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Research paper

Conformational analysis of protein secondary structure during spray-drying of antibody/mannitol formulations

Stefanie Schüle [a], Wolfgang Frieß [a,*], Karoline Bechtold-Peters [b], Patrick Garidel [b]

[a] *Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-University, Germany*
[b] *Boehringer Ingelheim Pharma GmbH & Co. KG, Process Science, Pharmaceutical Development, Biberach an der Riss, Germany*

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Abstract

Inhalation of spray-dried particles is a promising delivery route for proteins as an alternative to injection. Changes in the protein structure and aggregation have to be avoided. The effect of mannitol, a stabilizing agent typically used in both liquid and lyophilized protein formulations, on an antibody (IgG1) in a spray-dried powder was studied using different biophysical and chromatographic techniques. At first, different solutions composed of antibody (IgG1) and mannitol at a ratio between 20/80 and 100/0 IgG1/mannitol (100 mg/ml total solid) were investigated for their stability. Protein solutions containing the IgG1 showed mannitol-dependent aggregation. High amounts of mannitol (50–80%) exerted a destabilizing effect on the antibody and the aggregate 9level increased to 2.6–4.2%. In contrast, solutions with only 20–40% mannitol showed the same amount of aggregates as the pure antibody solution. The antibody mannitol solutions were investigated by circular dichroism (CD) and Fourier transform infrared spectroscopy (FTIR) to evaluate whether changes in the protein secondary structure can be correlated with aggregation. Considering the sensitivity of the used methods and data evaluation, FTIR spectra did not reveal structural changes in the IgG1/mannitol solutions compared to the pure antibody, despite varying aggregate levels. Thermal stress was reflected in perturbations of the secondary structure, but mannitol-dependent aggregation could not be correlated to detectable alterations in the FTIR spectra. Analyzing the CD spectra revealed no distinctive change in the shape of the CD curve, indicating that the protein secondary structure is mainly retained. This is in agreement with the infrared data.

Subsequently, the IgG1/mannitol solutions were spray-dried at T_{in}/T_{out} of 90/50 °C. Using ATR-FTIR for the investigation of the protein amide I band in the spray-dried powder revealed changes in the sub-components of the amide I band. This indicates that the peptide groups (C═O and N─H) of the protein are found in a different environment in the solid state, compared to the liquid protein formulation. After redissolution of the powders, the native structure of the pure antibody solution was found identical to the protein secondary structure before spray-drying, indicating that the protein secondary structure is not strongly altered in the dry state, and not affected by the spray-drying process. Thus, from the presented study it can be concluded that the formation of antibody aggregates in mannitol formulations cannot be correlated with significant perturbations of the protein secondary structure elements.

Keywords: Immunoglobulin; Circular dichroism; FTIR; Spray-drying; Mannitol

1. Introduction

Powders for inhalation comprise an innovative method for the application of protein molecules for inhalation. Exubera® could be the first approved biopharmaceutical product which will deliver a protein to the lung as a powder. In September 2005, Pfizer Inc., and the Sanofi-Aventis Group announced that the U.S. Food and Drug Administration Advisory Committee has recommended the approval of Exubera®, an inhalable, rapid-acting, dry powder insulin for the treatment of adults with Types 1 and 2 diabetes (www.sanofi-aventis.com).

* Corresponding author. Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-University, Butenantstr. 5 – 13, 81377 Munich, Germany. Tel.: +49 218077017; fax: +49 218077020.
E-mail address: Wolfgang.Friess@lrz.uni-muenchen.de (W. Frieß).

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For the preparation of pulmonary drug delivery systems with particle sizes in the range of 2–5 μm spray-drying is a suitable method. The long-term stability of protein pharmaceuticals can be enhanced by spray-drying of the protein with suitable stabilizing excipients such as trehalose, sucrose or mannitol. It is known that proteins adopt different secondary structures, which are extremely relevant for bioactivity. However, proteins are particularly sensitive molecules, so that various stress factors during spray-drying, e.g. thermal stress and/or shear stress at the outlet of the spray nozzle, may induce protein denaturation and aggregation and/or loss of activity. In the literature it is described that aggregate formation can often be correlated with the formation of β-sheet structures [1–3].

Different biophysical methods are available to determine the protein secondary structure in an aqueous environment. Multidimensional nuclear magnetic resonance spectroscopy yields particularly accurate information with regard to the secondary structure of the protein in solution. However, it is an extremely time consuming method and mostly limited to a protein size of approximately 30 kDa. To avoid these constraints, vibrational spectroscopic techniques like Raman or infrared (IR) spectroscopy can be used. Valuable information is also obtained from circular dichroism (CD) spectroscopy. These methods are very straightforward, but provide only a global view into the overall protein secondary structure [4]. However, especially for the purpose of preformulation of spray-dried formulations these methods may give valuable information for the discrimination of changes due to the manufacturing process [5,6].

