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Research paper

Development of HSA-free formulations for a hydrophobic cytokine with improved stability

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Abstract

The goal was to characterize a hydrophobic cytokine with respect to oxidation and aggregation, as well as its adsorption to the container at different pH and ionic strength conditions. The tendency of the cytokine to adsorb on surfaces and its low solubility at physiological pH were the main challenges during the development of HSA-free formulations for the cytokine. When the formulation pH exceeded 5.5 precipitation led to significantly higher turbidity. This turbidity increase and elevated aggregation as determined by HP-SEC and DLS was more pronounced at higher glycine and NaCl concentrations. With rising pH protein adsorption was more distinct compared to pH 3.0. However, protein adsorption could be minimized by polysorbate 20 or the use of glass type I^+. FTIR revealed a reduced thermal stability at higher pH values indicated by a declining denaturation temperature. Five liquid formulations in the pH range 3.5–4.5 and five lyophilized formulations at pH 4.0–5.0 were stored for 6 months and the stability was evaluated with respect to aggregation and chemical modification. Liquid formulations at pH 3.5–4.0 and lyophilized formulations at pH 4.0–5.0 were most stable during 6 months at 2–8 °C.

Keywords: Hydrophobic cytokine; HSA-free formulation; Protein adsorption; Stability

1. Introduction

One critical step for the application of proteins as pharmaceutical products is the development of stable formulations. It is a great challenge to maintain the native and functional structure of a protein during pharmaceutical processing, production, storage and the final application to the patient. Many proteins that are used as pharmaceuticals, e.g., interferons, interleukins or growth factors, are considered as hydrophobic, with the consequences of limited solubility, e.g., 1.5 mg/ml for G-CSF in PBS at pH 7.0 [1] or 0.05 mg/ml for rhINF-β-1b at physiological pH [2]. Although these proteins are often employed in therapeutical doses of a few micrograms per milliliter the low solubility and the tendency of hydrophobic proteins to adsorb to surfaces are major challenges during formulation development. For example, the use of higher concentrated bulk solutions is not possible and protein adsorption to filters, tubes or containers during manufacturing, as well as to the primary packing material, can lead to loss of active protein. One possible way is the formulation of hydrophobic proteins with Human Serum Albumin (HSA), which is an excellent stabilizer in liquid and lyophilized formulations [3]. Besides its function as lyo- and cryoprotector, it can minimize the loss of active protein due to adsorption, which poses a problem especially for low dose formulations of hydrophobic proteins [3–5]. However, HSA as excipient is generally obtained from human plasma and bears the problems associated with human blood derived products e.g., the risk of blood born pathogens. Recombinant HSA (rHA) as possible alternative is not routinely used in the field of protein formulation, due to the still high

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material costs. HSA itself exhibits a low risk of immunogenicity, whereas in presence of a second protein the formation of mixed aggregates which was e.g., shown for recombinant human interferon-α [6] can lead to immunogenicity reactions. Other issues emerging for HSA-containing formulations are the difficulties occurring during the development of analytical methods for the active protein in the formulations. With HSA, a second protein typically in excess compared to the active protein is present, which makes selective analytics of the active protein, as well as its aggregates and degradation products difficult and often impossible. For these reasons, HSA is avoided in the development of new formulations as much as possible.

We used a non-glycosylated, recombinant human cytokine with a molecular mass of about 19 kDa and an isoelectric point of 9.2 for the experiments. The cytokine is characterized by a GRAVI score of 1.8, which is the average hydropathy of all amino acids in the sequence, with a value of 4.5 for maximum and a value of −4.5 for minimum hydropathy [7]. Our objective was to characterize the aggregation, oxidation and adsorption behavior of a hydrophobic cytokine with the goal to stabilize the cytokine in a HSA-free formulation. In preformulation studies formulation buffer and pH were optimized to assure solubility and structural stability of the cytokine. Changes of the cytokine as a function of pH, ionic strength and temperature were evaluated by high pressure size exclusion chromatography (HP-SEC), dynamic light scattering (DLS) and nephelometry, as well as FTIR-spectroscopy. The impact of pH, NaCl, glycine and the presence of surfactants on the degree of protein adsorption was rated for different glass types. Depending on the results of the preliminary studies five liquid formulations and five lyophilized formulations were to be selected and their stability was evaluated over six months at 2–8 °C, 25 °C/60% RH and 40 °C/75% RH.

2. Materials and methods

2.1. Materials

The starting material contained 1.2 mg/ml cytokine in 20 mM glycine at pH 3.0. For the final formulations the starting material was diluted to a cytokine concentration of 0.25 mg/ml with 20 mM glycine, pH 3.0, which contained the particular excipients. Subsequently, the pH was adjusted with NaOH and HCl.

Sucrose from Suedzucker (Mannheim, Germany), mannitol from Caelo (Hilden, Germany), polysorbate 20 from Serva (Heidelberg, Germany) and glycine and NaCl from Sigma (Steinheim, Germany) were used without further purification.

2R glass vials (standard borosilicate glass type I and SCHOTT type I plus®) were a donation of Schott AG (Mainz, Germany). The vials were closed with unsiliconized butyl-lyophilization stoppers (Firma West, Eschweiler, Germany). BD Hypak SCF™ glass prefilled 1 ml syringes were a donation of Becton Dickinson GmbH (Heidelberg, Germany).

2.2. Nephelometry

The NEPHLA turbidimeter (Dr. Lange, Düsseldorf, Germany), which operates at 860 nm and detects the scattered light at 90° angle, was used for nephelometry. The turbidity of the samples was measured without filtration or dilution steps. The system is calibrated with formazine as standard and the results are given in formazine nephelometric units (FNU), which is equivalent to the units NTU (Nephelometric Turbidity Unit), FAU (Formazine Attenuation Units) or FTU (Formazine Turbidity Unit). To evaluate the degree of turbidity the reference solutions I–IV of the European Pharmacopoeia method 2.2.1 (clarity and degree of opalescence of liquids) were used [8]. A stock solution was prepared by mixing 10 mg/ml hydrazine sulfate (Sigma–Aldrich, Steinheim, Germany) and 100 mg/ml hexamethylenetetramine (Sigma–Aldrich, Steinheim, Germany) at a ratio of 1:1. After 24 h the stock solution was diluted with water to concentrations of 0.075 mg/ml hydrazine sulfate and 0.75 mg/ml hexamethylenetetramine. The reference solutions were prepared with the diluted solution according to Table 1 and the turbidity in FNU was measured.

2.3. Determine protein adsorption using UV-spectroscopy

To determine the degree of protein adsorption 1.0 ml of the different cytokine formulations at a concentration of 0.25 mg/ml was stored at 2–8 °C in the respective container. The loss of protein was determined by UV-spectroscopy using the Thermo Spectronic UV 1 (Thermo Electron Cooperatrion, Dreieich, Germany). The UV-absorption was measured in standard quartz cuvettes (pathlength 10 mm, fill volume 2 ml) at 280 nm and the protein content calculated by dividing the measured absorption by 1.7, the extinction coefficient for the cytokine. To avoid adsorption effects onto the quartz cuvettes, the cuvettes were saturated with cytokine solution in advance. To achieve this, the cuvettes were filled with 2 ml of 0.25 mg/ml cytokine solution of the particular pH for about one hour. Between the measurements the cuvettes were rinsed with buffer only.

Table 1
Composition of the opalescence reference solutions according to Ph. Eur. and corresponding turbidity

Reference solution	Diluted stock solution (ml)	Water *R* (ml)	Turbidity (FNU)	Degree of opalescence
I	5	95	3.2	Clear (⩽Ref I)
II	10	90	6.1	Slightly opalescent (⩽Ref II)
III	30	70	17.8	Opalescent (⩽Ref III)
IV	50	50	29.4	Very opalescent (⩽Ref IV)

2.4. High pressure size exclusion chromatography (HP-SEC)

Protein aggregation was determined by HP-SEC on a HP 1100 (Agilent Technologies, Waldbronn, Germany). A TSKgel G3000SWXL column (Tosoh Biosep, Stuttgart, Germany) with an adequate guard column was used. Twenty microliters of the protein sample was injected into the HPLC. The samples were not filtered or diluted prior to the analytics. Within the HPLC a 0.2 μM online filter was used. The running buffer was composed of 175 mM NaH_2PO_4 and 0.1% SDS at pH 6.8. The analytics were performed at a flow-rate of 0.5 ml/min with UV-detection at 210 nm.

