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Research Paper

A Critical Evaluation of $T_{m(FTIR)}$ Measurements of High-Concentration IgG_1 Antibody Formulations as a Formulation Development Tool

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Purpose. Fourier-transform infrared (FTIR) spectroscopy was applied for the determination of protein melting temperature ($T_{m(FTIR)}$) and to assess the stability predictability of a 100-mg/mL liquid IgG_1 antibody formulation.
Methods. $T_{m(FTIR)}$ values of various formulations (different pH, buffers, excipients) were compared to the results of a stability study under accelerated conditions (40°C/75% relative humidity), using size-exclusion high-performance liquid chromatography (SE-HPLC) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for the detection of soluble aggregates and covalent modifications.
Results. The highest $T_{m(FTIR)}$ was achieved at pH 5.5, and, similarly, SE-HPLC and SDS-PAGE results suggested a pH optimum between 5.5 and 6.0. Transition temperatures were comparable for all tested buffers. However, the decrease in the monomer fraction upon thermal storage was the lowest for citrate buffers. Whereas sugars and polyols resulted in an increase in $T_{m(FTIR)}$ and enhanced monomer fraction after storage, amino acids showed a destabilization according to SE-HPLC analysis, albeit no change or even an increase in the melting temperature was observed.
Conclusions. All examples gave evidence that $T_{m(FTIR)}$ values did not necessarily correspond to the storage stability at 40°C analyzed by means of SE-HPLC and SDS-PAGE. T_m values, e.g., determined by FTIR, should only be employed as supportive information to the results from both real-time and accelerated stability studies.

KEY WORDS: formulation development; FTIR; highly concentrated antibody formulations; melting temperature; protein.

INTRODUCTION

In 1986, the first recombinant monoclonal antibody Orthoclone® OKT 3 (Muromonab-CD3) was approved by the US Food and Drug Administration (FDA) for the prophylaxis of acute kidney transplant rejections (1). Since then, the steady progress of the market of therapeutic biologics was inexorable, and nowadays, therapeutic proteins, particularly the segment of monoclonal antibodies, are set to drive market growth across a broad range of therapeutic indications in the next years. Actually, antibody blockbuster sales are forecast to almost double from US$7.7 billion in 2004 to US$13.2 billion in 2008 (2,3).

Nevertheless, the market of recombinant antibody therapeutics is still moving forward, and novel antibody formats, including polymeric immunoglobulins possessing multiple antigen-binding sites, as well as antibody fragments, such as monovalent, bivalent, or even tetravalent single-chain scFv, antigen-binding Fab fragments, and single-fold domain antibodies (dAbs), are expected to rise in importance (4–7). Moreover, monoclonal antibodies conjugated with chemotherapeutic drugs, immunotoxins, radioisotopes, or cytokines might be employed to deliver cytotoxic payloads to tumor cells or to approach the synergistic effect of the toxic agents and the antibody molecule (6–8).

Stabilization of the sensitive protein molecules is the major formulation challenge for biologics (9–14). Despite the fact that lyophilization is often used to preserve biopharmaceuticals, an international working group on proper storage and handling of biopharmaceuticals recommended developing biological therapeutics as convenient ready-to-use, aqueous formulations (15). Additional production technology and formulation challenges evolve when high-concentration antibody formulations have to be designed to cope with the frequently elevated dose requirements and the preference to administer those by subcutaneous injection. In general, formulation development is regarded as an integrated approach whereby a stable formulation in a marketable dosage form with acceptable shelf life is developed that can also be successfully administered and economically manufactured (16). Hence, in terms of designing a suitable liquid high-concentration formulation, numerous interrelating

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parameters have to be balanced, such as the formulation conditions (protein concentration, pH value, type and amount of excipients) and the viscosity of the final drug product as well as the protein stability, recovery, and manufacturability during the particular concentration process (17,18). Proteins behave differently depending on their concentration because of, e.g., thermodynamic changes (19,20). Therefore, formulation development has to take place at the target protein concentration. Hence, an "upscaling" of the optimal formulation identified at a lower concentration due to material restrictions may not necessarily correspond to the best stabilizing formulation conditions required at higher protein concentrations and could impose new challenges not being prepared for.

Considering the common protein instabilities, special emphasis should be spent on an increased level of native, reversible self-association as well as aggregation processes in high-concentration formulations (18). For these formulations the effects of thermodynamic nonideality (also referred to as macromolecular crowding) have to be taken into account (19,20). Related to the fact that an increased volume fraction occupied by the protein molecules is prevailing at higher protein concentrations, the decrease in the effective volume available and, in turn, the higher apparent protein concentration biases the reaction equilibrium of protein self-association toward the maximally associated state (18–20). However, due to the larger and/or more asymmetric form of the denatured state, the equilibrium of the protein unfolding reaction is driven toward the compact native conformation by means of the mechanism of volume exclusion (19).

In general, most common analytical techniques applied in the formulation development and quality control of monoclonal antibody therapeutics could be transferred to high-concentration preparations by the introduction of a simple dilution step leading to the required concentration range of the distinct analytical method. Nevertheless, when analyzing concentration-dependent instability reactions, such as association and aggregation reactions or the physicochemical protein behavior in high-concentration formulations, dilution of the samples should be minimized or even avoided to measure the actual solution conditions and to prevent artifacts by potentially disturbing the protein's physical state (17,18).