The structurally repeat unit of proteins is the infrared active peptide group, which leads to nine characteristic absorption modes termed amide bands in vibrational spectroscopy. The most prominent bands in an infrared spectrum of a protein are located between 1700 and 1450 cm^{-1}. The band with its maximum at 1645 cm^{-1} is the so-called amide I and the band with its maximum at 1555 cm^{-1} refers to the so-called amide II band. The amide I band originates mainly from the carbonyl stretching vibration of the amide group. This vibrational mode is directly related to the backbone conformation and is thus conformational sensitive. From the analysis of the amide I band, information with respect to the secondary protein structure (e.g. α-helical structure, β-sheet structure, turns, loops) is obtained. The exact position and shape of the amide I band depend on the carbonyl binding strength, which can be altered by, e.g. the involvement of the carbonyl group in a hydrogen bonding network [7,8].

From CD spectra, similar information can be derived. CD spectroscopy measures differences in the absorption of left-handed polarised versus right-handed polarised light which arise due to structural asymmetry. The absence of regular structure results in zero CD intensity, while an ordered structure results in a spectrum which contains positive and negative signals [9–11]. Alterations in the secondary structure are measured in the region of 180–250 nm, the so-called Far-UV CD. This region is dominated by contributions of the peptide bonds, although some side chains may also be involved. The CD bands originating from aromatic amino acids and cystine in the near-UV (250–300 nm) can be utilized to determine the tertiary structure [10,12]. Like all spectroscopic techniques, the CD signal reflects an average of the entire molecular population.

An objective of this study was to evaluate prepared spray-solutions containing an antibody (IgG1) and mannitol as excipient for their stability and for possible alterations of the secondary structure due to the interaction with the excipient. The main aspect was to investigate whether a relation exists between aggregation in the liquid state and changes in the secondary structure. The analysis of the secondary structure was based on FTIR as well as on CD to obtain comprehensive results. Additionally, the structural environment of the protein of the spray-dried powder in the solid state was investigated, because such protein containing powder particles are ultimately directly applied to the lung epithelium. The appearance of a β-sheet structure upon protein denaturation has been described for a number of proteins and has been correlated with protein aggregation [13,14]. However, whether the appearance of aggregates is always related to changes in the secondary protein structure is unclear.

In the literature, divergent results concerning the secondary structure of immunoglobulins after spray-drying were presented. As described previously [15] the reaction of protein to the removal of water can be classified in different categories: (i) dehydration can lead to an irreversible destruction of the secondary structure, (ii) the structural changes can be reversible upon rehydration, or (iii) the native conformation is maintained in the dried state. rhIgG was resistant to dehydration-induced changes in the overall secondary structure, since the spectra of the spray-dried powder measured in the solid sate as well as in the liquid state after redissolution were identical [16]. In contrast Maury et al., claimed structural changes in the dried state – measured with FTIR as KBR pellet – which disappear upon redissolvation indicating reversible alterations [17]. Based on these results the question arose which statement could be supported and whether possible alterations in the secondary structure result from the sample preparation. Therefore, this paper focuses on the relationship between protein structure and protein aggregation behaviour for spray-dried formulations.

2. Materials and methods

2.1. Materials

A humanized chimeric monoclonal antibody (IgG1) was provided by Boehringer Ingelheim Pharma GmbH & Co. KG. The initial aqueous immunoglobulin solution was dialyzed to obtain a solution containing 95.0–100.0 mg/ml antibody, 1.6 mM glycine and 25 mM histidine. The pH

value was adjusted to 6.25 with HCl. This pH and buffer composition was used throughout all experiments. The antibody solution contained between 0.3% and 0.9% of aggregates after diafiltration. During the storage at 2–8 °C the stability of the antibody solution was tested in regular periods by size-exclusion high performance liquid chromatography.

The immunoglobulin solution was stored at 2–8 °C and was stable for at least 2 years according to SEC and turbidity (data not shown). Mannitol was purchased from Caesar & Lorentz GmbH (Germany) and was added to the IgG1 solution at weight ratios up to 20/80 IgG1/mannitol.

2.2. Spray-drying

Spray-drying was performed with a Büchi Mini Spray Dryer B-290 (Büchi Labortechnik, Flawil, Switzerland) at 25 °C/50% RH. For the spray-drying process the inlet and outlet temperature (T_{in}/T_{out}) was 90/50 °C. The drying air volumetric flow rate was adjusted 600 l/min, a liquid feed flow rate of 3 ml/min was utilized at an atomizing air volumetric flow rate of 670 l/min. The resulting aspirator flow rate was 35 m^3/h.

