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Research paper

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

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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).

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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