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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

Table 2
Formulating conditionas and aggregation temperature.

	IgG (mg/ml)	NaCl (mM)	Phosphate buffer (mM)	pH	Tween 80 (%)	Sucrose (mM)	T_{agg} (°C)
No. 1*	1	0	10	5.4	0	0	76.9 ± 0.8
No. 2*	1	0	10	7.2	0.7	300	77.0
No. 3*	1	0	50	5.4	0.7	300	78.2
No. 4*	1	0	50	7.2	0	0	76.0
No. 5*	1	300	10	5.4	0.7	0	72.4
No. 6*	1	300	10	7.2	0	300	77.3
No. 7*	1	300	50	5.4	0	300	75.8
No. 8	1	300	50	7.2	0.7	0	76.3
No. 9*	50	0	50	5.4	0	300	76.7
No. 10*	50	300	10	5.4	0.7	0	74.5
No. 11*	50	300	50	7.2	0	300	75.2
No. 12*	50	0	10	7.2	0.7	300	75.3
No. 13	50	300	10	5.4	0.7	300	74.8
No. 14	50	300	10	7.2	0	0	76.0
No. 15	50	300	50	5.4	0	0	74.6
No. 16	50	300	50	7.2	0.7	300	77.5
No. 17*	1	0	50	7.2	0.7	300	78.8
No. 18*	1	0	10	7.2	0	0	76.4
No. 19*	1	0	50	7.2	0	0	75.7
No. 20*	1	0	10	5.4	0.7	300	76.8
No. 21*	1	0	10	7.2	0	300	77.9
No. 22*	1	0	50	5.4	0	0	75.7
No. 23*	1	300	50	7.2	0	300	77.5
No. 24	50	0	50	5.4	0	300	77.4
No. 25	50	300	10	5.4	0.7	0	74.3
No. 26	50	300	50	7.2	0	300	76.3
No. 27	50	0	10	7.2	0.7	300	77.3

27 (Nos. 1–27) and 18 (marked with *) kinds of solutions were examined in stress and accelerated testings, respectively.

compared to the total AUC × 100. The fractions of dimer and monomer were calculated using the same method. The total AUC of the SEC chromatograms remained constant during accelerated testing.

2.5. D-optimal design

DOE is a statistical method that is used to determine the relationship between factors (X) affecting a process and an output of that process (Y). D-optimal design, one such DOE technique, was used to evaluate and model the main effects of single factors and interaction effects of combined factors on protein stability. We were especially interested in evaluating whether combinations of factors enhance the stability of a protein drug. In the optimal model, the main effects of single factors and the interaction effects of combined factors are determined by fitting the data to the following equations using the JMP5 software (SAS, Inc., Cary, NC).

$$Y = \beta_0 + \sum \beta_i X_i \quad (2)$$

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i<j} \beta_{ij} X_i X_j \quad (3)$$

$$X = \frac{2x - x_H - x_L}{x_H - x_L} \quad (4)$$

where Y is T_{agg} or fraction of aggregates. X_i and X_j represent independent variables that correspond to the normalized concentrations of excipient i and j, respectively. x_H is the concentration of excipient at high level, while x_L is the concentration of excipient at low level. According to Eq. (4), X is equal to +1 when $x = x_H$. Also, X is equal to −1 when $x = x_L$. β indicates the model coefficients determined by stepwise regression analysis; β_0 is a constant term, β_i indicates the main effect of single factor attributed to an excipient i, and β_{ij} represents the interaction effect of combined factors attributed to excipients i and j.

We used JMP5 software to perform all statistical procedures. In the stepwise regression procedure, we set 'prob to enter' at 0.25 and 'prob to leave' at 0.1 in order to find significant factors in the fitting calculations. Eq. (2) was used for a linear model, in which six single factors were assumed to affect the aggregation independently. In contrast, Eq. (3) was used for an interaction model, in which a synergic positive/negative influence between two factors was also considered. The differences at $p < 0.05$ were considered as significant.