2.5. Reversed phase high pressure liquid chromatography (RP-HPLC)

To determine oxidized cytokine, RP-HPLC was performed using a Jupiter C4 column with 300 Å 5 μm 250 * 4.6 mm i.d. and a security guard C4, 4 * 3 mm (Phenomenex, Aschaffenburg, Germany). A flow-rate of 1.0 ml/min was used with UV-detection at 214 nm. An elution gradient was applied, using 10% acetonitrile with 0.1% trifluoroacetic acid and 100% acetonitrile with 0.1% trifluoroacetic acid as eluents according to Geigert at al. [9]. Within the RP-chromatogram a met-oxidized form of the cytokine is eluting prior to the main peak followed by several peaks, which can be assigned to oligomerized protein [9].

2.6. Dynamic light scattering (DLS)

DLS performed on a Zetasizer Nano (Malvern, Herrenberg, Germany) was used to characterize the protein molecules and particles in the range from 1 nm to 1 μm. The samples were not diluted or filtered prior to the measurements. The measurements were conducted at 25 °C in the manual mode using 20 sub runs of 10 s. The size distribution by intensity and by volume was calculated from the correlation function using the multiple narrow mode of the Dispersion Technology Software version 4.00 (Malvern, Herrenberg, Germany).

2.7. Attenuated total reflection-FTIR spectroscopy (ATR-FTIR)

FTIR-spectra were measured with the Tensor 27 (Bruker Optics, Ettlingen, Germany) using a Bio-ATR unit with a ZnSe crystal with 7 reflexions and a transmission cell. The spectra were recorded from 4000 to 850 cm^{-1} in the attenuated total reflectance (ATR) mode, respectively, transmission mode at controlled temperatures. Each spectrum represents the average of 240 scans. After the analysis, the particular buffer spectrum was manually subtracted from the protein spectrum. The absorption spectra were further processed by an off-set correction and the second derivatives additionally by vector normalization. To determine the melting temperature of the cytokine spectral changes were monitored from 20 to 90 °C for formulations with 1.2 mg/ml cytokine in 20 mM glycine between pH 3.5 and 5.5. Twenty microliters of the sample was placed into the BioATR unit and the system was hermetically closed to overcome evaporation. Temperature was ramped from 20 to 90 °C using 5 °C steps with an equilibration of time of 120 s at each temperature prior to the measurement. The structural similarity between the second derivatives of the spectra was calculated using the spectral correlation coefficient r according to Wang et al. [10,11].

2.8. Lyophilization

Thousand microliters of the formulations was lyophilized in 2R vials in the Epsilon 2–12D freeze-drier (Martin Christ, Osterrode, Germany). The samples were frozen to −50 °C with a standard cooling rate of 0.45 °C/min and kept at −50 °C for 2 h. Primary drying was conducted at a shelf-temperature of −15 °C with a pressure of 0.045 mbar for 20 h. For secondary drying the shelf-temperature was increased to 40 °C for 10 h. The vials were closed under nitrogen atmosphere at a pressure of 800 mbar.

2.9. Karl–Fischer titration

The residual moisture of the samples was analyzed by coloumetric Karl–Fischer titration using the Aqua 40.00 titrator with a headspace module (Analytik Jena AG, Halle, Germany). The headspace method was validated against conventional Karl–Fischer titration, where anhydrous methanol was used to extract water from the lyophilized products. For the measurement at least 10 mg of the lyophilized sample was heated to 80 °C for 10 min. The evaporated water was transferred into the titration solution and the amount of H_2O was determined. As reference material apura Water Standard Oven 1% (Merck, Darmstadt, Germany) was used.

2.10. X-ray powder diffraction (XRD)

The morphology of the lyophilized products was analyzed with X-ray powder diffraction (XRD) from 5° to 40° $2-\Theta$, with steps of 0.05° $2-\Theta$ and a duration of 2 s per step on the X-ray diffractometer XRD 3000 TT (Seifert, Ahrenburg, Germany), equipped with a copper anode (40 kV, 30 mA, wavelength 154.17 pm).

2.11. Long-term stability

Liquid formulations were stored in siliconized prefilled glass syringes (Becton Dickinson GmbH Heidelberg, Germany) and lyophilized formulations in 2R vials glass type I^+ (Schott AG, Mainz, Germany). The formulations were stored at 2–8 °C, 25 °C/60% RH and 40 °C/75% RH. After 1, 3 and 6 months all samples were analyzed

by HP-SEC, RP-HPLC and DLS. The lyophilized samples were furthermore analyzed by Karl–Fischer titration and XRD.

3. Results and discussion

3.1. Impact of pH, glycine and NaCl concentrations on cytokine aggregation

3.1.1. Nephelometry

The selection of the optimum buffer composition and formulation pH is a prerequisite for the subsequent formulation development. As protein solubility is influenced by pH and ionic strength this point is especially crucial for hydrophobic proteins due to their often limited solubility. For formulations with 0.25 mg/ml cytokine the glycine concentration was varied between 5 and 50 mM. Furthermore, it was evaluated how cytokine aggregation is influenced by NaCl, which is frequently used to adjust the isotonicity. The formulation pH was step-wise increased from 3.0 to 9.0 by adding NaOH and the turbidity was monitored (Fig. 1). When using 5–20 mM glycine, the turbidity ranged from 1 to 3 FNU at a pH between 3.0 and 5.0. The solutions were less turbid than reference solution I (3.2 FNU) of the European Pharmacopoeia and the degree of opalescence was evaluated as clear. At these conditions the cytokine exhibited an adequate good solubility. Precipitation of the cytokine, noticeable by a steep increase in turbidity, was observed when the pH was raised above 5.5 for all studied glycine concentrations. The turbidity increase was ascribed to the declining solubility of the cytokine, when the formulation pH approached the pI of the cytokine, which is located at pH 9.2 similar to the protein described by Hershen et al. [12]. At its isoelectric point a protein exhibits a net charge of zero and with it the lowest solubility in aqueous media [13]. With increasing glycine concentrations the onset of the turbidity increase was shifted to lower pH values and maximum values above 150 FNU were measured. While samples with 5–20 mM glycine reached maximum turbidity values between 100 and 150 FNU at pH 9.0, maximum values of 200 FNU were measured at 50 mM glycine (Fig. 1). When adding NaCl to formulations containing 20 mM glycine, as could be relevant for isotonicity adjustment, a similar turbidity profile over the pH range was achieved for 0.1% (17 mM) and 0.2% (34 mM) NaCl. However, with increasing NaCl concentration the onset of the turbidity increase was shifted to lower pH-values. Turbidity was exceeding 3.2 FNU at approximately pH 4.5 for 0–0.2% NaCl, whereas it exceeded 3.2 FNU already at pH 3.6 for 0.9% NaCl. Therefore, NaCl is not suitable for isotonicity adjustment of the formulations.

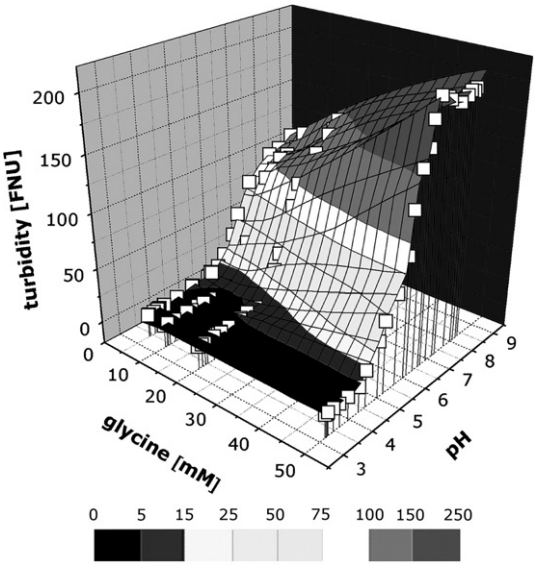

Fig. 1. Turbidity of 0.25 mg/ml cytokine in 5–50 mM glycine from pH 3.0 to 9.0.

