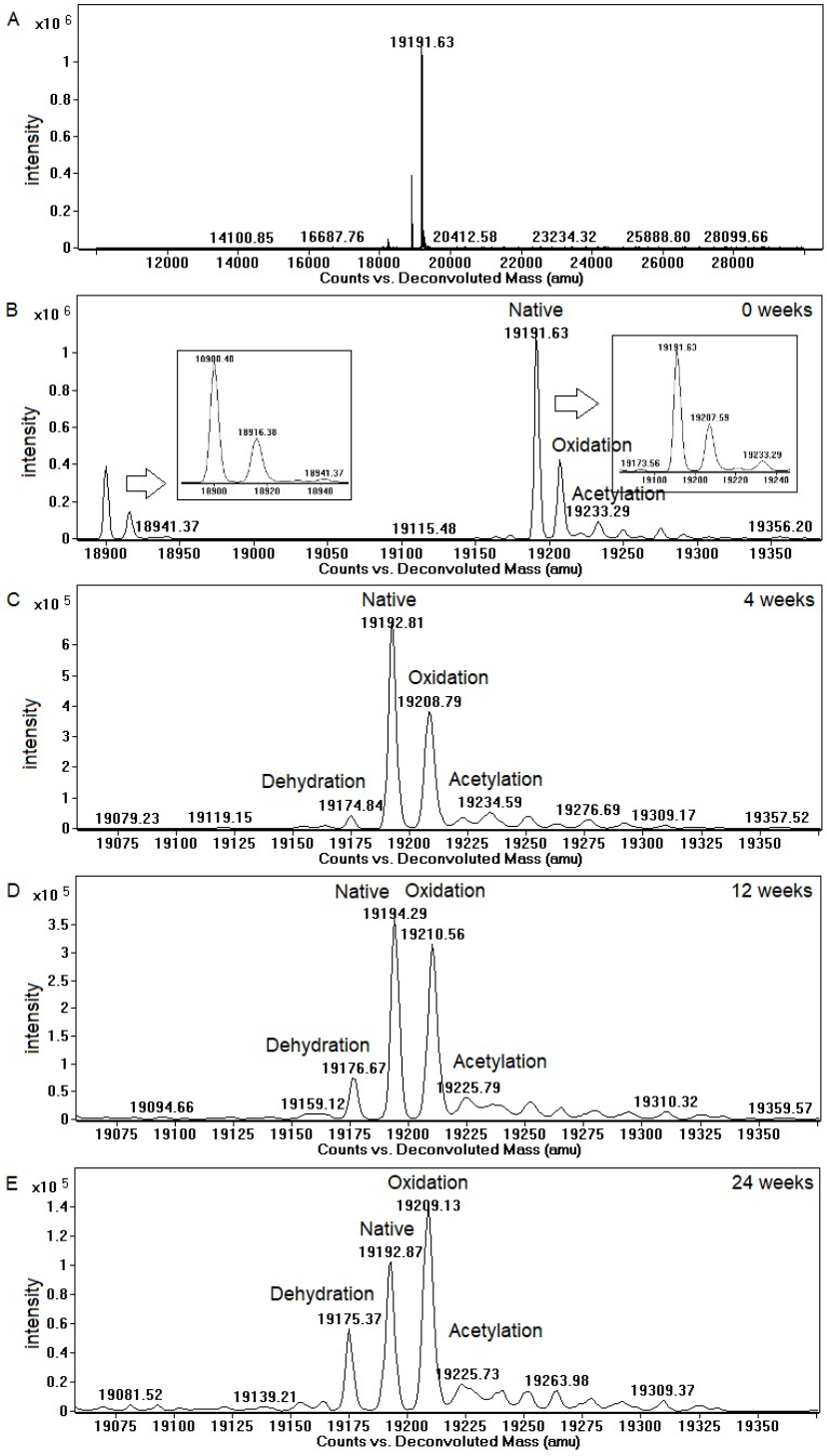

Figure 8: Mass spectrum of N-deglycosylated cytokine stored in COP vials at 40 °C after 0 weeks is displayed (**A**). The magnification (**B**) shows the two main peaks from (A) and enlargements of these two peak groups (marked with a frame) of the cytokine at week 0. Molecular alterations of the main isoform occurring over 4 to 24 weeks at a storage temperature of 40 °C are presented in (**C**) to (**E**). Peaks of interest are labeled based on the mass differences from the native protein.

The particle numbers increased from less than 125 particles/mL to a few hundred with the maximum at 1,500 particles/mL in all vial materials and storage temperatures. Overall, the particle count between the vial materials was comparable. The vial material had only a minor impact on the physical stability of the cytokine. The chemical stability, although differences exist, was to a similar extent negatively impacted in all three vial types. However, at 4°C and 25 °C glass vials showed in LC-MS analysis better results.