3. Results and discussion

3.1. Stress testing

An amide I band in IR spectra is frequently used to study protein conformations because its shape is sensitive to changes in secondary structure of proteins [12,13]. Fig. 1A shows the IR absorbance spectra of IgG in control buffer (150 mM NaCl) from 25 to 90 °C. The native IgG is predominantly β-sheet, which give rises to the strong absorbance around 1640 cm^{-1} at 25–55 °C. The thermal unfolding was exhibited by the spectrum recorded at 60–80 °C, where the band was shifted from 1640 to 1628 cm^{-1}. The thermally induced aggregation was indicated by the spectrum recorded at 80–90 °C. The observed band at 1628 cm^{-1} was assigned to the intermolecular β-sheet in the protein aggregates. The band at 1695 cm^{-1}, which is associated with the intermolecular β-sheet structures, was not clear in Fig. 1A, but it was observed at second derivative spectrum (Fig. 1B). The band at 1628 cm^{-1} was retained after cooling, indicating that the proteins were irreversibly aggregated. As for thermal perturbation of proteins, several FTIR studies have been reported previously [14,15]. These studies have demonstrated that IgG is inactivated by heat through a two-step mechanism: a thermal unfolding as a first step and a temperature-induced irreversible aggregation as a second step. Our observation was essentially consistent with these preceding investigations.

The temperature-induced aggregation causes a shift in wavenumber at the absorbance maximum in amide I band (Fig. 2). Fitting calculation of the sigmoid curve delivers a point of inflection, which is regarded as aggregation temperature T_{agg} in this

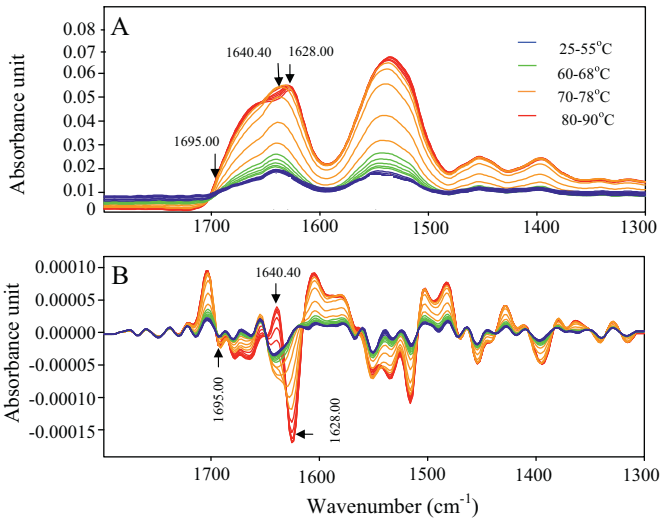

Fig. 1. IR absorbance spectra of human polyclonal antibody (IgG) solution during stress testing. Spectra were recorded at 25–55 °C (blue), 60–68 °C (green), 70–78 °C (orange) and 80–90 °C (red). IgG concentration: 1 mg/ml; buffer: 150 mM NaCl. (A) IR absorbance spectra; (B) second derivative spectrum. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

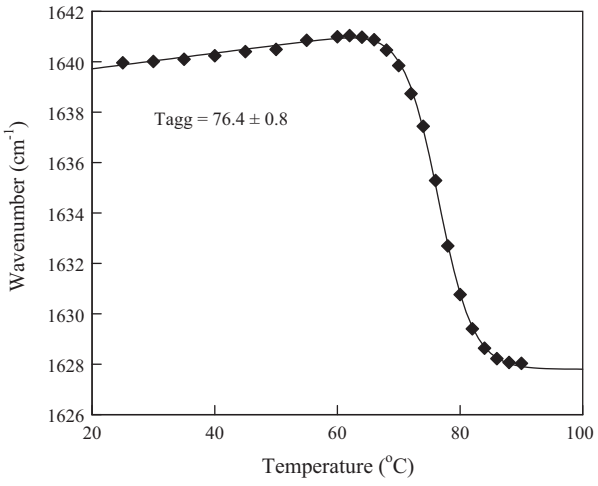

Fig. 2. Temperature dependence of maximum wavenumber for the amide I band during stress testing. Solid curve denotes a fitted theoretical curve that follows Eq. (1). IgG concentration: 1 mg/ml; buffer: 150 mM NaCl.