3.1.2. Determination of dimers and trimers by HP-SEC

The bulk solution of 1.2 mg/ml cytokine in 20 mM glycine at pH 3.0 contained about 3.8% dimers and trimers as determined by HP-SEC. For the formulations with 0.25 mg/ml cytokine in 20 mM glycine, aggregation determined by HP-SEC was augmented from 3.6% dimers and trimers at pH 3.0–4.4% at pH 5.5. The dilution step to a concentration of 0.25 mg/ml did not significantly increase the aggregation level at pH 3.0. However, it was obvious that higher pH values fostered the formation of dimers and trimers. Above pH 5.5 the fraction of dimers and trimers relative to the monomer peak further stepped up to 6.0%. At the same time, a substantial decrease of the total AUC of the HP-SEC chromatogram by 10% at pH 5.5 was monitored, indicating the presence of larger aggregates. Larger aggregates, as well as precipitated protein, are retained by the guard column and are therefore not detected by HP-SEC. The larger aggregates, on the other hand, were detected by nephelometry showing high turbidity values above pH 5.5 (Fig. 1). This reveals the weak point of HP-SEC in the detection of larger aggregates and shows that it is essential to use several methods to achieve a comprehensive characterization of the formulation.

For formulation development of the cytokine the pH range from 3.0 to 4.5 appeared to be suitable, with 5–20 mM glycine without further addition of NaCl being beneficial with respect to turbidity and aggregation determined by HP-SEC.

3.1.3. Dynamic light scattering

To gain comprehensive insight into aggregation within a particular formulation the use of more than one technique is obligatory [14]. Mahler et al. showed that DLS, Bionephelometry and light obscuration were capable to detect different types of aggregates in monoclonal IgG1 antibody formulations upon mechanical stress in relation to surfactant concentration [15]. Aggregation of the cytokine was therefore monitored by DLS as additional technique

besides the already described HP-SEC and nephelometry. Changes in the size distribution by volume from pH 3.0 to 4.5 were studied for formulations with 0.25 mg/ml cytokine in 20 mM glycine (Fig. 2a). The cytokine monomers were reflected by the main peak at a diameter of 5.1 nm in the volume-based size distribution. In comparison, for β-lactoglobulin, a protein with a similar molecular weight of 18.5 kDa the monomer peak was located at a diameter of 5.4 nm [16]. The first peak, representing primarily monomeric protein, is rather distinct as more than 95% of the protein is present in the monomeric state, according to HP-SEC. The second broader peak with a maximum at 13.5 nm could be assigned to aggregated protein [17]. Thereby, it has to be kept in mind that a well-defined separation of monomers and dimers, respectively, dimers and trimers is generally not possible with DLS, as the size resolution of the method is too low. The radii of particles have to differ by a factor higher than 2, the mass, respectively, by a factor of 8 in order to be resolved as two distinct peaks [18]. Therefore, the different aggregate sizes result in one broad peak within the size distribution. However, the change of the size distribution can be used to qualitatively evaluate cytokine aggregation e.g., at different pH conditions. When the pH was raised from 3.0 to 4.5, the intensity of the peak at 13.5 nm significantly increased from 1.0% at pH 3.0–4.0% at pH 4.5 (Fig. 2b). A similar increase from 1 FNU at pH 3.0–3.6 FNU at pH 4.6 was determined by nephelometry. HP-SEC only revealed a slight increase in aggregation for the cytokine formulations from pH 3.0 to 4.5. The data demonstrated that the tendency of increasing aggregation upon raising the pH from 3.0 to 4.5 was reflected in the DLS size-distribution by volume and nephelometry more clearly compared to HP-SEC. Generally, DLS appears to be a sensitive tool for formulation development comparable to nephelometry, detecting subtle changes of the formulation. Based on these results, DLS was selected as analytical method for the stability studies.

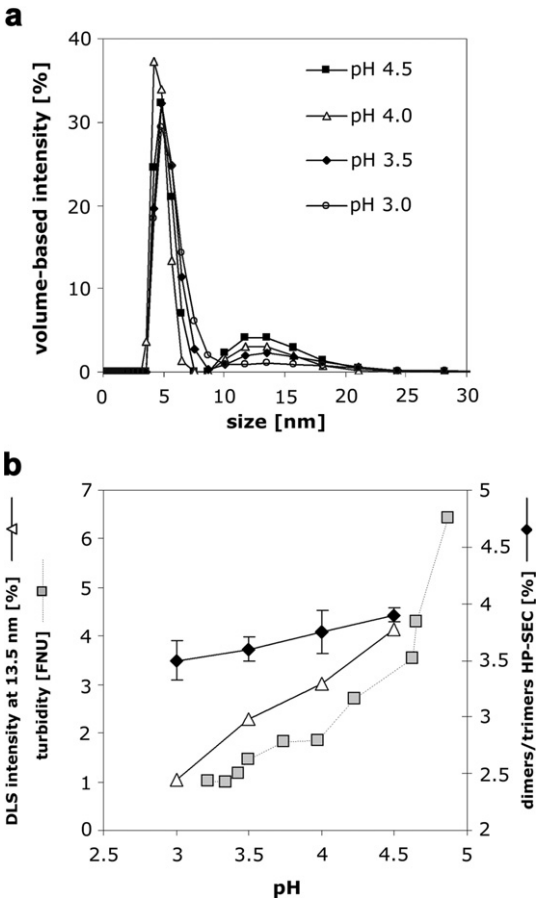

Fig. 2. DLS size distribution by volume (a) and maximum intensity at 13.5 nm determined by DLS, turbidity, respectively, dimers and timers determined by HP-SEC (b) of 0.25 mg/ml cytokine in 20 mM glycine for pH 3.0–4.5.

3.2. Temperature induced changes of the cytokine

Differential scanning calorimetry (DSC) is typically used in preformulation studies to determine the melting temperature (T_m) of proteins [19]. However, for the cytokine DSC was not capable to detect a melting temperature between pH 3.0 and 5.0, as the unfolding of the cytokine coincided with rapid aggregation. This aggregation interfered with the measurement and resulted solely in an exothermal shift of the baseline. Therefore, FTIR-spectroscopy was evaluated as alternative method to characterize temperature induced changes of the cytokine. For the FTIR measurement a higher cytokine concentration of 1.2 mg/ml was used as the intensity of the spectra was too low concentration of 0.25 mg/ml.

Initially, ATR-spectra of the cytokine were compared to transmission spectra and both set-ups resulted in similar spectral intensities at pH 3.0 (Fig. 3a). From the second derivatives, however, it became evident that the band intensity at 1619 cm^{-1} was more pronounced in the ATR than the transmission mode (Fig. 3c). This indicates structural differences at pH 3.0 between the adsorbed protein, which is mainly detected by the ATR mode, and the protein in solution measured by the transmission cell. At pH 5.0, ATR and transmission spectra differed only in their total spectral intensities. Here a 5-fold higher intensity was measured in the ATR-mode compared to the transmission mode (Fig. 3b). However, when comparing the second derivatives of the ATR and transmission spectra at pH 5.0 comparable results were seen (Fig. 3d) and adsorbed cytokine and the cytokine in solution exhibit similar conformations. Furthermore, the higher spectral intensity at pH 5.0 is indicative for more pronounced adsorption of the cytokine at pH 5.0 which is further discussed in Section 3.3. Adsorption per se can be measured with ATR-FTIR, which was described for human calcitonin by Bauer et al. who found that the intensity of the amide I band increased by a factor of 3.4 due to adsorption [20].

