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Aggregation factor analysis for protein formulation by a systematic approach using FTIR, SEC and design of experiments techniques

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ABSTRACT

A simple systematic approach using Fourier transform infrared (FTIR) spectroscopy, size exclusion chromatography (SEC) and design of experiments (DOE) techniques was applied to the analysis of aggregation factors for protein formulations in stress and accelerated testings. FTIR and SEC were used to evaluate protein conformational and storage stabilities, respectively. DOE was used to determine the suitable formulation and to analyze both the main effect of single factors and the interaction effect of combined factors on aggregation. Our results indicated that (i) analysis at a low protein concentration is not always applicable to high concentration formulations; (ii) an investigation of interaction effects of combined factors as well as main effects of single factors is effective for improving conformational stability of proteins; (iii) with the exception of pH, the results of stress testing with regard to aggregation factors would be available for suitable formulation instead of performing time-consuming accelerated testing; (iv) a suitable pH condition should not be determined in stress testing but in accelerated testing, because of inconsistent effects of pH on conformational and storage stabilities. In summary, we propose a three-step strategy, using FTIR, SEC and DOE techniques, to effectively analyze the aggregation factors and perform a rapid screening for suitable conditions of protein formulation.

1. Introduction

The past three decades have seen an explosive growth in the biopharmaceutical industry driven by advances in biotechnology. Currently, the global biotech industry raised a total of $53 billion in 2007, a 13% growth compared to the previous year [1]. However, a rapid commercialization of protein drug candidates has not been fully realized due to several technical difficulties, including protein aggregation.

Protein aggregation occurs readily in almost all biopharmaceutical processes. Indeed, aggregates can form during storage even though the protein preparation may have been aggregate-free after the last polishing step was completed. Aggregation levels as low as 1% over a 2 year shelf-life can render a product clinically unacceptable [2]. Aggregate formation, as the prevalent physical instability reaction in liquid protein formulations, is initiated by the intermolecular interaction of hydrophobic regions of at least two unfolded or partially folded protein molecules. Hydrophobic interaction is affected by temperature, ionic strength or shaking [3]. Chemical instability reactions can also directly crosslink protein chains or change the hydrophobicity of a protein, indirectly changing its aggregation behavior. Disulfide bond formation/exchange is probably the most common pathway of chemically induced protein aggregation, but non-disulfide cross-linking pathways also form covalent dimers or polymers of proteins. In addition, oxidation and Maillard reactions directly and indirectly induce protein aggregation during storage. Storage at low temperature is generally a safe way to protect a protein from aggregation although it is not always practical. An important strategy to protect protein preparations from aggregating during storage is the selection of an appropriate protein stabilizer and good buffering agent at a suitable concentration and pH. Numerous studies have demonstrated that protein aggregation can be significantly different in different buffer systems and at different concentrations [4,5]. Indeed, many protein stabilizers that inhibit protein aggregation have been studied. Common protein stabilizing excipients include sugars, polyols, surfactants, salts, PEGs, polymers, metal ions and amino acids. Among these stabilizers, sugars are most often used [5–8]. The commonly used salt, NaCl is known to play a critical role in the inhibition of aggregation of certain proteins [1,8]. Surfactants are also widely used to prevent protein aggregation, although they may actually promote aggregation of certain proteins during storage [9,10].

Various analytical techniques have been employed for identifying and monitoring soluble and insoluble aggregates in protein solutions. Spectroscopy including circular dichroism (CD), FTIR and fluorescence are common biophysical methods used to assess

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the protein secondary and tertiary structure and thus to detect unfolded and aggregated protein molecules. Light scattering and microscopy are physical methods for determining the particle size and identifying protein aggregation. Electrophoresis and chromatography are common chemical methods used to detect and quantify protein aggregation. Many studies have indicated that a single analytical method is generally not sufficient to evaluate protein aggregation, and a combination of physical and chemical analytical methods to assess the protein stability will assist in the search for the optimal formulation [3,5,11].

At present, around 60 common stabilizing excipients have been used to enhance the stability and activity of protein formulations. However, these stabilizing effects are usually concentration and protein dependent. Moreover, high concentration of excipients may not be necessarily more effective, and in some cases, can have negative effects. Thus, each protein formulation needs to be developed independently [9]. Screening all excipients by all analytical techniques is ideal for determining the best protein stabilizer and assisting in the search for the optimum formulation. Nevertheless, this is almost unrealizable due to limited time and spending. Therefore, development of a time-conscious and cost-effective approach to screen for stabilizing excipients and evaluate the stability of protein formulation is an important goal for biopharmaceutical industries. This paper proposes a simple systematic approach for analyzing aggregation factors in protein formulations and rapidly determining the suitable condition of protein formulation. The approach includes designing the suitable formulating conditions using DOE technique, determining the protein stability in stress and accelerated testings using FTIR and SEC, and analyzing the main effects of single factors and interaction effects of combined factors on aggregation. We used two orthogonal techniques, FTIR and SEC, in this approach. Although the combination of FTIR and SEC is not sufficient to fully characterize a heterogeneous protein population and its stability profile, but they are well established tools to quickly and objectively detect changes in protein conformation in stress testing and monitor the process of protein aggregation in accelerated testing [5,11,12]. We also chose three most important protein's environment's factors (protein concentration, formulation pH and buffer concentration), and three commonly used stabilizing excipients (sugar, salt and surfactant) as evaluating factors in DOE analysis. This approach could perform a simultaneous screening for multiple stabilizing excipients and other formulation conditions, such as buffer concentration, protein concentration and pH. Our results suggest that the approach would be useful for effectively analyzing the aggregation factors and quickly determining the suitable conditions of protein formulation.