Table 2 summarizes the stability changes occurring to the cytokine at all storage conditions.

Table 2: Overview of the quantity of cytokine alterations at 4 °C, 25 °C and 40 °C in three different vial types as determined with nine different methods over the storage period of up to 48 weeks

	Glass Vial	COP Vial	Multishell® Vial
		4 °C	
Visual	O	O	O
MFI	+/++	+	+/++
DLS	O	O	O
HP-SEC	O	O	O
SDS-PAGE	O	O	O
HP-IEX	O	O	O
RP-HPLC	O	O	O
ABS	O	O	O
LC-MS	O	O/+	O/+
		25 °C	
Visual	O	O	O
MFI	+/++	+/++	+
DLS	O	O	O
HP-SEC	O	O	O
SDS-PAGE	O	O	O
HP-IEX	O/+	O/+	O/+
RP-HPLC	O/+	O/+	O/+
ABS	O/+	O	O/+
LC-MS	O/+	+	+
		40 °C	
Visual	O	O	O
MFI	+/++	+	+/++
DLS	O	O	O
HP-SEC	++	++	++
SDS-PAGE	++	++	++
HP-IEX	+/++	++	+/++
RP-HPLC	+/++	++	+/++
ABS	++	+/++	+/++
LC-MS	++	++	++

O = no or minor changes; + = changes; ++ = major changes

5.3.7 Oxygen permeation into nitrogen filled vials

The permeation of oxygen into nitrogen filled glass and plastic vials was monitored for 24 weeks at 4 °C and 40 °C and is listed in Table 3. The oxygen concentration was at its highest in COP vials and at its lowest in glass vials. An elevated temperature led to an increased amount of oxygen within the vial during the same incubation time.

Table 3: Overview of the increase of the oxygen concentration [%] in nitrogen filled glass, COP and Multishell® vials which were stored in at least 75 % oxygen atmosphere over time

4 °C	**Glass**	**COP**	**Multishell®**
0 weeks	0.96 ± 0.34	1.21 ± 0.23	1.13 ± 0.59
1 week	0.80 ± 0.31	9.24 ± 0.23	1.14 ± 0.50
2 weeks	0.64 ± 0.29	15.87 ± 0.18	1.36 ± 0.38
4 weeks	0.58 ± 0.11	26.11 ± 0.23	1.68 ± 0.24
8 weeks	1.39 ± 0.13	41.50 ± 0.34	3.49 ± 0.35
24 weeks	1.63 ± 0.14	53.62 ± 0.31	7.04 ± 0.49

40 °C	**Glass**	**COP**	**Multishell®**
0 weeks	0.96 ± 0.34	1.21 ± 0.23	1.13 ± 0.59
1 week	1.24 ± 0.43	20.38 ± 0.30	2.66 ± 0.60
2 weeks	1.32 ± 0.19	29.55 ± 0.12	3.70 ± 0.28
4 weeks	2.20 ± 0.44	40.68 ± 0.17	6.30 ± 0.28
8 weeks	3.37 ± 0.31	49.93 ± 1.44	10.69 ± 0.54
24 weeks	8.36 ± 0.95	68.12 ± 0.40	21.13 ± 0.89

5.4. Discussion

The study assessed new vial materials for the long-term storage of biopharmaceuticals in comparison to the commonly used glass vials. The impact of the packaging material on the chemical and physical stability of a mAb and a cytokine was determined.

First, the particle level of the protein solutions in the three different vial types is discussed. Second, the focus is on the chemical alterations, in particular of those detected by LC-MS, on the mAb and then on the cytokine. Next, the oxygen barrier properties of plastic vials and possible improvements regarding their oxygen permeability are outlined.

Based on the particle counts of the protein solutions, it is not possible to define the most promising vial material. The number of particles in mAb solutions remained similarly low to the starting point values. In contrast, cytokine solutions had an increase in the particle count by factors up to 26. However, less than 150 particles/mL were quantified in the cytokine solution directly after vial filling and no more than 1,500 particles/mL were found later. In general, the measured particle numbers were low in all three vial materials, also in

comparison to other protein formulations [38-40]. Unlike syringes which can differ in their particle count due to different siliconization techniques or the absence of silicone oil in some polymer syringes [41-43], the vial material apparently had no major impact on the particle count of the protein solutions.