Fig. 4 provides an overview on the cytokine absorption spectra measured at 20 °C. At 20 °C the absorption spectra at pH 3.5 exhibited a distinct peak at 1619 cm^{-1} in the amide I region, while only a shoulder was present at pH

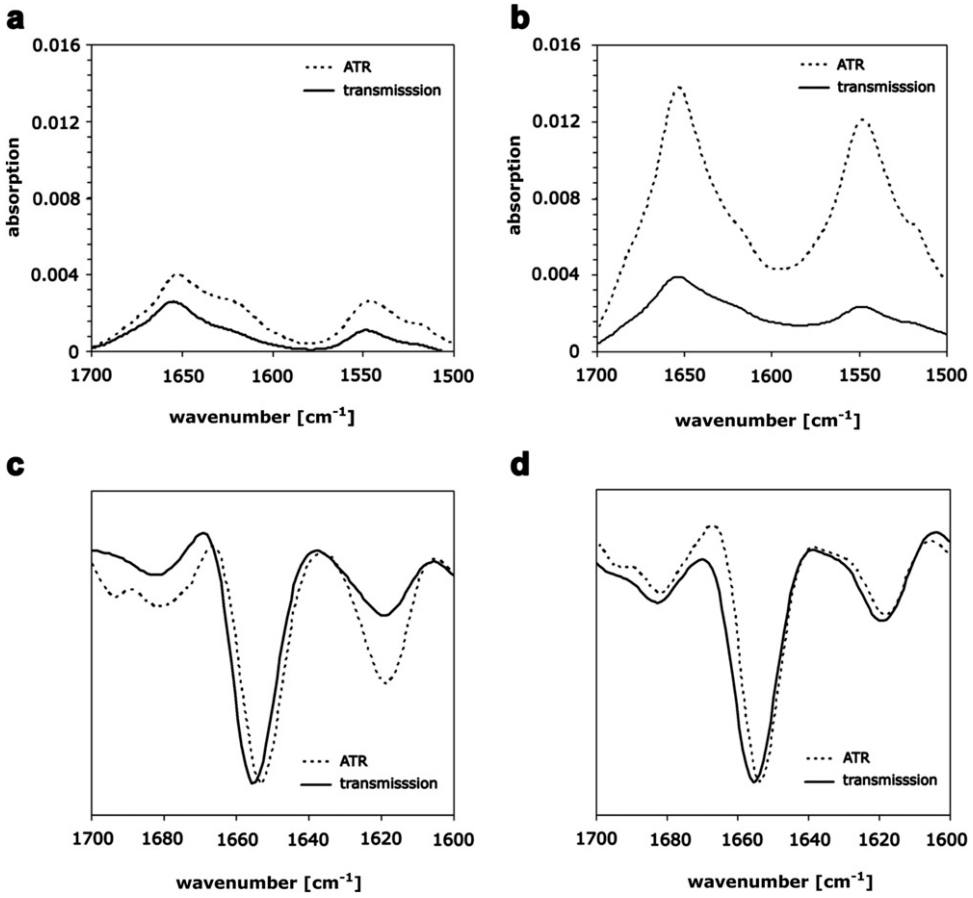

Fig. 3. Comparison of ATR-FTIR and FTIR-transmission measurements of 1.2 mg/ml cytokine in 20 mM glycine showing the absorption spectra at pH 3.0 (a) and 5.0 (b), as well as the second derivatives of the amide I band at pH 3.0 (c) and 5.0 (d). The ordinate of (c) and (d) is comparable.

4.2 and 5.5. A peak at 1619 cm^{-1} can be typically assigned to intermolecular β-sheet structures often due to aggregation, while a peak at 1653 cm^{-1} can be ascribed to α-helical structures [21–23]. An intensity increase at 1618 cm^{-1} for myoglobin [24], at 1615 cm^{-1} for BSA [22], at 1620 cm^{-1} for HSA [10] and at 1614 cm^{-1} for interferon-γ [25] was detected when intermolecular β-sheets were formed within aggregation processes.

For the cytokine ATR-FTIR detected an elevated intensity at 1619 cm^{-1} at pH 3.0 (Fig. 3a). However, HP-SEC pointed at a higher degree in aggregation with 4.5% dimers and trimers at pH 5.5, compared to only 3.5% dimers and trimers at pH 3.0, which was supported by turbidity and DLS analysis. Therefore, the band at 1619 cm^{-1} at pH 3.0 could not solely be ascribed to intermolecular aggregation of the cytokine in solution but most likely to adsorbed material. This was furthermore supported by the differences between ATR and transmission spectra at pH 3.0 (Fig. 3c). Above pH 3.8 ATR and transmission mode offered comparable results (data not shown).

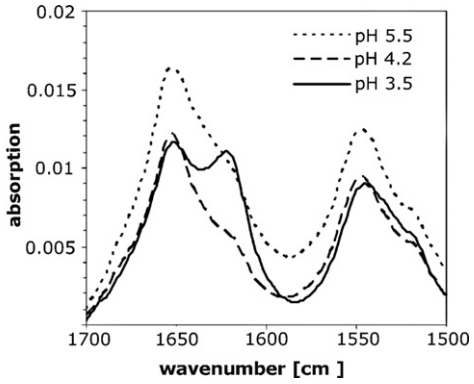

Fig. 4. ATR-FTIR absorption spectra of 1.2 mg/ml cytokine in 20 mM glycine at pH 3.5, pH 4.2 and pH 5.5 determined at 20 °C.

At pH 3.5 the intensity at 1619 cm^{-1} decreased to a lesser extent than the peak at 1653 cm^{-1} with increasing temperature, pointing at the formation of new β-sheet structures, potentially due to aggregation (Fig. 5a). At pH 4.2 the intensity at 1619 cm^{-1} was increased, while a slight reduction of overall intensity was measured in the areas of α-helical peak at 1653 cm^{-1} and the amide II band of the adsorption spectra when the temperature was raised from 20 to 90 °C (Fig. 5b). At pH 5.5, the spectral intensity raised with temperature demonstrating that protein was precipitating which was confirmed by the turbid nature of the solutions after the measurement (Fig. 5c). In particular, a tremendous increase of the peak at 1619 cm^{-1} comparable to pH 4.2 was obvious at pH 5.5.

Different methods can be applied to determine the denaturation temperature of a protein from the FTIR spectra