2.3. Fourier transform infrared spectroscopy (FTIR)

FTIR measurements were conducted with a Tensor 37 spectrometer (Bruker, Karlsruhe, Germany). Solutions were analyzed either by application of the Confocheck system which uses the technique of transmission (optical path length 5.9 μm, 120 scans, resolution 4 cm^{-1}) or in attenuated total reflection with the BioATR II cell (120 scans, resolution 4 cm^{-1}). The protein concentration ranged from 10 to 100 mg/ml. The powders were either dissolved to a defined concentration and measured by using the transmission cell or characterized by using the attenuated total reflection technique. A protein free blank spectrum was subtracted from the sample spectrum using the Bruker software. Each spectrum was minimum-maximum normalised and a 9-point smoothing followed.

2.4. Circular dichroism spectroscopy (CD)

The circular dichroism spectra were recorded with a Jasco J 710/720 CD spectrometer at a constant temperature of 25 °C. For Far-UV CD measurements at 180–250 nm the path length was 0.1 mm and the protein concentration was 0.1–0.2 mg/ml. Wavelength scans were obtained by accumulation of 4 scans with a wavelength step of 0.1 nm. The spectra were background corrected and converted to the mean residue ellipticity (deg cm^2 $dmol^{-1}$) using the molecular mass of 148 kDa and a total number of 1314 amino acids. For the estimation of the secondary structural compositions of the proteins, the CD spectra were deconvoluted.

2.5. Size exclusion high performance liquid chromatography (SE-HPLC)

Size exclusion high performance liquid chromatography was used to determine the amount of soluble protein aggregates in the spray solutions and of the spray-dried powders after dissolution. The measurements were performed on a HP 1090 and on a HP 1100 instrument (Agilent Technology, Waldbronn, Germany) in connection with a TSK3000SWXL column (300 × 7.8 mm, Tosoh Biosep, Stuttgart, Germany). The mobile phase consisted of 0.1 M di-sodium hydrogen phosphate dihydrate and 0.1 M sodium sulfate and was adjusted to pH 6.8 with *ortho*-phosphoric acid 85%. The flow rate was 0.5 ml/min and the injection volume was 25 μl with a protein concentration of about 2–10 mg/ml.

2.6. Turbidity

Turbidity measurement was performed with a Nephla turbidimeter (Dr. Lange, Düsseldorf, Germany) in triplicate. The measurement is based on 90° scattered light photometry. The system is calibrated with a formazin standard and the results are specified by formazin nephelometric units (FNU) according to Ph. Eur., Protein powders were dissolved at 2 mg/ml.

2.7. Photon correlation spectroscopy (PCS)

The PCS measurements were performed with a Zetasizer Nano ZS (Malvern, Herrenberg, Germany) in the non-invasive-backscatter method at an angle of 173°. PCS data were calculated from a unimodal modelling algorithm. The protein concentration was adjusted to 2 mg/ml. The results are specified as average size (*z*-average). The size distribution is described by the polydispersity index.

3. Results and discussion

3.1. FTIR spectroscopy of IgG1/mannitol solutions

Before spray-drying the prepared IgG1 solutions containing mannitol as stabilizing excipient were analyzed

Table 1
Aggregation, turbidity and PCS measurements of the IgG1/mannitol solutions in the range of 20/80–100/0 immediately after preparation

IgG1/mannitol	Aggregation in spray-solutions (%)	Turbidity (FNU)	PCS peak 1, volume (%)
20/80	2.6	6.5	88.4
30/70	4.1	7.4	89.4
40/60	3.0	6.8	93.1
50/50	4.2	7.3	93.8
60/40	1.1	3.6	93.7
70/30	0.9	2.4	99.0
80/20	0.7	3.0	100.0
100/0	0.9	4.5	100.0

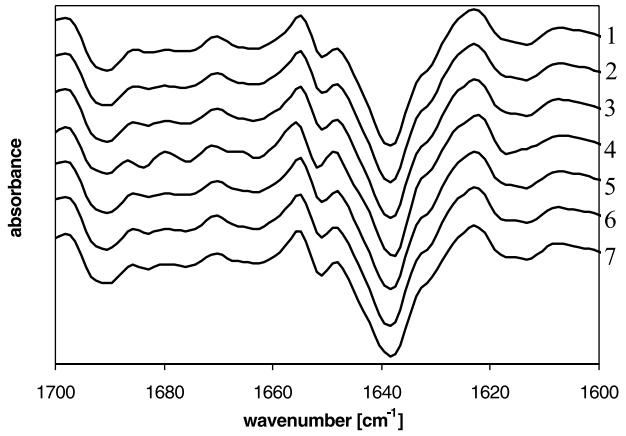

Fig. 1. FTIR second derivative spectra of the amide I band of IgG1/mannitol solutions 20/80 (1), 30/70 (2), 40/60 (3), 60/40 (4), 70/30 (5), 80/20 (6) and 100/0 (7) immediately after preparation.

for their initial aggregation level. The investigation by SE-HPLC revealed that 2.6–4.2% aggregates were present in solutions containing 50–80% mannitol (Table 1). With higher protein concentrations, the monomeric state is fully retained and comparable to the IgG1 in solution (0.9% aggregates). The evaluation of the IgG1/mannitol solutions with PCS showed that the solutions with 60–80% protein displayed only one population of particles (≈12 nm). Increasing amounts of mannitol induced a second peak, indicating the formation of a second particle population (100–200 nm). The turbidity slightly increased parallel to the aggregate level with higher mannitol concentrations.