Circular dichroism, Fourier-transform infrared (FTIR), absorption, and fluorescence spectroscopy belong to the common biophysical methods available to assess the protein secondary and tertiary structure and thus to detect unfolded protein molecules (21–23). Micro- or nanodifferential scanning calorimetry (μDSC/nDSC) is used to determine the transition temperature T_m of the unfolding reaction and to obtain data on the thermodynamics of protein in the folded and unfolded state (24), but spectroscopic techniques and particular FTIR analysis could also be used (25–28). As FTIR analysis is not restricted by an upper protein concentration limit—as is the case for μDSC/nDSC measurements—and can be applied to distinguish association and aggregation processes as well as to elucidate protein conformational stability in the presence of different formulation conditions, this technique seems to have a great advantage in the development of high-concentration formulations. Because the structural transition to intermolecular β sheets occurs regardless of the initial secondary structure composition of the native protein, these bands can be used to monitor denaturation and aggregation reactions in both aqueous and solid states (14). Thus, determination of a $T_{m(FTIR)}$ could be attained by calculation of the inflection point of a sigmoidal curve originating from the intensity–temperature plot of the thermally induced aggregation band (29). Further benefits of the use of FTIR for high-concentration antibody preparations are the small substance requirements, the rapid and easy-to-perform measurements, and the applicability to turbid liquids and different physical states of the drug product (22,23,30,31). Hence, FTIR analysis seems to be a "made-to-measure" method for the determination of transition temperatures of high-concentration protein solutions, especially of those containing an enormous amount of intramolecular β sheets elements, such as the IgG_1 antibody molecule (29).

Therefore, FTIR was evaluated in the following study as an analytical tool to support the formulation development of a 100 mg/mL liquid antibody formulation. The conformational stability of the IgG_1 antibody depending on different formulation conditions (pH value, buffer system, addition of excipients) was evaluated by determination of the $T_{m(FTIR)}$, using intensity–temperature profiles of the temperature-induced band at 1625 cm^{-1} as described previously (29). The $T_{m(FTIR)}$ values were compared to the level of aggregate induction upon short-term storage of the tested liquid protein solution at a concentration of 100 mg/mL at accelerated conditions [4–8 weeks of storage at 40°C and 75% relative humidity (RH)] and determined by analytical methods such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion high-performance liquid chromatography (SE-HPLC). Finally, a comparison of the FTIR analysis was performed to evaluate its predictive value as an analytical tool for the development of high-concentration antibody formulations.

MATERIALS AND METHODS

Materials

IgG_1 Antibody

A recombinant, chimeric mouse/human monoclonal antibody of the IgG_1 subclass (IgG_1, Merck KGaA, Darmstadt, Germany) composed of four polypeptide chains [two identical heavy chains (gamma, γ), each consisting of 449 amino acids, and two identical light chains (kappa, κ), each consisting of 214 amino acids] was used. The four chains are held together by a combination of covalent disulfide and noncovalent bonds. The N-terminal residue of the heavy chain is cyclized as pyroglutamate. It contains two N-linked carbohydrate sites on both heavy chains and has an approximate molecular weight of 154 kDa.

Sample Preparation

FTIR was evaluated as an analytical tool in the development of a high-concentration monoclonal IgG_1 antibody formulation with a protein concentration of 100 mg/mL. The antibody solutions were concentrated and buffer

exchanged by use of a standard tangential flow filtration (TFF) equipment (Labscale™ TFF System, 500 mL scale, Millipore Corp., Billerica, MA, USA), as described elsewhere (S. Matheus, W. Friess, and H. C. Mahler, in preparation).

To study the influence of pH on conformational stability the IgG_1 antibody was formulated in a broad-range multibuffer system containing Tris, citrate, and phosphate (TCPS; at 10 mM each) as buffering agents and saline as isotonicity agent (125 mM) in water for injection (WFI). The pH value of the antibody solution was adjusted to 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, or 8.0. The TCPS multibuffer system can be used in a wide range for pH screening and allows determination of the pH value independent of confounding qualitative buffer component effects when using different buffers at different pH values for screening. To investigate the influence of different buffer systems, the monoclonal antibody was buffered in acetate, citrate, histidine, or phosphate buffer (pH 6.0 ± 0.2). To evaluate the potentially stabilizing effect of excipients, the IgG_1 antibody was formulated in a phosphate buffer (pH 6.0 ± 0.2) with the addition of different excipients. For sugars or polyols, either 150 or 300 mM sucrose, trehalose, or mannitol, respectively, were added to the antibody formulation; out of the class of amino acids, 50 or 100 mM glycine or arginine–HCl, respectively, were used. All reagents used in this study were of pharmacopoeial grade and were obtained from Merck KGaA.

After filtration using a 0.2-μm PES syringe filter (Millipore), 2.0 mL of the IgG_1 solution was transferred into clean and sterile Fiolax 6-mL injection vials (Münnerstädter Glaswarenfabrik, GmbH, Münnerstadt, Germany), sealed with Teflon-faced injection vial stoppers (West Pharmaceuticals Services, Eschweiler, Germany), crimped, and incubated at 40°C/75% RH. After 4 and 8 weeks, two vials per formulation condition and time point were removed from the stability chamber and analyzed for protein stability by SE-HPLC and SDS-PAGE. Two freshly prepared vials were analyzed as control and one unstressed vial was used for FTIR analysis.

Methods

Fourier-Transform Infrared Analysis

Infrared spectra of the protein solutions were recorded by using a Tensor 27 spectrometer (Bruker Optik GmbH, Ettlingen, Germany). Protein samples were prepared in a BioATR™ II attenuated total reflectance cell (Harrick, Ossining, NY, USA), which is connected to a thermostat (DC30-K20, Thermo Haake, Paramus, NJ, USA). The BioATR sample cell can analyze protein samples either in solution or in suspension. To determine the melting temperature ($T_{m(FTIR)}$), temperature-dependent spectra were acquired at 2°C intervals in the temperature range from 60 to 90°C. For each spectrum, a 128-scan interferogram was collected at a single-beam mode with a 4 cm^{-1} resolution. Reference buffer spectra were recorded under identical conditions. The collected interferograms for the protein and the buffer system were Fourier transformed. Afterward, the spectrum of the protein was corrected for the spectrum of the corresponding buffer system for each temperature (for original spectra see (29)). Recorded infrared spectra were analyzed by the software Protein Dynamics for Opus 4.2 (Bruker Optik) and displayed as vector-normalized second derivative amide I spectra [calculated with 25 smoothing points according to the Savitzky-Golay algorithms (32)].