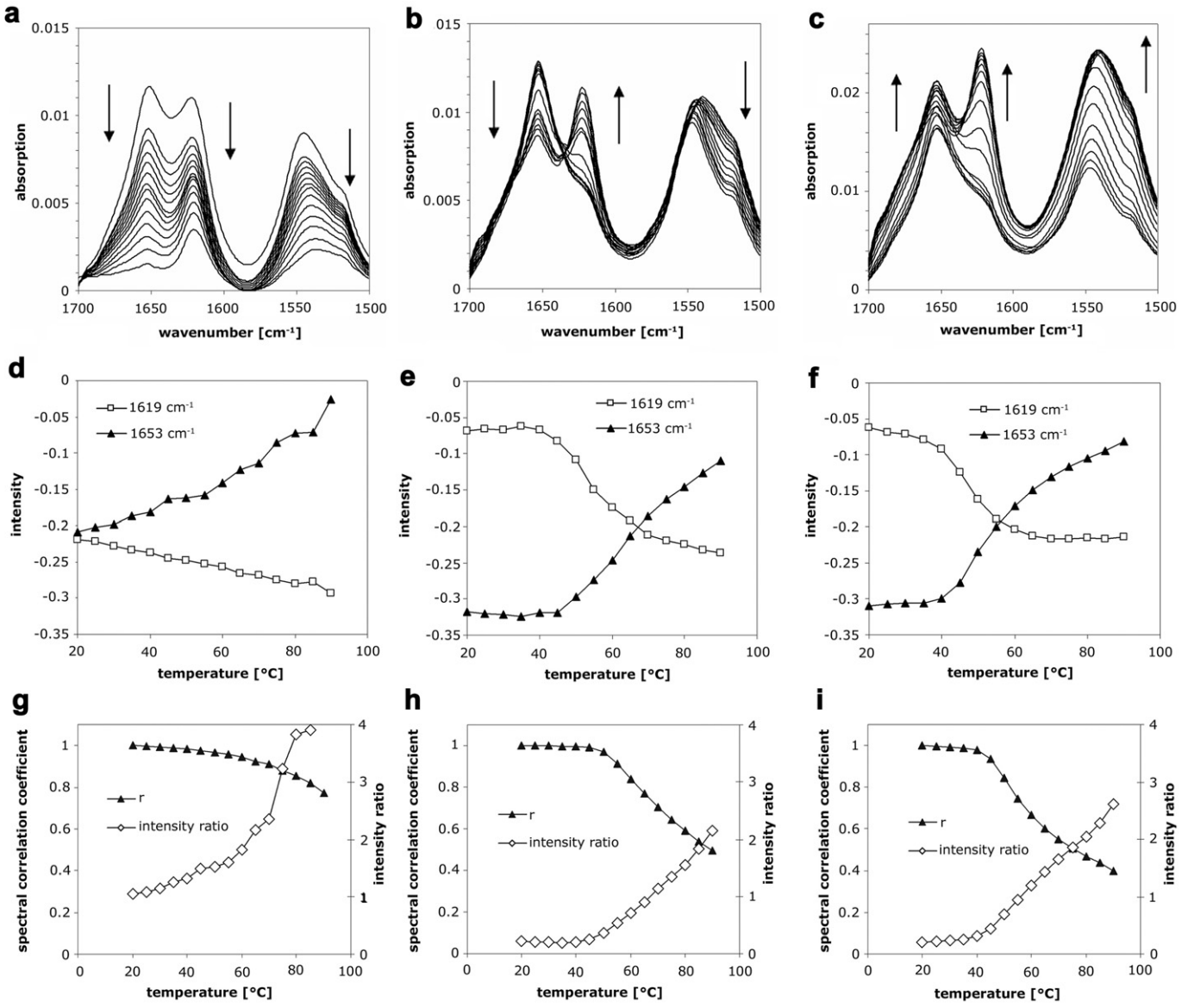

Fig. 5. ATR-FTIR absorption spectra of 1.2 mg/ml cytokine in 20 mM glycine at pH 3.5 (a), pH 4.2 (b) and pH 5.5 (c). The arrows mark the changes upon temperature increase from 20 to 90 °C. Change of the intensity of the second derivative of the ATR-FTIR spectra at 1653 cm^{-1}, respectively, at 1619 cm^{-1} at pH 3.5 (d), pH 4.2 (e), and pH 5.5 (f) and intensity ratio of 1619–1653 cm^{-1}, respectively, the spectral correlation coefficient *r* at pH 3.5 (g), pH 4.2 (h) and pH 5.5 (i) in the temperature range from 20 to 90 °C.

[26]. One possible approach is plotting the intensities of the α-helical, respectively, β-sheet bands over the changing temperature. Generally, the infliction point of the plotted curves is considered as the denaturation temperature of the protein. This was described by Meersman et al. for Myoglobin using the bands at 1624 and 1560 cm^{-1} [24] and by Dong et al. for Factor XIII using the bands at 1641 and 1626 cm^{-1} [27]. For the cytokine the intensity of the bands at 1619 and 1653 cm^{-1} in the second derivative was monitored from 20 to 90 °C (Fig. 5d–f). Wang et al. used the spectral correlation coefficient to rate the similarity between different spectra [10,11]. According to their method, the spectral correlation coefficient *r* was calculated for the amide I band of the cytokine using the wavenumbers from 1700 to 1600 cm^{-1}, setting the spectra at 20 °C as reference spectra and the spectra at higher temperatures as sample spectra. Furthermore, the intensity ratio of 1619–1653 cm^{-1}, comparable to Goosens et al. [25], was plotted and compared to the spectral correlation coefficient (Fig. 5g–i). A summary of the denaturation temperatures resulting from the different approaches is shown in Table 2.

At pH 3.5 the initial intensities of the α-helical band at 1653 cm^{-1} and the β-sheet band at 1619 cm^{-1} were similar (Fig. 5d). With increasing temperatures a continuous, almost linear, intensity decrease at 1653 cm^{-1}, respectively, intensity increase at 1619 cm^{-1} was monitored. A higher initial intensity was measured for the band at 1653 cm^{-1} compared to 1619 cm^{-1} at pH 4.2 and pH 5.5 (Fig. 5e and f). After a constant phase between 20 and 40 °C, the

Table 2
Denaturation temperatures (T_m) for the cytokine at pH 3.5, 4.2 and 5.5 determined with different approaches by FTIR

	Intensity 1619 cm^{-1} (°C)	Intensity 1653 cm^{-1} (°C)	Ratio 1619 cm^{-1} to 1653 cm^{-1} (°C)	Spectral correlation coefficient r (°C)	T_m (average) (°C)
pH 3.5	–	–	74	–	74
pH 4.2	53	60	55	60	57
pH 5.5	50	47.5	50	50	49

shape of the curve changed to sigmoid with a clear inflection point. This revealed a higher degree of temperature induced structural alteration at pH 4.2 and 5.5 compared to pH 3.5. At pH 3.5 it was possible to detect an inflection point for the intensity ratio offering a value of 74 °C as denaturation temperature (Fig. 5g). For pH 4.2 and pH 5.5 the spectral correlation coefficients, respectively, intensity ratios remained unchanged from 20 to 45 °C, which is relevant for the storage of the formulations (Fig. 5h and i). When increasing the pH from 4.2 to 5.5 the structural changes were more distinct and the melting temperature declined from 57 °C at pH 4.2–49 °C at pH 5.5. The melting temperature can be indicative for the stability of the protein during stability studies, especially at 25 °C and 40 °C storage. The results showed that FTIR spectroscopy can be used as an alternative method to determine the melting temperature of a protein, e.g., when the use of DSC is not possible. It is furthermore advantageous that FTIR also provides information on the structural changes of the protein with increasing temperature.

3.3. Cytokine adsorption to vials

Protein adsorption is a complex process [28] and can be impacted by protein concentration, temperature, pH, ionic strength, the presence of further excipients and the selection of the container material [29–31]. Due to the hydrophobicity of the cytokine its adsorption behavior was evaluated within the preformulation. Our intention was not to elucidate the adsorption mechanism of the cytokine but to evaluate the degree of adsorption under various conditions and its importance for formulation development.

Fig. 6a shows that the decline in protein concentration at pH 3.0 was less distinct for 20 mM glycine than for 10 mM glycine. In glass type I^+ vials the cytokine concentration dropped to 97.2% for 20 mM and 90.8% for 10 mM glycine after 72 h. About 3% more protein was lost in glass type I vials compared to glass type I^+ vials for the same cytokine formulations. After 24 h the adsorption process reached an almost constant level. The surface of glass type I^+ is coated with an extra layer of silicia resulting in a rougher surface which is expressed by a line roughness Ra (Ra = arithmetic average of the absolute values of the measured profile height deviations) of about 0.4 nm for glass type I^+ compared to only 0.1 nm for normal glass type I determined by atomic force microscopy (AFM) [29]. Furthermore, Schwarzenbach et al. demonstrated with AFM that the adhesion force of interferon-α-2a (INF-α-2a) was reduced by 40% when using glass type I^+ instead of normal borosilicate glass type I [29]. Typically glass surfaces are negatively charged due to the dissociation of silicol groups in aqueous media. A positively charged glass surface is only existent at very acidic pH below 2 [32]. In the pH range from 3.0 to 4.5 a slight increase in negative charge of the glass surface can be assumed. The cytokine, with a pI of 9.2, should tend to a slightly higher positive charge when the pH is lowered from 4.5 to 3.0. The same tendency of an increased positive net charge density at pH 3.0 can be hypothesized for glycine. Thus, a possible explanation for the less pronounced adsorption at higher glycine concentrations could be a shielding of the negatively charged glass surface by glycine.