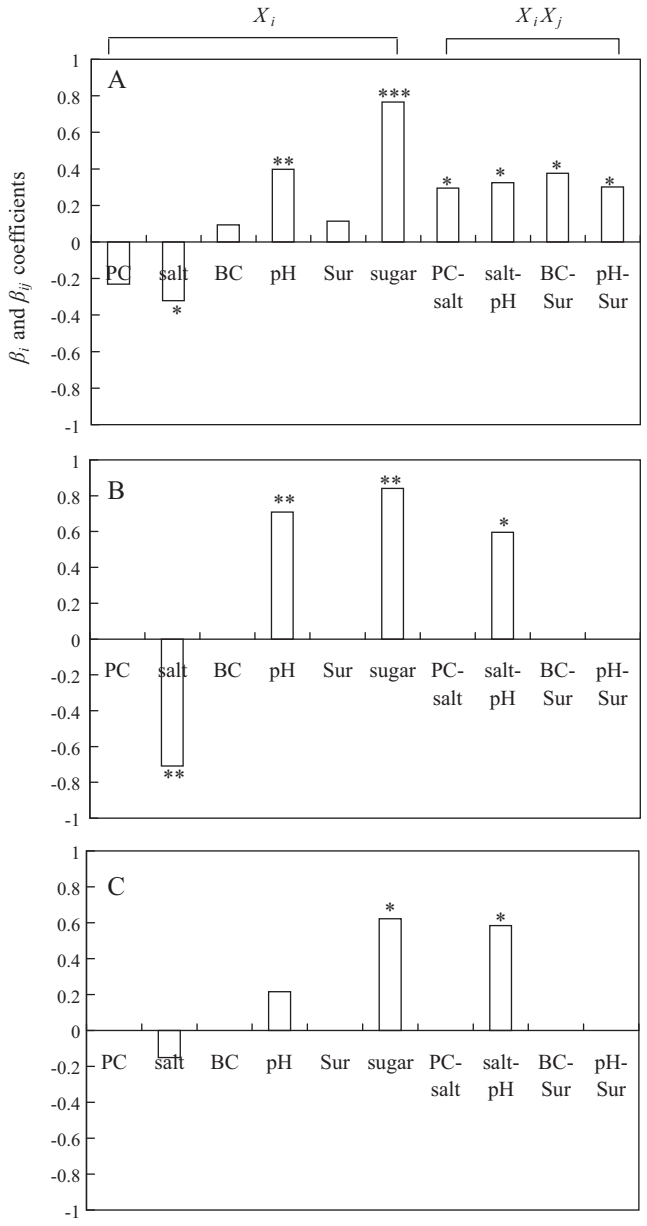

Fig. 3. The coefficient (β_i) of main effect of single factor and coefficient (β_{ij}) interaction effect of combined factors calculated from Eq. (3) during stress testing. (A) All samples including low and high concentrations of IgG formulations; (B) low concentration of IgG formulations (1 mg/ml); (C) high concentration of IgG formulations (50 mg/ml). PC, protein concentration; BC, buffer concentration; Sur, surfactant; X_i, individual factors; X_iX_j, combined factors; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

study. After the series of FTIR measurements, the values of T_{agg} for 27 kinds of IgG formulations were determined (Table 2). The average T_{agg} is 76.3 °C and the range is from 74.3 to 78.8 °C. The pH, salt and sugar contents were found to have a significant impact on protein stability (T_{agg}) for all IgG formulations ($p < 0.05$) (Fig. 3A). While increasing the pH from 5.4 to 7.2, an increment in aggregation temperature (ΔT_{agg}) was expected to be positive ($\Delta T_{agg} = 2.6$ °C). Sugar significantly increased the aggregation temperature ($\Delta T_{agg} = 3.0$ °C), while salt had the opposite affect ($\Delta T_{agg} = -2.4$ °C). Other factors, including buffer concentration and surfactant content, were not significantly associated with T_{agg} for all IgG formulations.

As to the low concentration IgG formulation, the pH and salt as well as sugar content were also found to have a significant impact on conformational stability (T_{agg}) (Fig. 3B). In all IgG formulations, an increase of pH from 5.4 to 7.2, T_{agg} significantly induced a positive increase in aggregation temperature ($\Delta T_{agg} = 2.5$ °C). Sugar significantly increased the aggregation temperature ($\Delta T_{agg} = 2.8$ °C), while salt had the opposite affect ($\Delta T_{agg} = -2.0$ °C). Other factors, including buffer concentration and surfactant, were not significantly associated with protein stability in low concentration IgG formulations.