Methionine sulfoxide formation in the Fc region of the mAb was observed at 25 °C after 48 weeks and in particular at 40 °C after 24 weeks as determined by Protein A Chromatography. The ABS assay detected tyrosine and phenylalanine oxidation at 40 °C from week 12 onwards. It is possible to say that the elevated fluorescence intensity measured in the ABS assay is an indicator for an increased amount of oxidized methionine and therefore confirmed the results obtained by Protein A Chromatography. It has already been shown that a quantitative correlation between the amount of methionine sulfoxide, which was quantified by LC-MS, and the concentration of oxidized tyrosine and phenylalanine exists [44]. Further, oxidation was only detected by LC-MS in the heavy chain of the mAb and not in the light chain independent of the storage condition. The mass difference between the native and oxidized species of the mAb, as analyzed by LC-MS, led to conclusions which amino acids were affected by oxidation. This is based on the fact that tyrosine oxidation products with a molecular weight increase of + 14 Da, + 16 Da and + 18 Da can be formed according to a tyrosine oxidation mechanism proposed by Steinmann et al. [36]. LC-MS data matched with the results of the other methods at higher temperatures. Only LC-MS showed major differences between the three vial materials at 4 °C. Generally, for all temperatures glass vials protected the mAb best, followed by Multishell® and COP vials.

All methods beside LC-MS showed a similar chemical stability of the cytokine stored in the three different vial types at lower temperatures. However, at 40 °C cytokine solutions stored in COP vials showed the highest amount of altered protein species in HP-IEX and RP-HPLC analytics. Oxidation of tyrosine and phenylalanine were also indicated by an increase of the fluorescence intensity of the ABS assay. LC-MS revealed at all storage temperatures molecular changes of the cytokine. Besides oxidation of a methionine or a carbohydrate [45], it was in particular at 40 °C noted that deacetylation and dehydration occurred to a high extent. Both mechanisms, deacetylation and dehydration, are temperature dependent [46-48]. Deacetylation of probably an O-acetylated sialic acid took place in the course of longer storage periods and higher temperatures [37]. The observed mass loss of - 18 Da was attributed to dehydration of aspartate [47]. An aspartate-serine motif, as can be found in the amino acid sequence of the cytokine, is known to promote the dehydration of aspartate [47]. The obtained LC-MS data for the cytokine correlated well with literature [47, 48]. The highest

stability of the cytokine was obtained in glass vials at 4 °C and 25 °C. The performance of Multishell® and glass vials at 40 °C was similar to each other and both performed better than COP vials.

Oxidation occurring over the storage period can be caused by oxygen that either derives from the headspace within the vial or permeates into the vial. The container closure integrity of glass vials was excellent as the oxygen permeability study showed. Over 24 weeks at 4 °C the oxygen concentration in nitrogen filled glass vials increased only from 0.96 ± 0.34 % to 1.63 ± 0.14 %, whereas in COP vials the oxygen amount was multiplied by a factor of 44.3. For Multishell® vials the amount of oxygen rose from 1.13 ± 0.59 % to 7.04 ± 0.49 % in 24 weeks. Such a high oxygen permeability of the primary packaging cannot be accepted for oxygen sensitive biopharmaceuticals. That was already concluded by Qadry et al., who calculated a 15 day half life time of the increase of the oxygen concentration in COP vials [23, 29]. Therefore, nitrogen blanketing of the headspace would not work well for COP vials, but could be an option for glass vials. However, other possibilities to use plastic vials as primary packaging materials for oxygen sensitive biopharmaceuticals exist. First, the gas barrier properties can be improved by coating the interior with a silicon dioxide layer [34]. Second, multilayer vials with an incorporated oxygen absorbing resin may prevent oxygen permeation into the container [49]. Third, oxygen absorbers in the secondary packaging can control the amount of oxygen in the container and its packaging [50, 51]. Finally, vials could simply be stored in gas tight aluminum pouches, which provide additional protection from light and could be filled with nitrogen.

5.5. Conclusion

New vial materials as primary packaging materials were evaluated for the long-term storage of biopharmaceuticals and compared to commonly used glass vials. The plastic vials consisted either of a COP monolayer or a COP/polyamide/COP multilayer. The chemical stability of a mAb and a cytokine was best preserved in glass vials. Even at 4 °C, which is the standard storage temperature for biopharmaceuticals, oxidation processes were more pronounced in plastic vials than in glass vials as LC-MS measurements revealed. Regarding the physical stability the particle level was similar and low in all vial types over the investigated period. Plastic vials can provide an alternative for the storage of biopharmaceuticals. However, multilayer vials showing higher gas barrier properties or vials stored in secondary packaging (e.g. aluminum pouches) with controlled oxygen levels should be used.

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Summary

Nowadays, a correlation between non-native protein species and immunogenicity is commonly accepted. Multiple factors such as transportation, storage, handling, drug formulation or primary packaging have been shown to influence the formation of protein aggregates and particles. As protein particles can adversely affect the health of patients, it is of great importance to prevent the formation or to diminish the existent protein aggregates in the final protein drug product. In the present thesis novel concepts were investigated and evaluated to improve the quality and safety of biopharmaceuticals further. First, the application of bedside filtration should lead to increased safety, because virtually any protein particle formed during the shelf life of the drug larger than the pore size of the filter could be removed. Second, the primary packaging material has to maintain the product quality and should not impact the product. However, currently used container materials are not inert and may interact with the drug and its formulation.