The addition of surfactants presents an effective approach to reduce and inhibit protein adsorption on surfaces. By adding polysorbate 20 the adsorption of

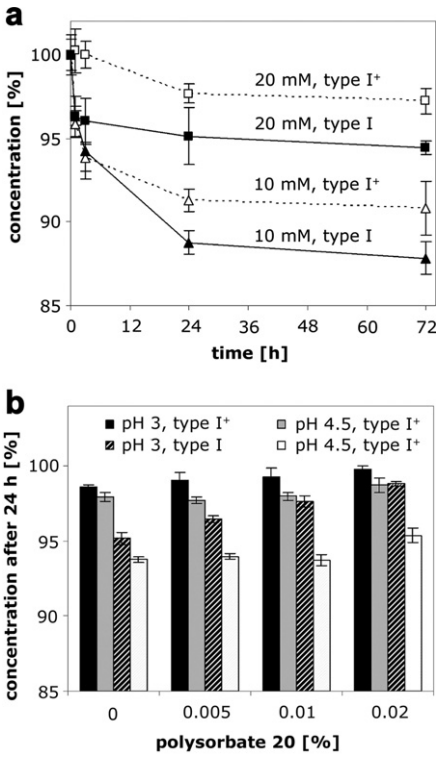

Fig. 6. Protein concentration for 0.25 mg/ml cytokine with 10 and 20 mM glycine at pH 3.0 stored in glass type I and I^+ vials for 72 h at 2–8 °C (a). Residual protein concentration after 24 h storage for 0.25 mg/ml cytokine in 20 mM glycine (pH 3.0 and 4.5) with 0–0.02% polysorbate 20 in glass type I and I^+ vials (b).

albumin onto silicon treated surfaces of different hydrophobicity was reduced by 15–90% depending on polysorbate 20 concentration and the hydrophobicity of the particular surface [33]. For the cytokine the use of polysorbate 20 to minimize protein adsorption was evaluated at pH 3.0 and 4.5 with 20 mM glycine in vials glass types I and I^+ (Fig. 6b). The adsorption on glass type I was inhibited to a greater extent by increasing the polysorbate 20 concentration compared to glass type I^+, especially at pH 3.0. However, without polysorbate 20 the initial adsorption was less distinct for glass type I^+ with 97.9% at pH 4.5 and 98.5% at pH 3.0 compared to glass type I with 93.7% at pH 4.5 and 95.2% at pH 3.0. Overall, glass type I^+ vials were superior to glass type I vials both with and without polysorbate 20 in the formulation. Especially for glass type I it became obvious that adsorption was more distinct at pH 4.5 compared to pH 3.0. This was in agreement with the ATR-FTIR spectroscopy results where less protein adsorbed on the ATR-crystal at pH 3.0 compared to pH 5.0. The best results with less than 1% protein loss after 24 h were achieved by adding 0.005% or 0.02% polysorbate 20 to formulations containing 20 mM glycine at pH 3.0 and by the use of glass type I^+. According to these investigations the use of HSA as adsorption preventing excipient could be avoided for formulations of the cytokine at pH 3.0–4.5.

3.4. Long-term stability of liquid and lyophilized formulations

Based on the results of the preformulation study the most promising conditions were selected for a six months stability study. The focus during the selection of the excipients and pH conditions was set on providing sufficient solubility of the cytokine and preventing aggregation and adsorption. A cytokine concentration of 0.25 mg/ml was used, as this is the typically used therapeutical concentration. The pH range 3.5–5.5 was selected as here the solubility of the cytokine was adequate. The addition of salt or the use of higher buffer concentrations was avoided, as aggregation and precipitation were favored at higher ionic strength (compare 3.1). Therefore, the isotonicity of the formulations was adjusted with mannitol in liquid formulations, respectively, combinations of mannitol and sucrose in lyophilized formulation. Furthermore, mannitol and sucrose slightly reduced cytokine aggregation the liquid formulation which was analyzed by HP-SEC in context of the preformulation studies (data not shown). Table 3 provides an overview of the composition of the liquid and lyophilized formulations used for the 6 months stability studies.

Table 3
Composition of the liquid and lyophilized formulations tested within the stability study over 6 months

Liquid formulations 4.8% mannitol		Lyophilized formulations 4.0% mannitol, 1.0% sucrose	
pH	Polysorbate 20 (%)	pH	Polysorbate 20 (%)
3.5	0.02	4.0	0, 0.005, 0.02
4.0	0, 0.005, 0.02	4.5	0.02
4.5	0.02	5.0	0.02

3.4.1. Stability of liquid cytokine formulations

The liquid formulations (for composition of the formulations compared Table 3) were stored in prefilled siliconized glass syringes. Due to the adsorption tendency of the cytokine the protein content in the formulations was monitored with UV-spectroscopy upon storage. When the cytokine is formulated in presence of Human Serum Albumin adsorption of the cytokine is prevented by the surplus of HSA. After 1 month storage of the HSA-free formulations at 2–8 °C a residual protein content of 96% for the formulation without polysorbate 20 was measured by UV-spectroscopy, while 99% were found at 0.005% polysorbate 20 and 102% at 0.02% polysorbate 20 at pH 4.0. A protein recovery of 99% at pH 3.5 and 101% at pH 4.5 in presence of 0.02% polysorbate 20 was determined after 1 month. This indicates that protein adsorption can be minimized by the addition of polysorbate.

After 6 months storage the level of dimers and trimers determined by HP-SEC was increased by 0.6% upon storage at 2–8 °C and by 1.4% upon storage at 25 °C for the formulation at pH 3.5 (Fig. 7a). An increase of about 1% in aggregates after 6 months was considered as an acceptable value. The most significant rise by 1.6% at 2–8 °C and by 5.2% for samples stored at 25 °C was determined at the highest pH value tested of 4.5. Aggregation stepped up drastically when the liquid cytokine formulations were stored at 40 °C. At pH 4.0 more than 40% and at pH 4.5 more than 50% dimers and trimers were formed, whereas only 11.5% dimers and timers were detected at pH 3.5. Comparable values of dimers and trimers within the HSA-containing formulations are not available, as it was not feasible to determine cytokine dimers and trimers in the presence of 10- to 50-fold surplus of HSA which is typically used to stabilize the cytokine. The pH-dependence of the stability of the HSA-free formulation was in agreement with the results of the FTIR-studies. For the cytokine formulated at pH 3.5 a denaturation temperature T_m of 74 °C was determined, whereas pH 4.2 resulted in a T_m of 57 °C. FTIR had shown significant structural changes at pH 4.2 already starting at 40–45 °C. Therefore, the storage temperature of 40 °C, which is only about 15 °C below T_m, was already too high to assure the integrity of the cytokine at pH 4.5. The addition of 0.005% and 0.02% polysorbate 20 to the liquid formulation at pH 4.0 had no considerable impact on the formation of aggregates.

The improved stability at pH 3.5 compared to the higher pH values was further reflected in the size distribution by volume determined by DLS (Fig. 8a). For the formulations at pH 3.5 a main peak at 5 nm was present after 6 months at all storage conditions. For the samples stored at 40 °C a second peak at 10–30 nm appeared. This peak can be

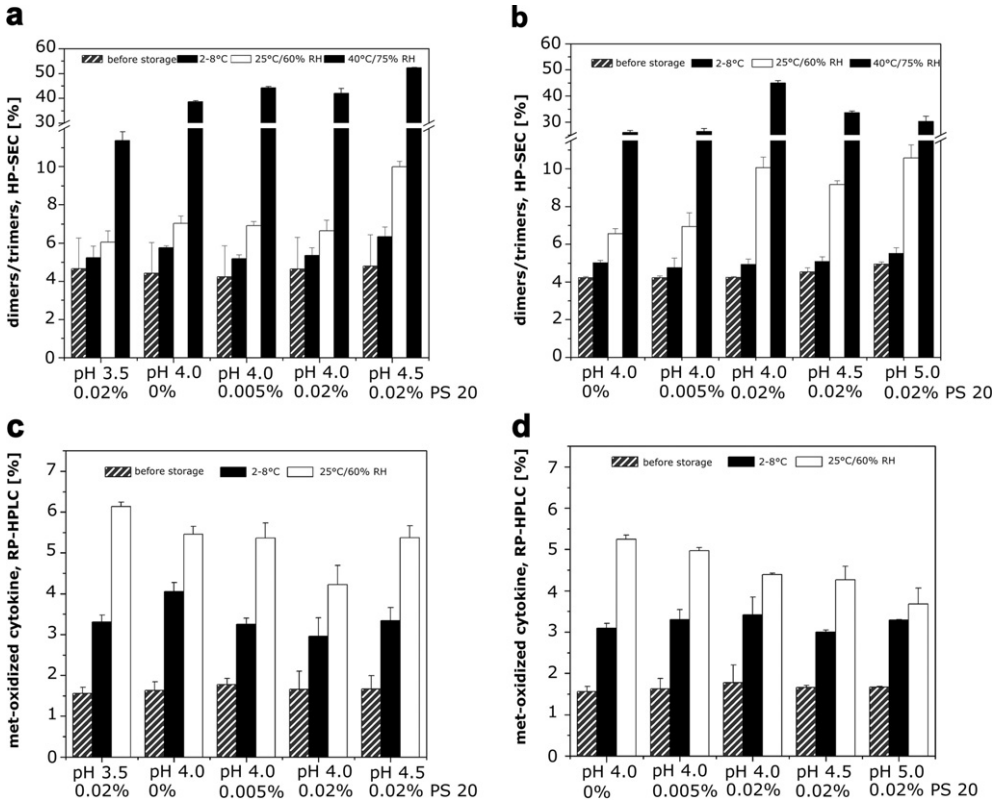

Fig. 7. Aggregation determined by HP-SEC of liquid (a) and lyophilized formulations (b) and met-oxidized cytokine determined by RP-HPLC of liquid (c) and lyophilized formulations (d) after 6 months storage.