2. Materials and methods

2.1. Materials

A human polyclonal antibody (IgG) that was purchased from Sigma–Aldrich (St. Louis, MO) was used as a model protein for analyzing the aggregation factors for protein formulation. Stock solutions of IgG were prepared as 50 mg/ml or 1 mg/ml by dissolving IgG directly in DW (distilled water). Six factors (protein concentration, pH, phosphate buffer concentration, salt, sugar and surfactant) were analyzed, each factor being set to two alternative values (see Table 1 for details). We used D-optimal design to choose 27 formulating conditions in this study (Table 2). Stock solutions of IgG were dialyzed overnight at 4 °C against the 27 different solutions using a Micro Dialyzer (TOR-14K, Nippon Genetics, Tokyo, Japan). The final compositions of these formulations are listed in Table 2.

Table 1
Levels for the factors examined in D-optimal design.

Factors		Levels	
		Low	High
Protein concentration (mg/ml)	IgG	1	50
Salt (mM)	NaCl	0	300
Buffer concentration (mM)	Phosphate	10	50
pH		5.4	7.2
Surfactant (%)	Tween-80	0	0.7
Sugar (mM)	Sucrose	0	300

2.2. Stress and accelerated testing

The stress testing was performed in the temperature range from 25 to 90 °C over a time period of 1.5 h using FTIR spectroscopy to monitor the conformational stability of proteins. Twenty seven IgG solutions (shown in Table 2) were used in this procedure. In accelerated testing, 18 different IgG solutions (Table 2, * mark) were prepared and stored at 40 °C. After storage for 4, 6 or 8 weeks, the solutions were analyzed by SEC at room temperature to evaluate storage stability.

2.3. FTIR spectroscopy

Infrared spectra of the protein solutions were recorded by using a Tensor 37 spectrometer (Bruker Optik GmbH, Ettlingen, Germany). Protein samples were filled in a BioATR II attenuated total reflectance cell (Harrick, Ossining, NY), which was connected to a thermostat (HAAKE K20, Thermo electron Haake, Paramus, NJ). Aggregation temperature (T_{agg}) is a measure of the stability of biopharmaceuticals against aggregation. Temperature-dependent spectra were observed at 2 °C intervals in the temperature range from 25 °C to 90 °C. For each spectrum, a 128 scan interferogram was collected at a single beam mode with 4 cm^{-1} resolution. Reference buffer spectra were recorded under identical conditions. The collected interferograms for the protein and the buffer solutions were then Fourier transformed, respectively, and the protein spectrum was obtained by subtracting the buffer spectrum at each temperature. Recorded infrared spectra were analyzed by the Protein Dynamics mode in OPUS software (Bruker Optik).

T_{agg} values were obtained by determining the inflection point of the thermal transition curves, which were acquired by plotting the wavenumber at the absorbance maximum in the amide I band *versus* temperature, and then calculated from the following formula based on the work of Zscherp et al. [13].

$$f(T) = \frac{(a_1T + b_1) + (a_2T + b_2)\ \exp(c(T - T_{agg}))}{1 + \exp(c(T - T_{agg}))} \quad (1)$$

where the parameters a_1, a_2, b_1 and b_2 characterize the linear parts of the function at temperatures sufficiently above and below the transition temperature, respectively. The parameter c describes the steepness of the transition.

2.4. SEC

SEC was used to determine the amount of soluble aggregate, dimer, monomer and cleaved fragments in the IgG formulations. The measurements were performed on an ÄKTA prime plus (GE Healthcare, Piscataway, NJ) using a Superdex™ 200/10/300 GC column (GE Healthcare). The column was pre-equilibrated in 150 mM NaCl, 50 mM sodium–phosphate buffer, pH 7.0. Samples (100 μl volume) were injected onto the column at a flow rate of 0.5 ml/min and the UV absorbance of the eluate was monitored at a wavelength of 280 nm. The soluble aggregate content in % was calculated as the AUC (total area under the curve) of the soluble aggregate