However, protein stability in high concentration IgG formulations showed a different tendency to that displayed in low concentration IgG formulations (Fig. 3C). Only sugar significantly induced a positive increase in the aggregation temperature ($\Delta T_{agg} = 2.9$ °C). Proteins behave differently depending on their concentration [13]. Matheus et al. [5] indicated that an up-scaling of the optimal formulation identified at a lower concentration due to material restrictions may not necessarily correspond to the ideal stabilizing formulation conditions required at the higher protein concentration. Indeed, an increase in protein concentration can impose new and unforeseen changes in terms of protein stability.

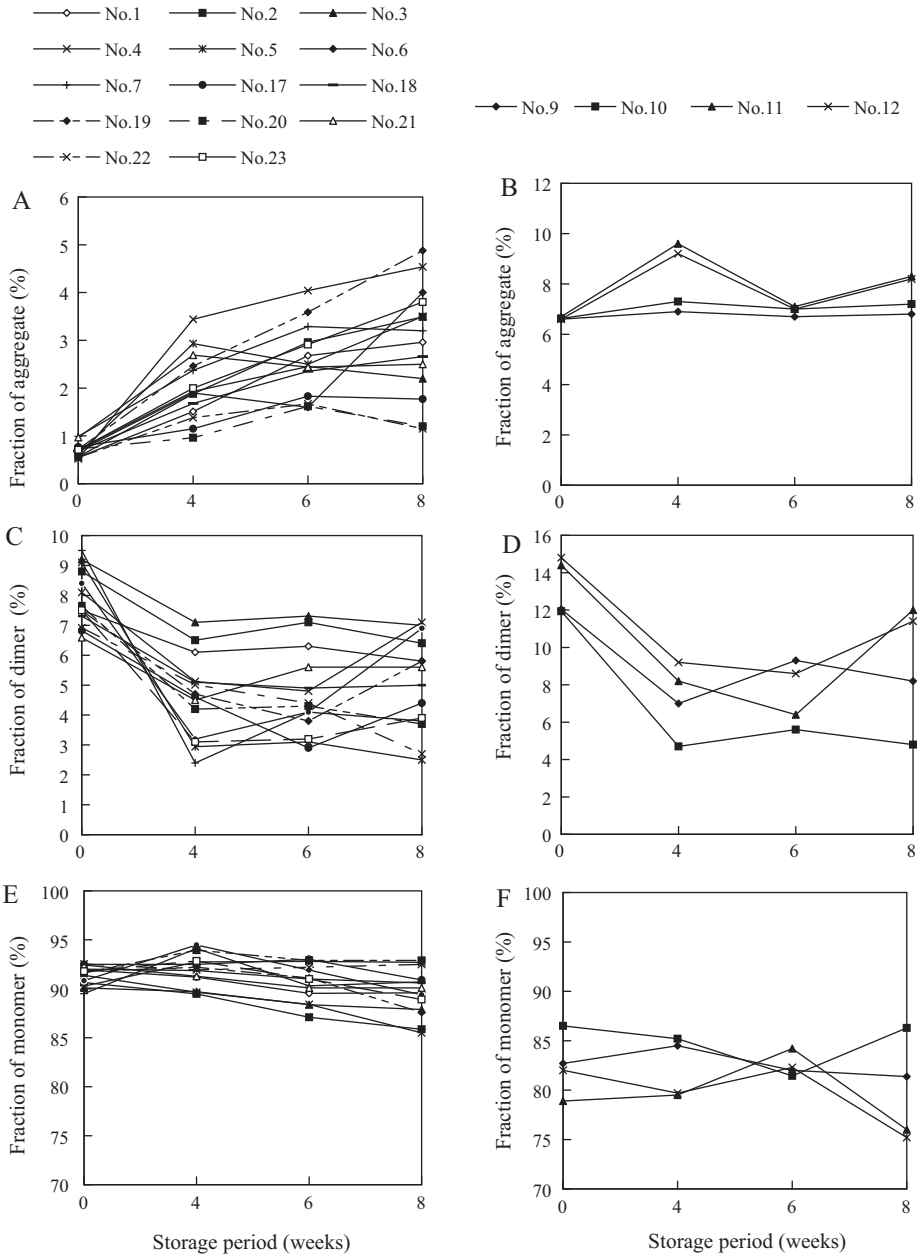

Fig. 4. Increase and decrease in the fractions of aggregate (A and B), dimer (C and D) and monomer (E and F) of IgG during accelerated testing at 40 °C. (A, C, and E) Low concentration formulations (1 mg/ml); (B, D, and F) high concentration formulations (50 mg/ml). The fractions of aggregate, dimer and monomer were calculated from SEC. Line number indicates a formulating condition shown in Table 2.