The $T_{m(FTIR)}$ values were obtained by the calculation of the inflection point of thermal transition curves, which were acquired by plotting the intensity of the increasing ("temperature-induced structural element") band of the vector-normalized second-derivative amide I spectrum *vs.* the temperature, using Microcal™ Origin™ software (version 5.0, Microcal Software Inc., Northhampton, MA, USA) and applying a sigmoid fit according to the Boltzmann model (29).

Size-Exclusion High-Performance Liquid Chromatography

The monomer content, soluble aggregates, and protein clippings due to hydrolysis were monitored by SE-HPLC. The analytical system employed consisted of an HPLC pump (L 6250 Intelligent Pump, Merck-Hitachi, Darmstadt, Germany), an autosampler (AS 4000 Intelligent Autosampler), a UV–Vis detector (L-4250 UV–Vis Detector), and an SE-HPLC column attached to a guard column (TSK-Gel® G3000SWXL column and a TSKguardcolumn® SWXL respectively, Tosoh Biosep, Stuttgart, Germany). For sample separation, phosphate-buffered saline (pH 7.2) was used as mobile phase at a flow rate of 0.5 mL. Samples were injected at a volume of 10 μL and UV detection was performed at a wavelength of 280 nm. The fraction of monomer (in percent) was obtained by the ratio of the area–percent antibody monomer peak to the total peak area. The fraction of monomer after storage reflects the difference (in percent) of the monomer peak (fraction of monomer in percent) after the described stress period compared to the monomer peak (fraction of monomer in percent) at the initial time point. Accordingly, the fraction of aggregation and degradation products in Fig. 2 was displayed as increase upon storage, referred to the initial time point.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Formation of aggregates and clipping products were analyzed by SDS-PAGE under reducing conditions. Gel electrophoresis was carried out in a XCell SureLock Mini-Cell electrophoresis chamber with a PowerEase™ 500 power supply using Novex® 4–20% Tris–Glycine precast gels (1.0 mm, 12 wells) and Novex® Tris–Glycine SDS Running Buffer (all from Invitrogen GmbH, Karlsruhe, Germany). The samples contained 0.4 mg/mL IgG_1 antibody, 50% Novex® Tris–Glycine SDS Sample Buffer (Invitrogen) and 10% of a 10% 1,4-dithiothreitol (DTT) solution (Merck KGaA). After heating at 95°C for 4 min, 10 μL of each sample containing 0.4 μg of the IgG_1 antibody was loaded per lane and focused. A standard Coomassie-staining protocol including a washing, fixing, staining, destaining, and drying step was applied for the detection of the resulting protein bands. Analysis of the stained SDS-PAGE gels was performed by use of Personal Densitometer SI (Amersham Biosciences Europe GmbH, Freiburg, Germany) and AIDA Image Analyzer software

(raytest GmbH, Straubenhardt, Germany). To calculate the molecular weight of the detected zones, a Mark12™ Unstained Standard (Invitrogen) was used. The sum of the area percent of the heavy and light chain at the initial time point and after the described stress period (time, temperature) was calculated.

RESULTS AND DISCUSSION

Screening of the pH Value

Initially, the impact of the pH value on the conformational stability of the high-concentration IgG_1 solutions was evaluated in the pH range 5.0–8.0 (Fig. 1). Resulting $T_{m(FTIR)}$ values were compared to the fraction of monomer remaining upon storage at accelerated temperature conditions analyzed by means of SE-HPLC and reducing SDS-PAGE (Fig. 2). Although solution pH is probably the most important factor regarding protein stability (33), only differences in the range of 2°C were obtained for the transition temperature by FTIR analysis with regard to the pH of the 100 mg/mL IgG_1 formulation. The highest $T_{m(FTIR)}$ value was achieved at a pH value of 5.5, associated with a significant diminishing of the transition temperature at the lower pH of 5.0 and a continuous, slight decrease with increasing pH value up to a pH of 8.0 (Fig. 1). ATR measurements seem not to lead to a disturbed spectrum due to surface effects of the crystal on protein stability or due to the overestimation of the protein molecules on or near the crystal surface (30), because the spectrum of the IgG_1 antibody measured by the transmittance mode was comparable to that of the spectrum obtained by measurements in the ATR cell (data not shown).

As cited in the literature, conformational stability could be reduced at pH values far from as well as near the isoelectric point (IEP) of proteins (34,35). At a pH far from the IEP, increased charge repulsion within the protein due to the increased number of charged groups on the protein surface destabilizes the folded protein conformation because the charge density on the folded protein is greater than on the unfolded protein. Thus, pH-induced unfolding leads to an enlarged surface area and, consequently, to a state of lower electrostatic energy (35). In contrast, at pH values close to the IEP the concomitant availability of positively and negatively charged groups could result in an anisotropic charge distribution, giving rise to dipole formation and energetically favoring the aggregation processes (34). Probably, the pH of 5.5 ensuring maximum conformational stability as detected by FTIR could be almost between the two effects described, as the IgG_1 IEP is between 8.3 to 9.5 (36).

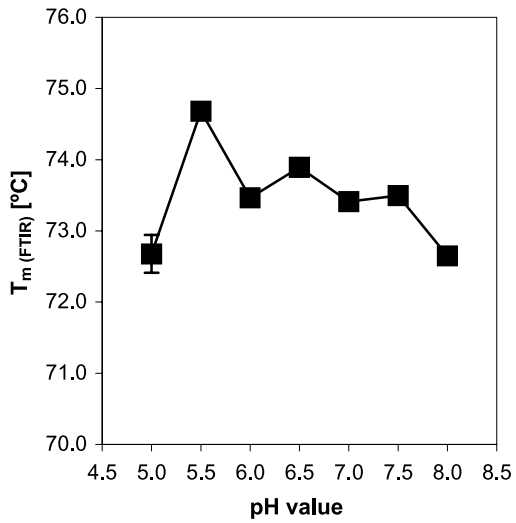

Fig. 1. $T_{m(FTIR)}$ values of the 100 mg/mL IgG_1 formulations with dependency on the pH value determined as inflection point of the second-derivative intensity–temperature plot of the amide I frequency at 1625 cm^{-1} that is assignable to intermolecular β sheets.