Fig. 1 shows the second derivative spectra of the amide I band measured in transmission obtained for the distinct IgG1/mannitol solutions. The major peaks in the second derivative spectra were found at 1612, 1638 and 1690 cm^{-1}. The bands can be assigned according to the scheme displayed in Table 2 [16–19]. The obtained spectra of the IgG1/mannitol solutions depicted great overall similarity regarding the peak position, the shape and the intensity and correlated with the spectrum obtained for the pure antibody solution (Fig. 1). The major band components at 1638 and 1690 cm^{-1} in addition to the components at 1612 cm^{-1} are indicative for a β-sheet folded protein [19,20]. The small band component at 1651 cm^{-1} represents the α-helical fraction. The band positions agree fairly well with those reported for a general IgG molecule [21], since antibodies are mainly composed of β-structures with a small portion of α-helix [16]. Using the protein data bank provided by the manufacturer for the secondary structure analysis of the FTIR spectra, the amount of β-sheet elements of the antibody was estimated at 48 ± 3%, the α-helical regions at 7 ± 3%, and the rest unordered, random coil structures. This corresponds to the characteristic secondary structure of antibodies [16].

Table 2
Band assignments from FTIR spectra in H_2O [16–19]

Secondary structure elements	Average (cm^{-1})	Range (cm^{-1})
β-Sheet	~1617	1615–1620
	~1639	1628–1645
	~1690	1686–1697
Non-ordered structures	~1645	1640–1650
α-Helical	~1652	1650–1658
Turns	~1670	1660–1667
		1676–1680

The addition of mannitol at high concentrations influenced the aggregation behaviour of the antibody, but relevant alterations in the secondary structure of the protein were not obvious. Apparently, aggregation is not the consequence of changes in the secondary structure which could be picked up by FTIR. There are several explanations for these results: firstly, the formation of aggregates may be due to changes in very small sections or sequences of the antibody molecule that cannot be detected by FTIR (under the used evaluation method the resolution is ~5% of changes in the secondary structure). Secondly, according to Fink and van de Weert, the protein can unfold via a "molten globule" intermediate, retaining its native secondary structure elements, but with changes in the tertiary structure [22,23]. Due to the fact that with spectroscopic techniques in general only overall information with regard to the protein secondary structure can be obtained, one could speculate that just very small populations of protein molecules may have undergone a change in the secondary protein structure, and that these molecules may induce protein aggregation. However, up to now, a clear scientific justification supporting this possibility is still missing.

Finally, it is also possible that aggregation is not related to changes in the secondary protein structure at all. Again, it has to be mentioned that with spectroscopic techniques just global information with regard to the protein secondary structure can be detected. It has been shown that IgG suspensions precipitated e.g. with PEG, retain the protein secondary structure [24], as well as associate in highly concentrated formulations [25].

3.2. CD spectroscopy of IgG1/mannitol solutions

In order to further investigate the phenomenon of mannitol-induced aggregation and possible changes in the secondary structure, CD was used as a complementary method. The spectra of the IgG1/mannitol solutions showed a minimum at 217 nm which is assigned to n–π^* transition in the amide bond of the protein backbone and a maximum at 201 nm which is assigned to π–π* transition of electrons in the valence shell of the oxygen atom in the amide bond [26,27]. The minimum and maximum correlate with a structure that consists mainly of β-sheet elements (see Fig. 2) [12,20], analogous to the results achieved with FTIR-analysis of the antibody solution. The different IgG1/mannitol solutions were measured as equi-concentrated solutions with different mannitol quantities, using the corresponding mannitol–buffer solution as blank. The spectra revealed the same shape as the pure antibody solu-

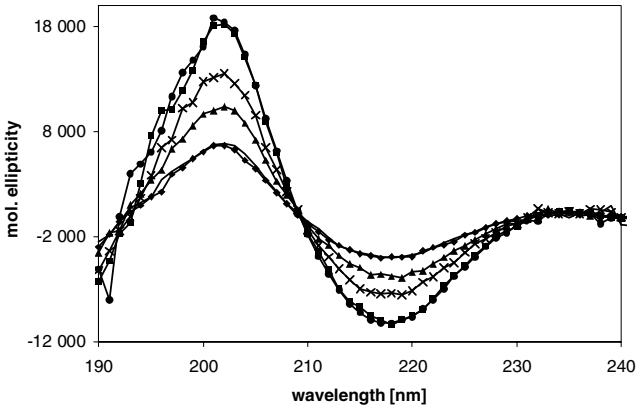

Fig. 2. CD spectra of IgG1/mannitol solutions (0.1 mg/ml) in the ratio of 20/80 to 100/0; 20/80 (–), 30/70 (▲), 40/60 (3), 50/50 (♦), 70/30 (×), 80/20 (■) and 100/0 (●) immediately after preparation.