Like the results of Matheus et al. [5], our findings strongly indicate that investigation of stress testing at low concentrations of protein do not always reflect the actual behavior at higher concentrations. Therefore, a high concentration-stress testing is indispensable for a high concentration-formulation of biopharmaceuticals.

The main effects of protein concentration, buffer concentration, pH and stabilizing excipients on protein conformational stability have been reported in numerous studies [5,10], but very few of these reports have investigated the interaction effects among these factors. In this study, all factors were also tested on an interaction model. Our results show that combinations of protein concentration and salt positively enhance protein stability. In contrast, protein concentration did not significantly enhance protein stability as a single factor, while salt displayed a significant negative effect as a single factor. Positive interaction effects were also statistically significant between buffer concentration and surfactant, and pH and surfactant, although buffer concentration and surfactant did not individually show a significant effect on protein stability. Combining salt and pH had a significant positive effect on protein stability. Intriguingly, salt showed a negative effect whereas pH showed a positive effect. Sugar displayed the strongest significant positive effect on protein stability, but other factors combined with sugar had no major impact. These results indicated that the evaluation of the main effects of single factors is insufficient for choosing appropriate formulation conditions. Indeed, protein formulations always include several excipients to protect against protein degradation. The adjusted R^2 values were 0.53 and 0.98 for fitting the linear and interaction models using stepwise regression analysis, respectively. The result showed that the interaction model is a statistically suitable model for evaluating aggregation factors in IgG formulations, but the linear model is unsatisfactory. Thus, our results indicate that the interaction effects of combined factors

are essential for determining the suitable protein formulation of a biopharmaceutical agent.

3.2. Accelerated testing

3.2.1. Effect of protein concentration

18 kinds of sample solutions (Nos. 1–7, 9–12 and 17–23 in Table 2) were selected for examination in accelerated testing by D-optimal design. The fraction of aggregate in low concentration-IgG formulations (1 mg/ml) increased from 0.5–1.0% to 1.2–4.9%, but the increasing tendency was significantly different depending on the formulating conditions (Fig. 4A). Six formulations (Nos. 1, 2, 4, 18, 19, 23) displayed an increased fraction of aggregate after an extended period of storage. In four formulations (Nos. 7, 17, 20, 22) the fraction increased during storage for 4 and 6 weeks but then decreased after 6 weeks. A further four formulations (Nos. 3, 5, 6, 21) showed a complex pattern of results, with aggregation increasing at 4 weeks incubation then reducing at 6 weeks and finally increasing again at 8 weeks. The fraction of dimer decreased from 5–10% to 3–7% during the same period (Fig. 4C). Intriguingly, the decreased level of dimer formation almost mirrored the increasing tendency to generate aggregates ($R^2 = 0.97$). The fraction of monomer showed a slight decrease from 91.5 to 89.8% (Fig. 4E). Ventrella et al. [16] reported that increasing the temperature enhances the aggregation process and that the aggregation kinetics is faster for dimers than for monomers. Based on these results, the increase in the level of aggregate can be considered to be correlated to the decrease of dimer.

High concentration-IgG formulations (50 mg/ml) contained an elevated level of aggregation prior to storage, showing a 6-fold higher level than seen in low concentration IgG formulations at week zero (Nos. 9–12 in Fig. 4B). The fractions of aggregate increased from 6.6–6.7% to 7.2–8.3%. The increasing tendency did not coincide with that observed in low concentration IgG samples (Fig. 4B). The fraction of dimer dramatically decreased from 12–15% to 5–12% (Fig. 4D), while the fraction of monomer slightly decreased from 82.5 to 79.7% (Fig. 4F).