Concurrently, the decrease in the fraction of the monomer as compared to the initial monomer fraction (determined by means of SE-HPLC analysis) after 4 and 8 weeks storage at accelerated temperature conditions is minimized at a pH of 5.5 and 6.0 (Fig. 2A). However, in contrast to FTIR analysis, the SE-HPLC data for the pH values exceeding 6.0 show a significant decrease in IgG_1 stability, i.e., an enormous decrease in the monomer fraction. Thereby, the decrease in the monomer fraction is mainly at the expense of the formation of soluble aggregation products. The fraction of aggregates increases steadily up to pH 8.0 (Fig. 2B). In contrast, the formation of degradation products occurred only to a less degree. The lowest rate of degradation products resulted in the pH range 6.0–7.0, accompanied by a slight increase at acidic and basic pH values (Fig. 2C). In addition, the total areas under the curve (AUCs) were monitored over the whole study and no significant changes occurred.

A slightly acidic pH of 6.0 is often considered appropriate for IgG_1 formulations to avoid acid- and base-catalyzed degradation reactions, as hydrolytic cleavage of peptides can take place in proteins maintained in an extremely acidic environment, and under basic conditions, further reactions—among others, peptide bond hydrolysis, β elimination, and racemization—can occur (14,37). The fraction of the sum of the heavy and light chain referred to the total area of all zones detectable in the reducing SDS-PAGE revealed an analogous pH profile (Fig. 2D) and a stability maximum at pH 5.5 as compared to SE-HPLC results.

Hence, using T_m determinations by the FTIR technique was in part capable of providing information about the pH value of maximum stability (pH 5.5), although the pH of 6.0 could also be chosen and was preferred due to the results of SE-HPLC and SDS-PAGE as well as of further aggregate-indicating methods, such as light obscuration methods, nephelometric determination, visual inspection methods, and other techniques exploited after applying shaking stress to the protein samples (data not shown). However, the use of $T_{m(FTIR)}$ value as a supportive tool in pH screening was not successful in all terms, as it was not able to show the enormous extent of instabilities occurring in the neutral to slightly alkaline pH range. The different mechanisms triggering conformational stability on the one hand and chemical stability on the other hand do possibly account for the dissimilar outcome of the analytical methods.

Screening of Buffer Systems

A buffer used to formulate proteins should exhibit little or no change in pH with temperature and have maximum