tion (Fig. 2), thus indicating no obvious changes in the secondary structure. However, the CD spectra showed a variation in ellipticity, despite equal protein concentrations. Solutions with 20–40% mannitol displayed spectra similar to the one of pure antibody solution. With an increasing amount of mannitol (80%) the intensity decreased. The isodichroic point at about 209 nm indicates an equilibrium between two states [28,29]. This decrease in intensity leads to the question whether it can be traced back to changes in the secondary structure, although no band shifts are visible. A potential reason would be changes in the polarity of the medium by addition of mannitol, which were observed to produce large changes in the intensities as seen for lysozyme [30]. Furthermore, aggregate formation can lead to a decrease in the intensity due to varied interaction with light and the resulting light scattering. An increase in the molar ellipticity has been described with a shift of the native-state to more ordered and compact protein conformations without detectable changes in the secondary structure [30,31]. The analysis of the secondary structure elements from CD revealed almost the same results as FTIR did. The bulk solution showed about 12% α-helix, 44% β-sheet and 37% random coil (Table 3). The antibody/mannitol solutions revealed comparable secondary structure compositions and the differences of 3–4% are within error of the measurement technique [11].

Table 3
Secondary structure analysis of IgG1/mannitol solutions in the range of 20/80 and 100/0 (from CD), accuracy ±3%, $n = 3$

	10/90	20/80	30/70	50/50	70/30	80/20	100/0
Helix	8.3	8.6	7.2	8.5	10.1	11.8	11.5
Antiparallel	41.3	41.0	43.3	40.7	38.6	36.4	36.4
Parallel	5.8	5.9	5.3	5.9	6.5	7.1	7.0
β-Turn	15.7	15.5	18.3	15.6	13.0	11.5	11.4
Random coil	34.2	34.3	32.4	34.4	36.0	37.0	37.3

3.3. Spectroscopic investigations of stored IgG1/mannitol solutions

In a next step, the IgG1/mannitol solutions were stored for one week at 40 °C to investigate an additional influence of storage on the protein secondary structure and to analyze whether a destabilizing effect could be triggered by time and temperature. As determined by SE-HPLC analysis, the aggregate content of the pure antibody solution and the IgG1/mannitol formulation 70/30 was 0.4–0.5% before storage. After storage for 1 week at 40 °C the aggregate level of the formulation 70/30 IgG1/mannitol was not changed and that of the pure antibody solution increased slightly to 1.5%. In contrast to these two solutions, the solution containing 30/70 IgG1/mannitol formed about 3.0% aggregates in solution and 6.5% after storage. The FTIR transmission spectra revealed no alterations in the secondary structure in the stored solutions (Fig. 3). All formulations showed the typical immunoglobulin spectrum with bands at ~1616, at ~1639, at ~1665, and at ~1690 cm^{-1} as the bulk solution did [16]. To conclude, the aggregation status did not correlate directly with changes in the FTIR spectra as seen for the different IgG1/mannitol solutions and from the stored IgG1/mannitol samples.

3.4. Influence of forced denaturation

To get a further understanding how the secondary structure can be altered, the antibody was denatured by temperature. The pure antibody solution (10 mg/ml) was heated stepwise for 10 min at 40, 50, 60, 70, 80 or 90 °C. Turbidity measurements showed that the solutions did not differ in turbidity after heating at 40, 50, 60 or 70 °C. The solutions were clear according to the European Pharmacopoeia [32]. There was a slight increase in turbidity after heating the solutions to 80–90 °C and the measured turbidity values

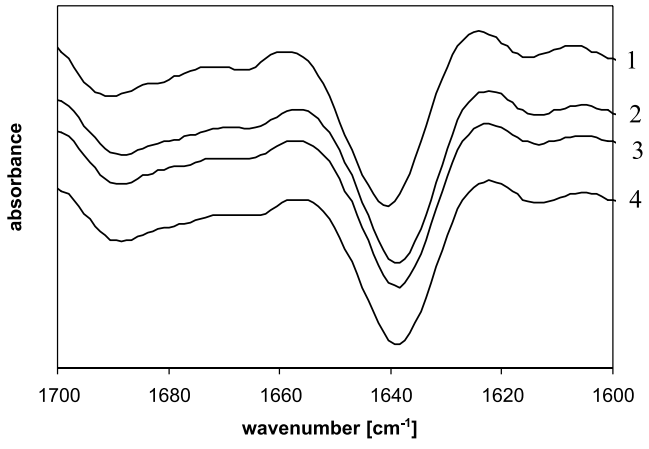

Fig. 3. FTIR second derivative spectra of IgG1/mannitol solutions stored at 40 °C for 1 week, measured in transmission [100/0 IgG1 solution (1), 100/0 IgG1 solution stressed (2), 70/30 IgG1/mannitol solution stressed (3), 30/70 IgG1/mannitol solution stressed (4)].