In Chapter 1 all approved biopharmaceuticals in Germany have been listed and analyzed for the first time regarding their filtration recommendations. The focus is in particular on the dosage form, the filter membrane and pore size recommendations, the time of filtration – during drug preparation and administration – and the protein concentration, dosing and route of administration. This review revealed that about 16 % of approved biopharmaceutical drugs are filtered during drug preparation or administration. For the majority of these filtered biopharmaceuticals, the manufacturers do not recommend a specific filter membrane. However, if specific filtration instructions are suggested, filters with a polyethersulfone membrane and a pore size of 0.2 µm are predominantly applied. Two thirds of all filtered drugs are lyophilisates with most of them being filtered during drug preparation, whereas solutions are primarily filtered during drug administration.

Chapter 2 shows that bedside filtration with different filters could be expanded to a broad range of protein drug products including proteins with different molecular weights or concentrations. This was substantiated by assessing multiple aspects such as filtration efficacy, filter cleanliness or protein loss due to adsorption. All investigated filters effectively eliminated protein particles. After filtration with a 0.2 µm filter the particle count was always at a low level, independent of thousands of particles in the non-filtered solution. In general, the investigated filters did not contribute to the particle burden as tested with placebo buffers.

Moreover, the concentration of the detected leachables was on a very low level, yet, some filters show higher amounts than others. Protein adsorption did not occur in the majority of all cases, but if a loss of protein was observed it was in the low µg/mL range. Hence, adsorption needs only to be considered for very low dosed biopharmaceuticals. On the other hand rather large hold-up volumes of the filters were found, making most filters used nowadays not applicable to filtration of low volume products. Bedside filtration did not alter the protein structure. Furthermore, measurements showed that the addition of a filter to a syringe did not require substantially more ejection force. Based on these findings an expansion and routine application of filtration of biopharmaceuticals can be proposed.

It was reported in the literature that elevated and elongated intraocular pressures have been observed in patients after injection of aflibercept, bevacizumab and ranibizumab. A higher rise of the intraocular pressure has been detected in particular for ranibizumab in comparison to aflibercept. An increased particle burden in the ranibizumab product was assumed to be the cause for this. To test, if different particle levels in the three biopharmaceuticals can be responsible for the varying levels of the intraocular pressure, a direct comparison of the particle burden of these drugs was performed and the results are presented in Chapter 3. Comparable particle counts were observed for all three proteins, except for one of the two repackaged bevacizumab products.

Silicone oil free cyclic olefin polymer syringes might be beneficial for the storage of biopharmaceuticals, when silicone oil which can foster protein aggregation is absent. In Chapter 4 such polymer syringe was evaluated for the first time over a longer storage period for its application as primary packaging container for biopharmaceuticals. This evaluation revealed the following three findings. First, the major benefit of the silicone oil free system was a significant lower particle count in comparison to glass syringes. The particle level in cyclic olefin polymer systems remained stable over the investigation period, whereas in glass syringes the particle number increased over time. Second, at 4 °C the chemical stability of the two proteins was comparable in polymer and glass syringes. At higher temperatures in particular at 40 °C glass syringes showed the best results regarding the chemical stability of the mAb, whereas polymer syringes stored in nitrogen filled aluminum pouches were identified to be the best packaging configuration for the cytokine. Third, if silicone oil free cyclic olefin polymer syringes were stored in nitrogen filled aluminum pouches, the oxidation problem can be resolved and they could be used as primary packaging for oxygen sensitive biopharmaceuticals.

In Chapter 5 monolayer and multilayer silicone oil free plastic vials were investigated as alternatives for glass vials as primary packaging materials for biopharmaceuticals. Chemical alterations, in particular oxidations, of the protein of interest are stronger pronounced in plastic vials than in glass vials, even at 4 °C. The plastic vials also did not show a benefit regarding lower particle counts. If plastic vials are considered as primary packaging container for oxygen sensitive drugs, multilayer vials with lower gas permeability or oxygen controlled secondary packaging should be used.

The results of this thesis show that bedside filtration and novel primary packaging systems have the potential to contribute significantly to an improvement of the product quality and safety of biopharmaceuticals. Both concepts minimize effectively the number of protein aggregates and therefore protect patients. Hence, they should be considered and implemented in future applications.

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Publications

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Schargus, M.*, Werner, B.P.*, Gerrling G., Winter, G., **CONTAMINATION OF ANTI-VEGF DRUGS FOR INTRAVITREAL INJECTION: How Do Repackaging and Newly Developed Syringes Affect the Amount of Silicone Oil Droplets and Protein Aggregates?.** 2017, RETINA, 9000. Publish Ahead of Print.
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