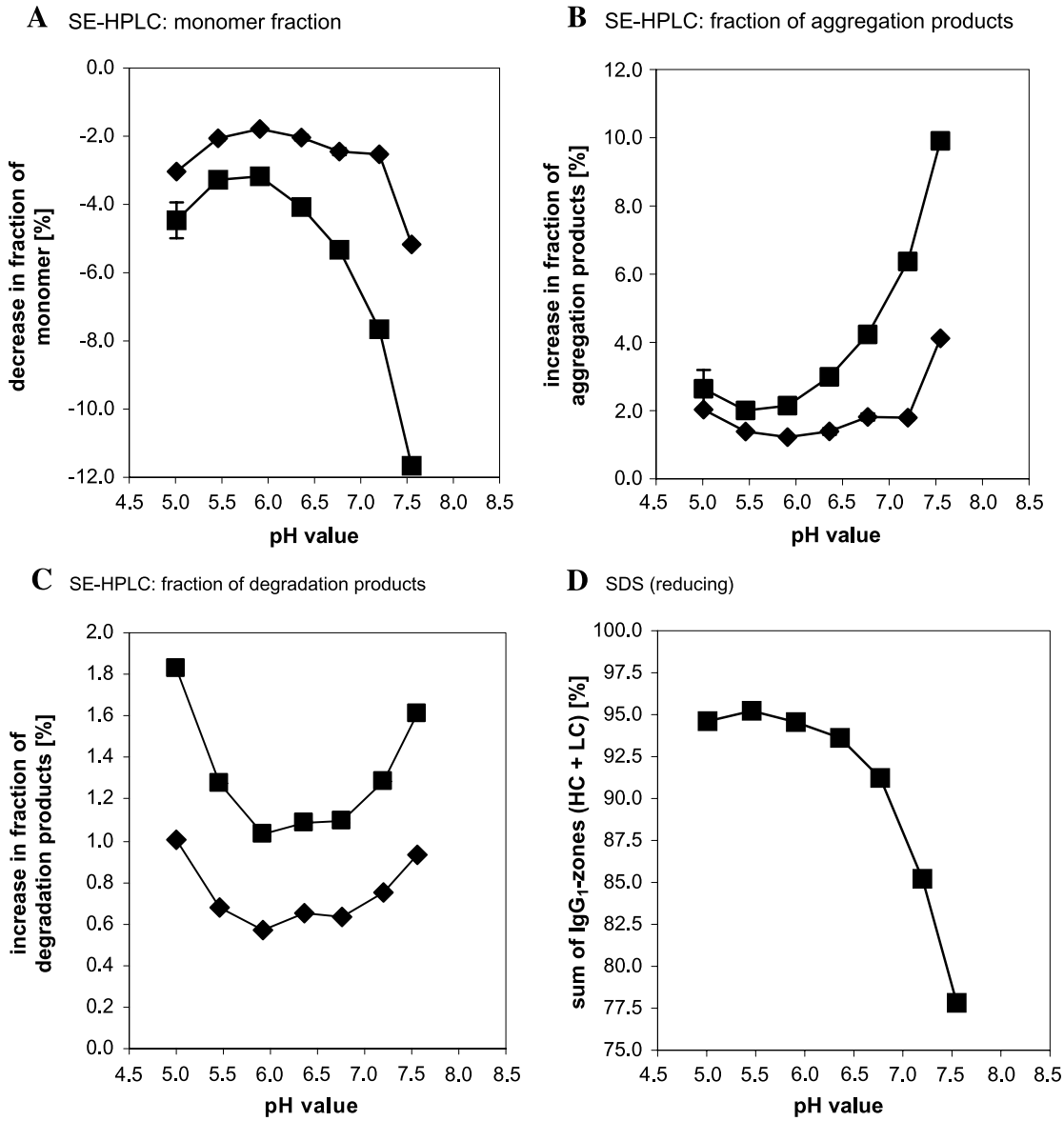

Fig. 2. Fraction of monomer (A), aggregation products (B), degradation products (C), determined by SE-HPLC, and fraction of monomer (D), determined by reducing SDS-PAGE, of the 100 mg/mL IgG_1 formulations with dependency on the pH value upon storage at 40°C/75% RH. For the SE-HPLC analysis, the percentage decrease in the monomer fraction or increase in the fraction of aggregation or degradation products, respectively, referred to the initial value, is shown after 4 (♦) and 8 weeks (■) of storage. For the reducing SDS-PAGE the sum of the area of the heavy and light chain after 8 weeks (■) of storage is used.

buffer capacity at a pH where the protein exhibits optimal stability and, of course, should not destabilize or rather even stabilize the protein. Therefore, acetate, citrate, histidine, and phosphate buffer were evaluated at two concentrations (10 and 50 mM) at a pH of 6.0 regarding their stabilizing effect on the 100 mg/mL IgG_1 formulation. The influence of these buffers on the conformational stability of the IgG_1 antibody is shown in Fig. 3. Obviously, the $T_{m(FTIR)}$ values were in a comparable range (74.8–75.4°C), with the exception of the 50 mM histidine formulation revealing a transition temperature of only 74.0°C.

As Ugwu and Apte (38) distinguished three mechanisms for the profound effects of buffers on the conformational stability, i.e., binding effects at lower buffer concentrations, electrostatic charge screening at intermediate concentrations, and cosmotropic or chaotropic effects, respectively, at higher buffer concentrations, several points of discussion for these results could be mentioned. On the one hand, a stabilizing effect of the cosmotropic anions phosphate, citrate, and acetate would be conceivable. On the other hand, the hypothesis of Ugwu and Apte that the conformational stability would increase if using cationic buffers below the IEP as a result of repulsion between the cationic buffer and the protein could not be confirmed by our results. The cationic buffer histidine led to a decrease in the $T_{m(FTIR)}$ value at a pH of 6.0 below the IEP of the IgG_1 antibody.

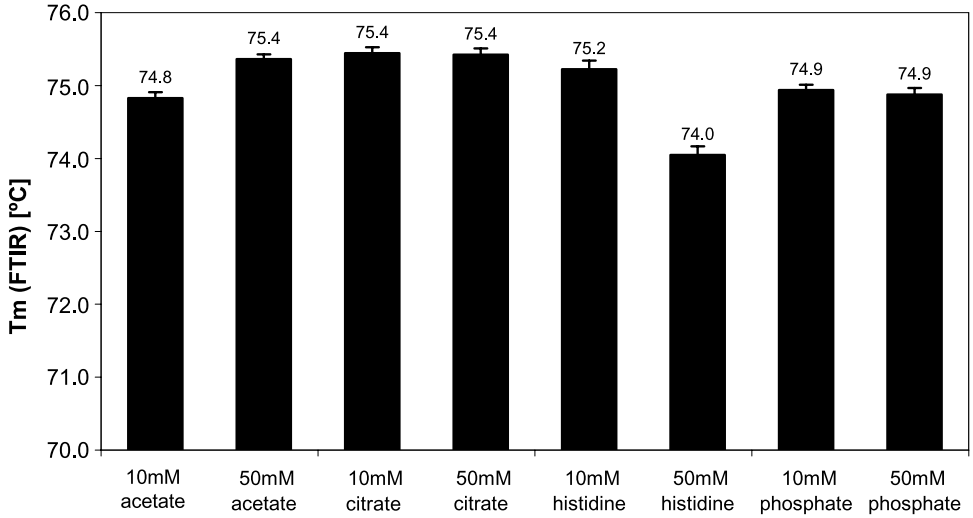

Fig. 3. $T_{m(FTIR)}$ values of the 100 mg/mL IgG_1 formulations (pH 6.0) with dependency on the buffer system used (10 and 50 mM acetate, citrate, histidine, or phosphate) determined as inflection point of the second-derivative intensity–temperature plot of the amide I frequency at 1625 cm^{-1} that is assignable to intermolecular β sheets.

Probably, an inverse shielding effect by the anionic buffers acetate, citrate, and phosphate confounding to the protein conformational stability would be conceivable. However, the ability of ionic compounds to cause stabilization of the protein by binding to specific residues has also been discussed (38).

The SE-HPLC results revealed that the decrease in the fraction of monomer (in reference to the initial value before storage at accelerated conditions) was the lowest for citrate and could be further reduced by a higher citrate concentration. In comparison, the other buffer components showed a more pronounced degradation of the IgG_1 monomer in SE-HPLC measurements, especially at the higher buffer strength (Fig. 4). Similar to the results of the pH study, the reduction of monomer fraction was mostly induced by the formation of soluble aggregates in case of histidine-, phosphate-, and citrate-buffered systems. However, a particularly pronounced fraction of degradation products could be monitored for the antibody solutions formulated in acetate buffer (data not shown). Several publications (11,39) discussed the general influence of the buffer anion, particularly the enhancing effect of the phosphate ion on the protein deamidation rate of the protein.