Table 4
Aggregation and turbidity of IgG1 solution before and after heating to 40 up to 90 °C

	Turbidity (FNU)	Aggregates (%)	AUC (%) referred to pure IgG1 solution
100/0	1.0	0.4	100.0
10 min, 40 °C	2.5	0.4	99.0
10 min, 50 °C	2.6	0.4	99.9
10 min, 60 °C	2.2	0.4	103.2
10 min, 70 °C	3.0	0.4	101.0
10 min, 80 °C	3.4	1.9	38.7
10 min, 90 °C	4.3	Not analysable	Not analysable

described a slightly opalescent solution. In SE-HPLC no increase in aggregates was detectable after heating for 10 min stepwise at 40–70 °C under similar temperature stress conditions as used for the spectroscopic investigations (Table 4). Exposure to 80 °C caused an increase in aggregates to 1.9% comparing the aggregate peak to the monomer peak. But analysis of the Area under the curve (AUC) revealed that the aggregation status must be higher, because the AUC after heating was only about 40% of the pure protein AUC which indicated formation of insoluble aggregates.

As the temperature was increased to 80–90 °C, two new bands at 1649 and 1657 cm^{-1} appeared in the infrared spectral regions of the amide I, which are characteristic for random coil and helical structures [33] (Fig. 4). The increase in random coil elements indicated the loss or the collapse of secondary structures upon heating. Moreover, a so-called red-shift of the band at 1638 cm^{-1} to a lower wavenumber of 1627 cm^{-1} occurred, which is correlated with increased formation of dehydrated β-sheet structure. The appearance of an absorption band at 1612 cm^{-1} correlates with the formation of intermolecular β-sheets and denatured structures going along with aggregation [2,20,34]. The intensity increase in the band at 1680 cm^{-1}

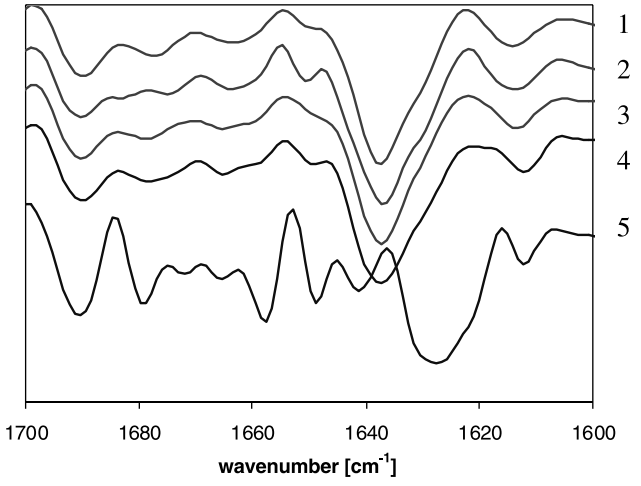

Fig. 4. FTIR second derivative spectra of IgG1 solutions heated (measured in ATR), [IgG1 solution (1), 60 °C (2), 70 °C (3), 80 °C (4), 90 °C (5)].

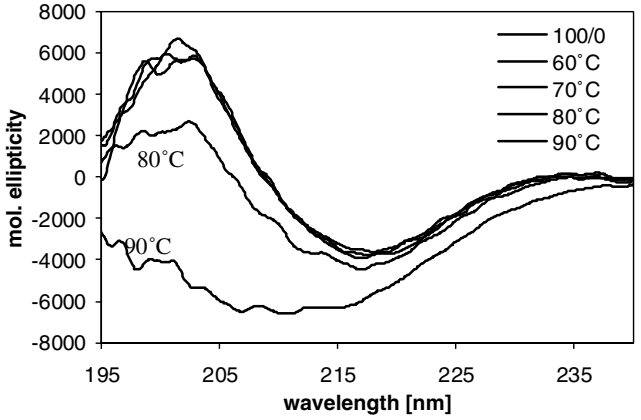

Fig. 5. Far-UV CD spectra of heated IgG1 solutions in the temperature range of 40–90 °C.

can be assigned to the antiparallel β-sheet formation. Murayama et al., found that the aggregation of BSA after heating to 70 °C was reflected in the appearance of two new bands at 1685 and 1615 cm^{-1} [34]. The heating of rhBMP-2 led to the similar results [35]. The temperature induced changes of the antibody were also investigated by CD. Up to 70 °C no change in the protein spectrum was detectable, indicating that the IgG1 secondary structure was stable within this temperature range and under the used thermal stress conditions (Fig. 5). After heating the protein up to 80 °C, the intensity of the maximum at 196 nm decreased and broadened [36]. A shift of the negative band from 219 to 210 nm is also obvious. Heating up to 90 °C led to a disappearance of the maximum at 196 nm, revealing that the secondary structure was greatly influenced by high temperature. A similar pattern of structural alteration by exposure to thermal stress was observed for bovine IgG and changes in the secondary structure took place above 70 °C [26,37].