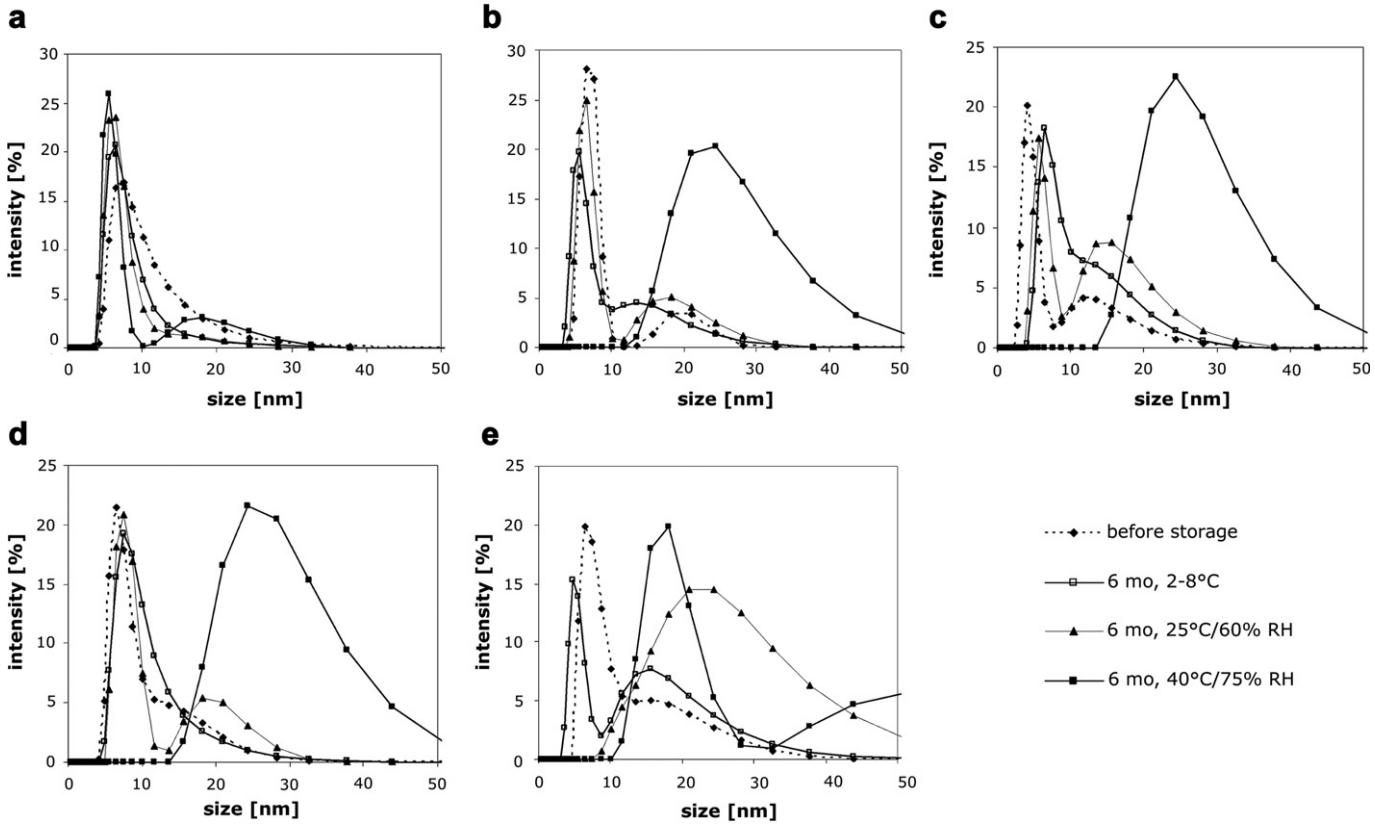

Fig. 8. DLS size distribution by volume of liquid cytokine formulations at pH 3.5, 0.02% polysorbate 20 (a), pH 4.0, 0% (b), 0.005% (c), 0.02% polysorbate 20 (d) and pH 4.5 0.02% polysorbate 20 (e) after 6 months storage.

attributed to a more pronounced aggregate formation and was in agreement with the increase in dimers and trimers detected by HP-SEC. The samples at pH 4.0 exhibited comparable changes in the size distribution at all tested levels of polysorbate 20 (Fig. 8b–d). Already before storage a distinct peak at 10–20 nm was present without and with 0.005% polysorbate 20 and a shoulder with 0.02% polysorbate 20. For the samples stored at 2–8 °C the size distribution changed less with 0.02% polysorbate 20, as no distinct peak at 10–30 nm could be assigned. This points at a stabilizing effect of polysorbate 20. For the samples stored at 25 °C more multimeric protein was formed at pH 4.0 as compared to the samples stored at 2–8 °C, which was reflected in a higher intensity in the size distribution by volume at 10–20 nm. Furthermore, a shift of the first peak from 5 to 30 nm was determined for samples stored at 40 °C. The decreased storage stability of formulations at pH 4.5 at elevated temperatures was reflected in the drastically changed DLS size distributions after storage at 25 °C and even more after storage at 40 °C, at which a second peak at 60 nm emerged (Fig. 8e).

Especially at low pH values chemical modification e.g., oxidation or deamidation is more likely to occur [34,35]. Before storage, the formulations contained an average of 1.6% met-oxidized forms which was determined by RP-HPLC (Fig. 7c). Met-oxidized cytokine formed as a function of storage time and temperature. For the samples stored at 2–8 °C a slight rise in met-oxidized cytokine by 0.2–0.6% was determined after 3 months, which further increased by 1.4–2.4% after 6 months compared to the levels before storage. Higher values of met-oxidized cytokine were determined for the samples stored at 25 °C. The data indicated a slight stabilizing effect of polysorbate 20 at pH 4.0, at which the lowest levels of met-oxidized cytokine were measured in the presence of 0.02% polysorbate 20. Generally, polysorbates are known for their potentially oxidizing effect on protein due to the formation of peroxides during storage which is more pronounced at higher temperatures and oxygen contents [36]. On the other hand, stabilization of protein against chemical degradation by polysorbate can be found as well which was for example described by Son and Kwon for human Epidermal Growth Factor [37]. For the cytokine the stabilizing properties of polysorbate at pH 4.0 appeared to be prevailing to a potential oxidizing effect. However, the formulations should be stored at 2–8 °C to keep the oxidation on a low level. As an alternative, the addition of methionine as antioxidant could be evaluated.