In the buffer screening study, $T_{m(FTIR)}$ values and the results of SE-HPLC analysis did not necessarily correspond to each other. In view of the fact that an ideal buffer should be able to inhibit both physical and chemical instabilities simultaneously and taking additionally the results of particle indicating methods, such as light obscuration methods, nephelometric determination, visual inspection methods,

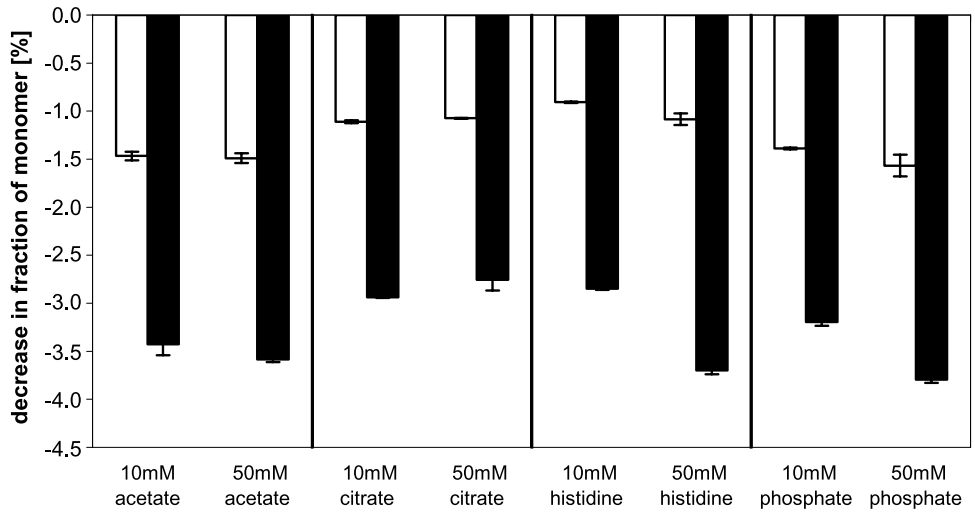

Fig. 4. Monomer content of the 100 mg/mL IgG_1 formulations (pH 6.0) with dependency on the buffer system (10 and 50 mM acetate, citrate, histidine, or phosphate) used upon storage at 40°C/75% RH determined by SE-HPLC. For the SE-HPLC analysis, the percentage decrease in the monomer fraction referred to the initial value is shown after 4 (□) and 8 weeks (■) of storage.

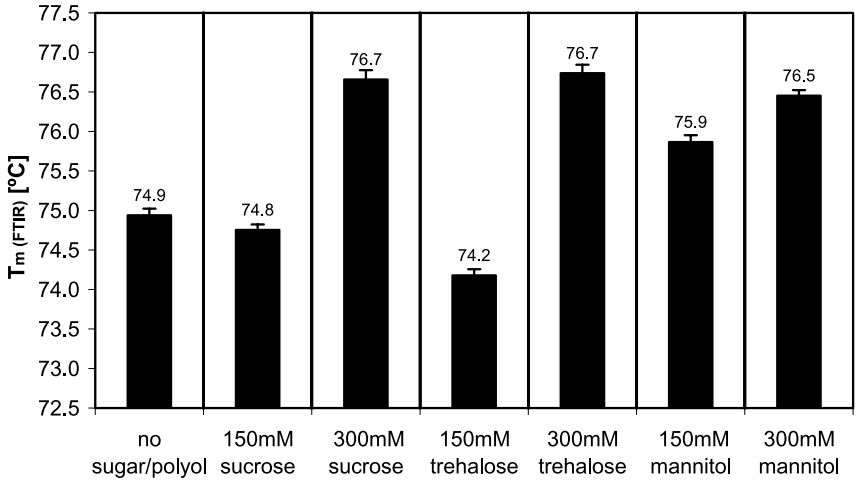

Fig. 5. $T_{m(FTIR)}$ values of the 100 mg/mL IgG_1 formulations in phosphate buffer (pH 6.0) with dependency on additional excipients (150 and 300 mM sucrose, trehalose, or mannitol) determined as inflection point of the second-derivative intensity–temperature plot of the amide I frequency at 1625 cm^{-1} that is assignable to intermolecular β sheets.

and others after application of shaking stress into account (data not shown), a 10 mM phosphate buffer (pH 6.0) was chosen as a suitable buffer system to maintain the IgG_1 antibody stability. Thus, neither FTIR nor SE-HPLC and SDS-PAGE were able to be used as sole methods to determine the optimal buffer system for this IgG_1 antibody.

Screening of Stabilizing Excipients

Following the evaluation of pH value and suitable buffer systems, several excipients were studied regarding their stabilizing effect on the liquid high-concentration IgG_1 formulations. It has been shown by several authors that the addition of a number of polyhydric alcohols, sugars, and some amino acids to aqueous solutions of proteins led to their stabilization (40–49). As sugars and polyols, either sucrose, trehalose, or mannitol were tested individually at a concentration of 150 and 300 mM (Fig. 5) regarding their influence on the transition temperature of the 100 mg/mL IgG_1 solution formulated in a phosphate buffer (pH 6.0). As amino acids, either arginine–HCl or glycine was tested individually at a concentration of 50 and 100 mM (Fig. 6). Whereas every excipient of the group of sugars and polyols resulted in an increase in the $T_{m(FTIR)}$ values at a concentration of 300 mM, the two amino acids acted inconsistently. On the one hand, glycine had no influence on the transition temperature; on the other hand, arginine caused even a decrease of the $T_{m(FTIR)}$.