The analysis of the secondary structure elements by CD indicated that the structural composition was stable up to 70–80 °C (Table 5). Heating to 90 °C induced a slight change in the amount of α-helix elements from 8% to 10%. The antiparallel structures were reduced from 41% to about 28%, the parallel β-sheet fraction decreased as well and the β-turn sequences increased to 22%. Vermeer and Norde have shown using CD spectroscopy that heating immunoglobulins result in an increase in random coil struc-

Table 5
Secondary structure analysis based on CD measurements of heated IgG1 solution

Secondary structure elements	Pure IgG1	40 °C	60 °C	80 °C	90 °C
Helix	8.5	8.4	8.6	9.0	9.9
Antiparallel	40.7	40.6	40.6	36.8	27.9
Parallel	5.9	5.8	5.9	5.5	4.7
β-Turn	15.7	16.1	15.9	17.5	21.6
Random coil	34.3	34.2	34.2	34.7	36.5

ture [36]. The increase in random coil and α-helix elements by heating was detected by both CD and FTIR.

It can be concluded that the structural changes induced by thermal stress were different from those resulting from the perturbations induced by addition of mannitol.

3.5. Spectroscopic investigations of redissolved spray-dried IgG/mannitol powders

Antibody 1/mannitol solutions with ratios between 100/0 and 20/80 were spray-dried at T_{in}/T_{out} of 130/75 °C and a total solid content of 10%. The resulting powders contained 0.7–6.4% aggregates. As described previously [15] the reaction of protein to the removal of water can be classified in different categories: (i) dehydration can lead to an irreversible destruction of the secondary structure (ii) the structural changes can be reversible upon rehydration or (iii) the native conformation is maintained. The reversibility of the protein unfolding during redissolving was recognized for lyophilized lysozyme and ribonuclease powders [38]. The prepared IgG powders were measured both in attenuated total reflection (ATR mode) as solids, as well as after reconstitution as aqueous solution in transmission. By using the ATR technique we can exclude potential negative effects deriving from KBr pellet, as described in the literature [39]. The transmission spectra of all re-dissolved antibody/mannitol powders were identical with that of the pure antibody solution (Fig. 6). Thus, FTIR spectroscopy of the reconstituted antibody powders did not show relevant perturbations of the secondary structure. These results were consistent with the data obtained for rehydrated spray-dried immunoglobulins and lysozyme described in the literature [16–18]. The reconstituted spray-dried powders were additionally characterized by CD spectroscopy. All spectra of the redissolved spray-dried powders showed the characteristic maximum and minimum at 207 and 217 nm, respectively, which is indicative for a protein that is mainly composed of β-sheet structure. The spectra showed no significant alterations in the secondary structure despite varying aggregation levels of the powders.

3.6. ATR-FTIR spectroscopy of spray-dried IgG/mannitol powders

The transmission spectra of redissolved powders displayed identical spectra for all antibody/mannitol solution indicating that the secondary structure of the antibody was either maintained during spray-drying or regained by rehydration, corresponding to literature [17,18,40]. In order to study whether changes in the solid state were induced by spray-drying, the powders were investigated in the dried state with ATR. The IgG1/mannitol powders 60/40, 70/30 and 80/20 IgG1/mannitol all showed the same spectra (Fig. 7). Comparison of these protein powder spectra with the antibody solution measured in ATR mode revealed that the shape of the amide I band was changed upon spray-drying. Compared to the IgG1 solution, the spray-dried mannitol powders showed a shift of the band from 1637 cm^{-1} to about 1639 cm^{-1} with an increase in band width, and the bands at 1615 and 1690 cm^{-1} nearly disappeared. Griebenow and Klibanow [40] have described similar results by examining the secondary structure of freeze-dried protein formulations. An increase of the β-sheet content was detected upon drying of the protein. It has also been mentioned that the existence of intermolecular β-sheet elements in a polypeptide is favoured in the solid state, since these can maintain a lower degree of solvation compared to other secondary structural elements such as α-helical or random coil [15]. The alterations in the spectra are caused by changes in