3.4.2. Stability of lyophilized formulation

After freeze-thawing, respectively, lyophilization no boosted aggregate formation was determined by HP-SEC and DLS for the five studied formulations (data not shown). The selected excipients offered sufficient protection of the cytokine against freezing and drying induced stresses. The approach of producing a lyophilized cake with highly crystalline mannitol in combination with amorphous sucrose as stabilizer in a ratio of 4:1 was used to stabilize the cytokine. Johnson et al. demonstrated that this combination of mannitol and sucrose could stabilize different therapeutical proteins [38]. The lyophilization cycle was optimized in a way to promote mannitol crystallization and to avoid the formation of mannitol hydrate [39]. Amorphous mannitol should be avoided as it can crystallize in an uncontrolled way upon storage which can be detrimental for the protein. Mannitol hydrate on the other hand can release its hydrate water upon the transformation into the anhydrous forms. This leads to an increase in residual moisture in the lyophilized cake, which can impair the storage stability. While it can clearly be stated that mannitol hydrate should be avoided, no evidence is available that one of the anhydrous mannitol modifications is superior for the stabilization of proteins. After lyophilization mainly δ-mannitol (peak at 9.7° $2-\Theta$) with a small fraction of β-mannitol (peak 14.6 and 23.5° $2-\Theta$) was present in all formulations exemplarily shown in Fig. 9 for 0 and 0.02% polysorbate 20 at pH 4.0. Without polysorbate 20 peaks of α-mannitol appeared at 13.6° $2-\Theta$ and 17.2° $2-\Theta$ after 6 months storage at 25 °C/60% RH and 40 °C/75% RH (Fig. 9a). It was further evident that the intensity of the δ-mannitol peak at 9.7° $2-\Theta$ decreased during 6 months at 40 °C. This could be ascribed to the fact that the δ-modification is the least stable modification of mannitol [40]. Samples with 0.02% polysorbate 20 were

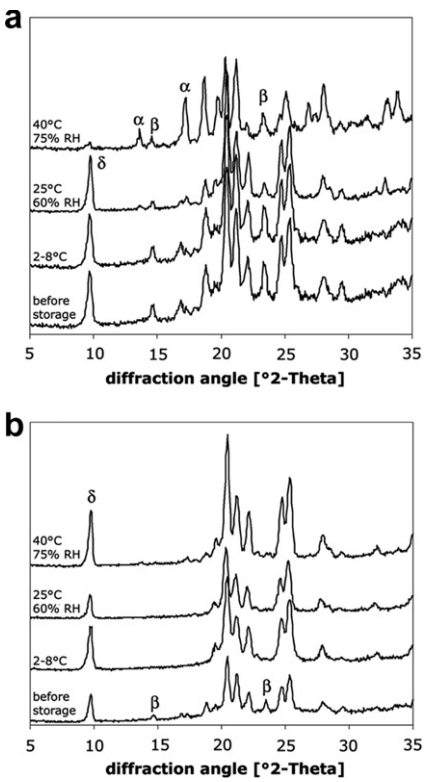

Fig. 9. X-ray diffraction pattern of lyophilized formulation at pH 4.0 without (a) and with 0.02% polysorbate 20 (b) after lyophilization and 6 months storage at 2–8 °C, 25 °C/60% RH and 40 °C/75% RH.

more stable regarding mannitol modifications with only a slight decrease in β-mannitol (Fig. 9b).

All formulations exhibited low residual moisture levels between 0.5% and 1.0% after lyophilization, which could be ascribed to the high content of crystalline mannitol in the formulations. Over the storage time of 6 months only a slight increase in residual moisture up to maximally 1.2% at 2–8 °C and 25 °C/60% RH and maximally 1.4% at 40 °C/75% RH was measured. Water can act as reactant in chemical reactions within the lyophilized sample and as plasticizer with the consequence that the molecular mobility of the system is altered [41]. Generally, a residual moisture level of about 1% is suggested for lyophilized protein formulations [42].

After 6 months at 2–8 °C the level of dimers and trimers increased by approximately 0.3–0.7% for all studied formulations, independent of formulation pH and the addition of polysorbate 20 (Fig. 7b). Storage at 25 °C/60% RH and 40 °C/75% RH resulted in higher contents of dimers and trimers, especially in presence of 0.02% polysorbate 20. XRD revealed that the formulations at pH 4.0 without polysorbate 20 were physico-chemically less stable and underwent morphological changes during storage. Concerning aggregation the formulations at pH 4.0 with 0% and 0.005% polysorbate provided improved stability compared to the formulations with 0.02% polysorbate 20 after 6 months storage at 25 °C/60% RH and 40 °C/75% RH. The improved stabilizing conditions without or with lower polysorbate 20 concentrations outbalanced a potential detrimental effect of the change in modifications during storage. Webb et al. showed that polysorbate 20 destabilized recombinant human interferon-γ during urea induced unfolding and fostered aggregation during refolding [43]. However, the mechanism by which polysorbate 20 increased aggregate formation in the lyophilized product during storage at 25 °C/60% RH and 40 °C/75% RH is unclear. During storage at 40 °C/75% RH the highest aggregation values were measured at pH 4.0, when comparing the formulations with 0.02% polysorbate 20 at the different pH values. For sucrose containing formulations a pH of 4.0 is critical due to the risk of the inversion of sucrose at low pH [44]. This was confirmed by the slightly brownish color of the reconstituted formulations stored at 40 °C, which was distinct at pH 4.0. The discoloration induced by the Maillard reaction occurs when sucrose undergoes inversion to fructose and glucose. The use of trehalose instead of sucrose would be an alternative approach to improve the stability of the lyophilized formulation, by avoiding the inversion of sucrose. The AUC of the HP-SEC remained at a constant level for the samples stored at 2–8 °C and 25 °C/60% RH indicating that no significant loss of protein due to adsorption or precipitation occurred. Storage at 40 °C/75% RH led to a decline in the AUC because of the formation of larger aggregates. DLS of the formulations confirmed the results from HP-SEC (data not shown). Here again the improved stability of samples without polysorbate 20 at pH 4.0 was evident. The size distribution by volume only changed to a moderate degree in polysorbate-free formulations when stored at 2–8 °C and 25 °C/60% RH indicating a slight increase in aggregation. The increase in met-oxidized cytokine in the lyophilized formulations was close to the liquid formulations upon storage at 25 °C/60% RH (Fig. 7d). All samples stored at 2–8 °C showed similar levels of approximately 3% met-oxidized cytokine after 6 months storage. At 25 °C/60% RH about 1% less met-oxidized cytokine was formed when 0.02% polysorbate 20 was added to the formulations a similar effect as in the liquid formulation.

3.4.3. Comparison of liquid and lyophilized formulation

In the stability study of the liquid formulation, pH 3.5 and 4.0 turned out to be most stable regarding aggregation. The formation of the met-oxidized cytokine was independent of the pH but increased with storage time and temperature. Lyophilized formulations showed the highest stability from pH 4.0–5.0, when stored at 2–8 °C. Again the formation of met-oxidized cytokine was independent of the formulation pH. In order to avoid protein adsorption the addition of polysorbate 20 to the liquid formulation was necessary, as the pre-filled syringes were manufactured of glass type I. Furthermore, in liquid formulation, polysorbate 20 was beneficial with respect to the DLS-size distribution by volume and the formation of met-oxidized cytokine. The reduced formation of met-oxidized cytokine in presence of polysorbate 20 was as well seen for the lyophilized formulations. However, here polysorbate 20 fostered the formation of aggregates. As glass type I^+ vials were employed for the lyophilized formulation, polysorbate 20 could be omitted with respect to adsorption.

4. Conclusions

For formulation development the focus was set on the pH range between 3.0 and 5.5 at which an adequate solubility of the cytokine at a constant aggregation level of between 3% and 4% was given. A characterization of the cytokine revealed an improved stability with respect to aggregation and turbidity below pH 4.5. Above pH 5.5 precipitation of the cytokine, indicated by an increased turbidity, impeded reasonable formulation development. It could be shown that a combination of DLS, HP-SEC and nephelometry was feasible to comprehensively characterize different types of aggregation in the cytokine formulations. Furthermore, a decline of thermal stability with increasing pH values could be demonstrated by FTIR. A concentration of 20 mM glycine was selected as appropriate because the tendency of the cytokine to adsorb on glass was reduced compared to lower glycine concentration. Possible approaches to further reduce the loss of protein by adsorption were the use of glass type I^+ and the addition of polysorbate 20 to the formulations, as more than 97% of the protein content could be recovered after 24 h at pH 3.0 and 4.5. Within the studied formulations the liquid

formulations at pH 3.5 and 4.0 with 0.02% polysorbate 20, as well as the lyophilized formulations at pH 4.0 without and with 0.005% polysorbate 20, offered the best stability. Summarizing, it can be concluded that it is possible to stabilize the cytokine without the use of HSA as stabilizer at low pH. Aggregation and oxidation were critical after 6 months at 25 °C/60% RH which in consequence made storage at 2–8 °C necessary. The issue of protein adsorption can be overcome by using polysorbate 20 as excipients, as well as by glass type I^+ vials.

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