The mechanism of protein structure stabilization by these compounds was elucidated by Timasheff (50), who evolved the mechanism of preferential exclusion of the cosolvents from the domain of the protein, resulting in

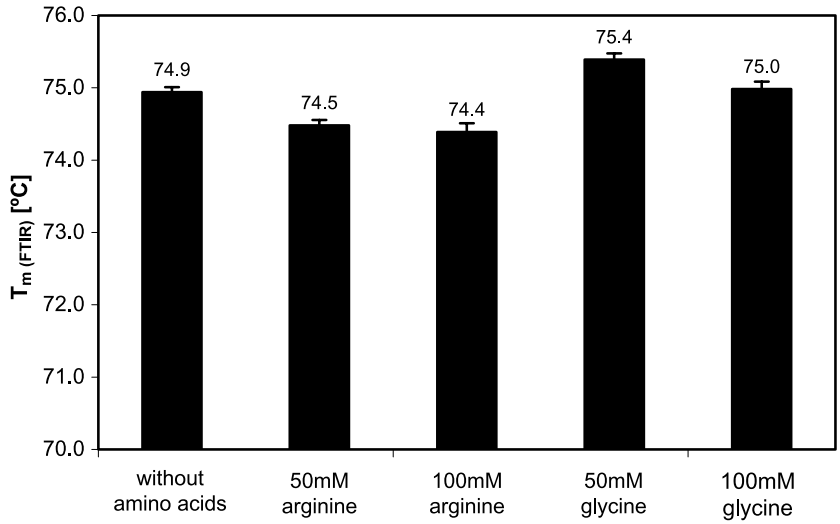

Fig. 6. $T_{m(FTIR)}$ values of the 100 mg/mL IgG_1 formulations in phosphate buffer (pH 6.0) with dependency on additional excipients (50 and 100 mM arginine–HCl, or glycine) determined as inflection point of the second-derivative intensity–temperature plot of the amide I frequency at 1625 cm^{-1} that is assignable to intermolecular β sheets.

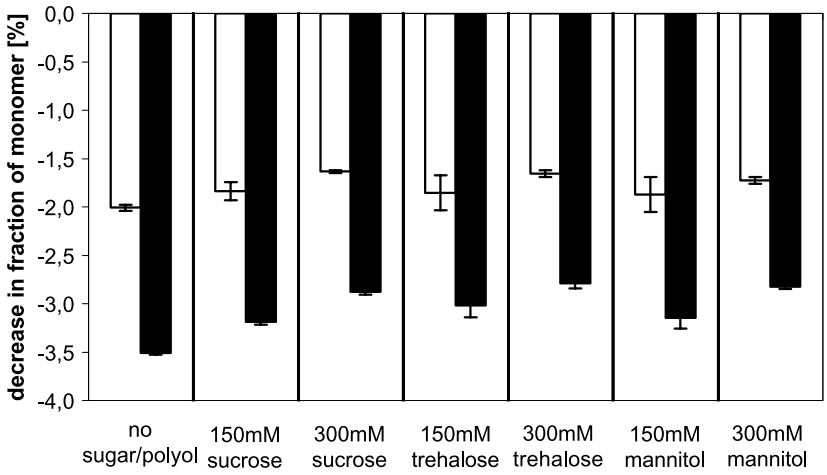

Fig. 7. Monomer content of the 100 mg/mL IgG_1 formulations in phosphate buffer (pH 6.0) with dependency on additional excipients (150 and 300 mM sucrose, trehalose, or mannitol) upon storage at 40°C/75% RH determined by SE-HPLC. For the SE-HPLC analysis, the percentage decrease in the monomer fraction referred to the initial value is shown after 4 (□) and 8 weeks (■) of storage.

preferential hydration of the macromolecules. Thus, protein stabilization is due to the ordering of water around the protein; the cosolvent molecules exert pressure to reduce surface contact between the protein and the solvent. Unfolding increases the surface area of the protein, and consequently the volume from which the cosolvent is excluded, ensuing an unfavorable energetic state.

Actually, the excipients acting according to the mechanism of preferential exclusion could be distinguished whether they interact with the protein molecules itself or not. Whereas sucrose and trehalose initiate the preferential hydration by an increase in surface tension and do not interact with the protein, binding of arginine, glycine, and mannitol to the protein are considered possible. Mannitol is subjected to the mechanism of preferential exclusion due to its solvophobicity, indicating that contacts between nonpolar regions of a protein and the water–mannitol mixture are entropically even more unfavorable than contact with water (48).

However, the addition of 300 mM of sucrose, trehalose, or mannitol generated a comparable increase of 1.5 to 2°C in the melting temperature. These results were corroborated by a diminished decrease in monomer fraction upon storage at 40°C, although a reduction of approximately 0.7% (Fig. 7) of the monomer fraction was observed. Correspondingly, the stabilization by preferential exclusion mechanism might become apparent only at relatively high cosolvent concentrations exceeding 0.3 M (51). The higher monomer fraction in the presence of the tested sugars or polyols could be attributed to a reduced temperature-induced formation of

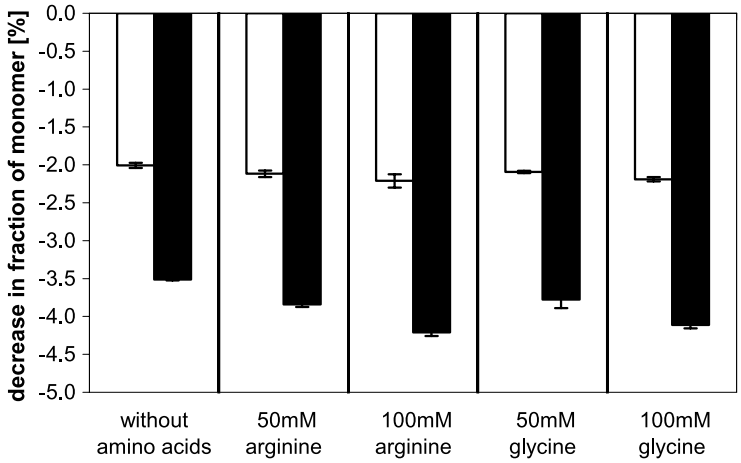

Fig. 8. Monomer content of the 100 mg/mL IgG_1 formulations in phosphate buffer(pH 6.0) with dependency on additional excipients (50 and 100 mM arginine–HCl or glycine) upon storage at 40°C/75% RH determined by SE-HPLC. For the SE-HPLC analysis, the percentage decrease in the monomer fraction referred to the initial value is shown after 4 (□) and 8 weeks (■) of storage.

soluble aggregates. For the evaluated excipients, the fraction of protein clipping products remained unchanged as compared to the excipient-free antibody preparation (data not shown).