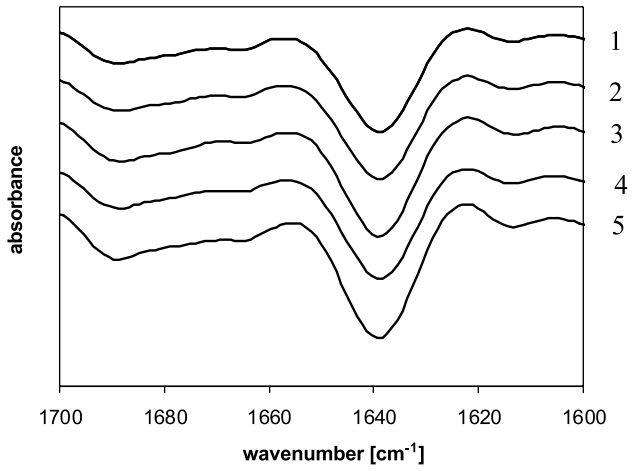

Fig. 6. FTIR second derivative spectra (transmission) of IgG11/mannitol powders spray-dried at a T_{in}/T_{out} of 130/75 °C after rehydration [100/0 IgG1 solution (1), 70/30 IgG1/mannitol (2), 60/40 IgG1/mannitol (3), 40/60 IgG1/mannitol (4), 20/80 IgG1/mannitol (5)].

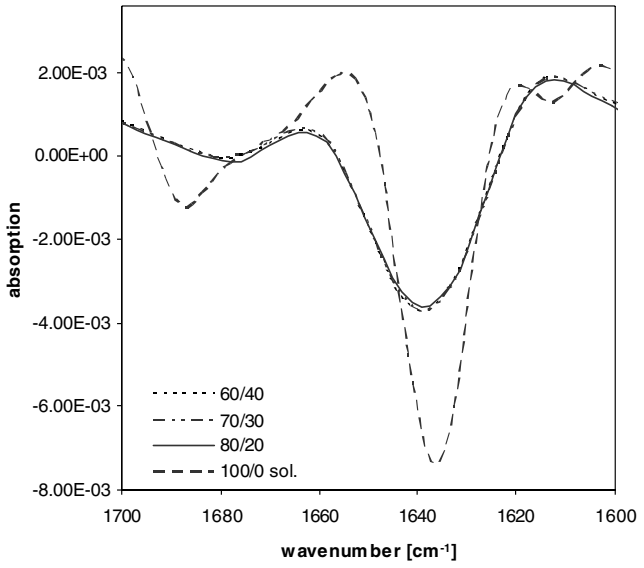

Fig. 7. FTIR second derivative spectra (ATR measurement) of spray-dried IgG1/mannitol powders (60/40 ■■■, 70/30 -■■-, 80/20 – IgG1/mannitol) in comparison to the IgG1 solution.

the polar environment of the carbonyl group of the peptide bond, which is involved in different hydrogen bonding networks. In the solid, dry state water molecules are removed and mainly replaced by OH groups from the sugar. This "new" hydrogen bonding environment of the protein can induce the observed change in the amide I band shape. However, changes of the secondary structure also have to be considered [17,38,41,42]. For most unprotected proteins the second derivative spectra for the dried solid are greatly altered, relative to the respective spectra for the native proteins in aqueous solutions. It appears that extended, random portions of the peptide backbone are not favoured in the dried solid, due to the propensity of such regions to form intermolecular interactions in the absence of water [38]. Assuming very strong changes in the protein secondary structure, one would expect that after redissolution changes of the protein secondary structure would be retained. However, it is observed that the original structure was regained after redissolution, and one can therefore conclude that the secondary structure is not much altered in the solid state or that the changes of the secondary structure are completely reversible upon hydration. Additionally, the different levels of aggregate formation were not reflected in a varying degree of alteration in the amide I region.

4. Conclusions

Addition of 60–80% mannitol (100 mg/ml total solid) induced antibody instability resulting in the formation of aggregates. Independent from the mannitol content the FTIR spectra of the antibody solutions depicted great overall similarity with band components at 1638, 1690 and below 1620 cm^{-1} indicative for β-sheet folded proteins. This is supported by CD data. Consequently, the aggregation cannot be correlated with significant changes of the protein secondary structure, under the consideration of the sensitivity of the used techniques.

During storage of IgG1/mannitol solutions, the aggregate level increased, but alterations in the secondary structure were not detectable by FTIR. Thermal induced denaturation revealed significant alterations in the antibody secondary structure as detected by FTIR and CD at temperatures >80 °C. However, the effects could not be correlated with the mannitol induced changes or aggregate formation. Neither FTIR nor CD could detect any changes which could be related to relevant changes in the protein secondary structure and structure of redissolved IgG1/mannitol independent of aggregate status. Studies of the powders in the dry state indicated a change in the protein environment in the solid state, mainly due to alterations of the polar environment of the peptide groups (C═O, N─H). However, upon redissolution the secondary structure of the antibody is totally regained. Thus the secondary protein structure is not altered during spray-drying.

Overall, FTIR and CD spectroscopy can provide further insight into the structural behaviour of antibodies upon spray-drying but the formation of the observed aggregates cannot be correlated with significant changes of the protein secondary structure.

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