The protein concentration was found to have a significant impact on protein aggregation for all IgG formulations ($p < 0.001$) (Fig. 5A). Indeed, protein aggregation is generally dependent on its concentration. Ruddon and Bedows [17] have suggested that increasing the protein concentration above 0.02 mg/ml may facilitate potential protein aggregation. Other studies have also reported accelerated aggregation of proteins at high concentration e.g., interleukin-1β (IL-1β) above 0.1–0.5 mg/ml [18], low molecular weight urokinase (LMW-UK) above 0.2–0.9 mg/ml [19]. Our results are in agreement with these conclusions and also with the results of stress testing by FTIR. In conclusion, based on the results obtained by FTIR and SEC, it is not appropriate to replace high concentration-testings with low concentration-testings.

3.2.2. Effect of pH

The fractional increase of aggregate was significantly higher at pH 7.2 than at pH 5.4 in all IgG formulations ($p < 0.001$) (Fig. 5A). IgG shows the greatest conformational stability at pH 7.0, but increased aggregation tendency appears to be due to the proximity of the isoelectric point of the major portion of the molecules [4]. Szenczi et al. [4] reported that the isoelectric point of polyclonal IgG varied between 4.7 and 7.5 in equal distributions. The optimum pH range for storage is 5.0–6.0, which is a compromise between conformational stability and the tendency for oligomerization. Our results showed that the averaged fractions of aggregate at pH 5.4 were significantly decreased 33% compared with at pH 7.2, and pH 5.4 was more suitable pH value than pH 7.2 for storage stability (Fig. 6).

On the contrary, T_{agg} determined by the FTIR indicated that the pH value of maximum conformational stability is pH 7.2, and an increment in ΔT_{agg} was 2.6 °C. These contradictory results obtained by FTIR and SEC can be considered to be due to the different mechanism of aggregation. During the heating from 25 to 90 °C, as applied in FTIR analysis, an increase in temperature strongly affects perturbation of the native protein structure, and fosters sufficient unfolding to promote aggregation. The perturbations in structure occur as a result of changes in the respective secondary structural amide I band, and induce the shift of absorbance peak. Accelerated testing that was carried out at a temperature below the T_{agg}, could induce the formation of an aggregate-competent species due to degradation mechanisms, like deamidation, peptide bond cleavage and oxidation, as well as unfolding. Thus, the soluble aggregate monitored by SEC would be influenced by chemical instability of proteins. On the other hand, pH has a significant effect on the preferred chemical degradation pathways. For example, deamidation and disulfide bond scrambling occur at neutral and basic pH, while peptide bond hydrolysis occurs at either low or high pH. As revealed by SEC analysis of the accelerated stability samples, aggregation was formed mainly from dimmer. FTIR really measured the monomer behavior. These results suggest that stress testings using FTIR cannot replace accelerated testings for analyzing pH effects. The effect of pH on the long-term stability of IgG formulations should be determined by accelerated testings.

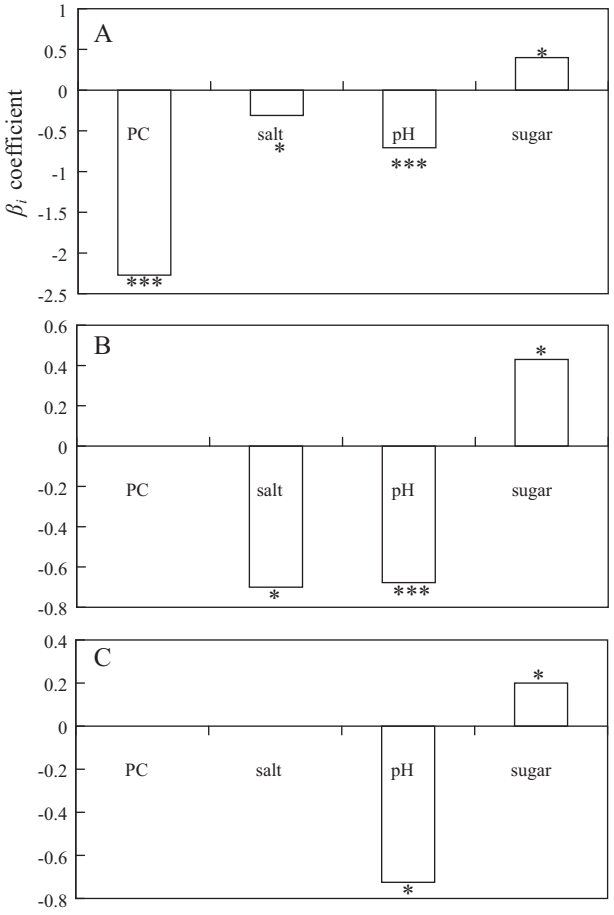

Fig. 5. β_i-Coefficient of main effect calculated from Eq. (2) during accelerated testing. (A) All samples including low and high concentrations of IgG formulations; (B) low concentration of IgG formulations (1 mg/ml); (C) high concentration of IgG formulations (50 mg/ml). PC, protein concentration. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3.2.3. Effect of buffer concentration