Glycine and arginine enable preferential hydration of macromolecules by an increase in surface tension, too. Moreover, a weak interaction of glycine with proteins and binding of arginine to negative charges are possible, probably causing perturbations of protein structure (50). Therefore, arginine is excluded from the group of osmolytes chosen by natural organisms to exist in an environment in which stresses either from high osmotic pressure or from freezing are prevalent (52). Reasons for the unchanged $T_{m(FTIR)}$ values in the case of glycine or for the decreased transition temperatures in the case of arginine might be due to these unfavorable interactions (although the IgG_1 antibody is positively charged at a pH of 6.0) or the low concentration range of the cosolvents used. Coevally, a destabilizing effect of the amino acids arginine and glycine could be identified in SE-HPLC analysis, becoming evident in a more intense decrease in the monomer fraction during the storage at elevated temperatures compared to control formulations not containing these excipients (Fig. 8). This was caused by an unfavorable greater increase in the percentage of aggregation products as compared to excipient-free samples, because no differences in the tested formulations with respect to the formation of degradation products could be monitored (data not shown).

Hence, all of the cosolvents acting according to the mechanism of preferential exclusion could also enforce protein–protein interactions because the increase in the free energy change between the native and denatured state is attained by making the denatured conformation thermodynamically more unfavorable than the native one. Yet, it is also shown that the protein's native state becomes thermodynamically unfavorable in the presence of cosolvents, thus fostering reduction of the surface area by aggregation (42). Consequently, the processes of protein solubility and protein salting out could be also described by Timasheff's model of preferential exclusion (50). Probably, this could be a reason for the increased occurrence of light-scattering aggregates determined by turbidity analysis in samples containing sugars and polyols or amino acids after mechanical stressing (data not shown).

CONCLUSIONS

All examples discussed gave evidence that the $T_{m(FTIR)}$ did not necessarily correspond to the stability upon thermal storage at 40°C and analyses by means of SE-HPLC and SDS-PAGE. The decrease in the monomer fraction (in relation to the initial value) as measured by SE-HPLC can be due to (soluble) aggregation and degradation processes, whereby denatured molecules could be prone to both mechanisms. Moreover, aggregation could be involved in further degradation reactions and vice versa. Carpenter *et al.* (53) distinguished the mechanism of aggregation processes induced by short-term or long-term thermal stress. Whereas perturbation of the native protein structure during heating ramps—as applied in FTIR analysis—can foster sufficient unfolding to promote aggregation, the formation of soluble aggregates and/or protein precipitates during a time frame of several months can arise under these conditions, greatly favoring the native state. FTIR could also be used for isothermal measurements. For the IgG_1 antibody tested, no change in the FTIR spectrum became apparent at 55°C incubation for 24 h, whereas incubation at 70°C for at least 1 h resulted in band shifts corresponding to the denaturation of the antibody (data not shown). Consequently, future work could focus on the use of FTIR under isothermal conditions.

Summing up the results of this study, it can be concluded that formulation conditions or excipients leading to an increase in the transition temperature must not necessarily cause a decrease in aggregation rate during thermal storage below the melting temperature. Because an increase in temperature also strongly affects reaction rate constants due to an exponential increase of rate constants for activated reactions with temperature (34), other degradation mechanisms like deamidation, peptide bond cleavage, etc. could induce the formation of an aggregate-competent species and explain the different results obtained by FTIR analysis and the standard analytical methods used to detect aggregates by long-term thermal stress. However, it should be mentioned that FTIR is not only capable of measuring $T_{m,}$ but also yields information on the protein secondary structure (22,30,31,54).

Nevertheless, in several examples from the literature, the melting temperature—determined by either μ/nDSC or FTIR analysis—corresponds to the results of standard protein analytics. In the evaluation of the influence of polyols on lysozyme by Singh and Singh (46), thermodynamic μDSC measurements and biological activity analysis correspond to each other because it was shown that polyols are capable of providing protection against various degradation mechanisms causing protein conformational destabilization associated with a decline in biological activity. Additionally, μDSC predictions of the stability of interleukin-1 receptor type I (IL-1R) in the presence of various preservatives could be verified by means of SE-HPLC data (55). In general, preferential exclusion excipients such as sucrose might inhibit the formation of aggregates if nonnative aggregation is the prevalent degradation reaction in liquid formulations (44). In contrast to the examples described but corresponding to the results of this study, Papadimitriou (56) observed a miscorrelation between the transition temperatures obtained by nDSC and covalent aggregates formation detected by SDS-PAGE under reducing conditions developing a liquid formulation of an erythropoietin derivative.

Finally, it has to be kept in mind that FTIR is only capable of describing formulation conditions with respect to their perturbing or stabilizing effect on the native secondary structure of the protein. However, maintaining the secondary structure itself may not be sufficient for the long-term stability of an antibody formulation. Protein stability is the sum of overall physical stability, including conformational stability as well as chemical stability, and is thus affected by a diversity of instability reactions. Thus, inappropriate comparisons between long-term or thermodynamic stability data should be avoided in the formulation development of proteins (57). Besides that, if a consensus between the different methods exists, particularly when thermally induced aggregation is the predominant pathway for protein degra-

dation, it could not be assessed which extent in increase or decrease of the transition temperature $T_{m(FTIR)}$ would be extremely relevant to an improved or declined protein stability.

Therefore, it is strongly recommended to use T_m values as supportive information only in combination with results from real-time and accelerated temperature and mechanical stress studies, applying a battery of other analytical methods including SE-HPLC, SDS-PAGE, IEF/IEC, particle measurement by either light obscuration techniques, nephelometric measurements, visual particle determinations, and finally, potency measurements.

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