Both the type and concentration of buffers may affect the physical and chemical stabilities of a protein. Phosphate buffer

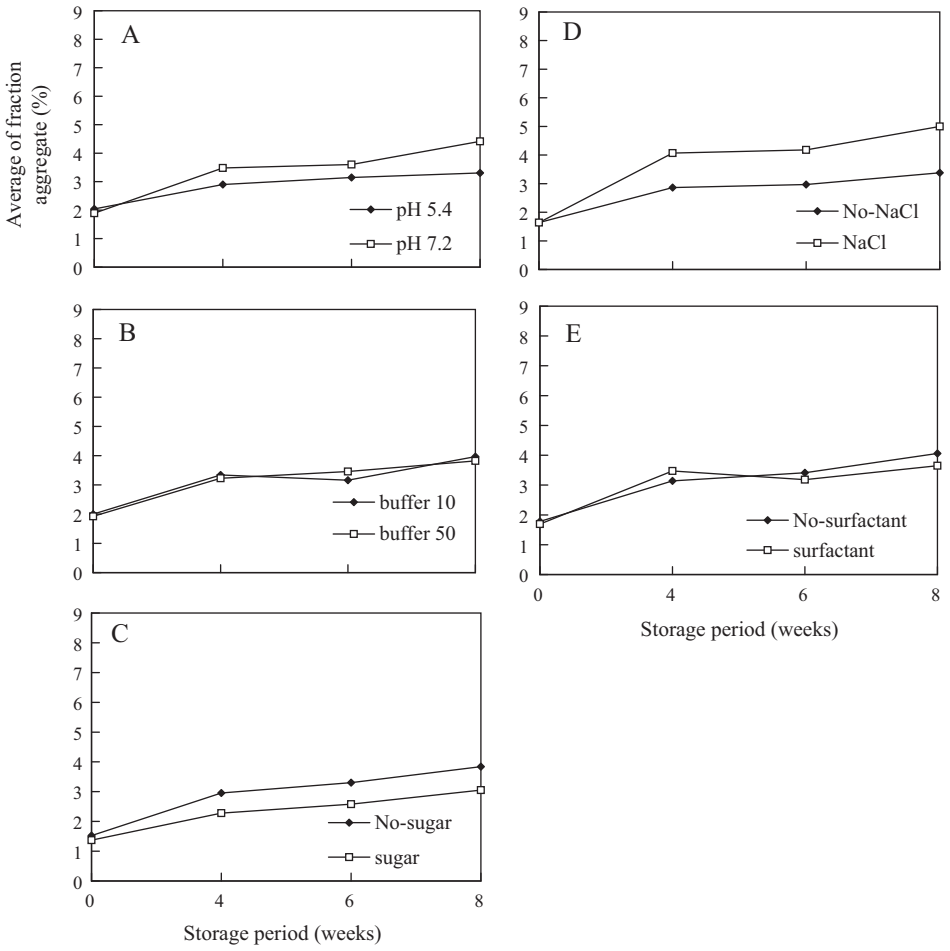

Fig. 6. Averaged fractions of aggregate during accelerated testing at 40 °C. Dependences on pH (A), buffer concentration (B), sugar (C), NaCl (D) and surfactant (E) are illustrated.

is commonly used in the formulation of antibody drugs such as Simulect (Novartic), Remicade (Centocor Ortho Biotech Inc.) and so on. In this study, phosphate buffer was used and evaluated at two concentrations (10 and 50 mM) to investigate the potential stabilizing effect on the 1 and 50 mg/ml IgG formulations. The results revealed no significant change in protein storage stability (Fig. 5). Matheus et al. [5] reported that increasing phosphate buffer concentration from 10 to 50 mM increases the rate of IgG_1 aggregation. However, Won et al. [20] showed the opposite affect i.e., increasing phosphate buffer concentration from 4.6 to 9.2 mg/ml significantly decreases the rate of an acidic fibroblast growth factor (aFGF) aggregation. Pikal et al. [21] reported that maximum aggregation of human growth hormone (hGH) occurs at 0.23 mg sodium phosphate/mg-hGH among three phosphate concentrations (0.11, 0.23 and 0.45 mg). These conflicting results suggest that phosphate buffer concentration may affect storage stability of a protein, but no general rules can be established for buffer concentration. The stress testing also showed that the buffer concentration did not significantly affect protein conformational stability of both low and high concentration IgGs.

3.2.4. *Effect of stabilizing excipients*

Sugar was shown to significantly affect stabilization of all IgG formulations. In 300 mM sugar, 21% of averaged fraction of aggregate of IgG was decreased as compared to sugar free samples ($p < 0.05$) (Fig. 6). Sugar is a commonly used nonspecific protein stabilizer that acts by initiating preferential hydration but it does not interact directly with a protein [8]. Several studies reported that concentration of 300 mM sugar is thought to be the minimum amount required for observing significant protein stabilization [5,22]. The stress testing by FTIR also showed that 300 mM sugar significantly increased the aggregation temperature ($\Delta T_{agg} = 3.0\,^{\circ}C$). Therefore, it can be considered that as high as 300 mM sugar is a good stabilizing excipient for inhibiting IgG aggregation.

Salts may stabilize, destabilize, or have no effect on protein stability depending on the type of salts, concentration, mode of ionic interactions, and charged residues in a protein [6,23]. Our SEC study showed that NaCl, most commonly used salt in antibody drugs, promotes aggregation of IgG significantly. In 300 mM NaCl, 48% of averaged fraction of aggregate was increased in all IgG formulations as compared to NaCl free samples (Fig. 6). The stress testing using FTIR also showed that salt had negative effect ($\Delta T_{agg} = -2.4\,^{\circ}C$). Wang and Roberts [1] suggested that at low concentrations, salts weaken ionic repulsion/attractions as counter ions. Therefore, this electrostatic shielding effect may either stabilize a protein when there are major repulsive interactions leading to protein unfolding, or destabilize it when there are major stabilizing salt bridges or ion pairs in the protein. The shielding effect is saturated at high concentrations, so that the dominant effect of salt, like other additives, is on the solvent properties of the solution. Chen et al. [23] reported that the T_m (67.4 °C) of recombinant human deoxyribonuclease (rhDNase) decreased to 65.3 °C in the presence of 1.2 M of NaCl but increases to 70.1 °C at 3.4 M. These results suggested that salt, such as NaCl, is one of the major factors in stabilizing or destabilizing a protein during storage, though its effect depends considerably

Table 3
Approaches to managing protein aggregation during storage.

Factors	Level	Number of conditions	Analytical methods	Data analysis	References
Traditional stability screening					
5 (pH, buffer, sugar, polyol, amino acid)	2–7	27	SEC, FTIR, SDS-PAGE	Impact of single factor, no statistical analysis	[5]
4 (sugar, polyol, amino acid, salt)	3–5	25	DLS, DSC	Impact of single factor, no statistical analysis	[6]
4 (pH, sugar, polyol, amino acid)	3–7	61	SEC, Spectrophotometer	Impact of single factor, no statistical analysis	[7]
1 (pH)	5	>43	DSC, CD, SEC	Impact of single factor, no statistical analysis	[25]
3 (pH, buffer, salt)	5	20	DLS, SEC, FFF, CD	Impact of single factor and a combination of factors, no statistical analysis	[26]
High-throughput screening					
2 (pH, buffer)	17	>100 using 96-well plate	Automated liquid handing systems, UV fluorescence microplate reader	Impact of a combination of factors, no statistical analysis	[27]
4 (buffer, sugar, amino acid, salt)	6	>288 using 96-well plate	DLS, Flow cytometry	Impact of single factor and a multiple factors, no statistical analysis	[28]
4 (pH, sugar, amino acid, salt)	2–4	>25 using 384-well plate	DSF, DSLS, HPSEC	Impact of single factor and a multiple factors, no statistical analysis	[29]
Statistical screening					
5 (protein concentration, pH, sugar, amino acid, salt, excipitents)	2–3	81	DSF, DLS	Main effect of single factor and interaction effects of combined factors	[30]
6 (protein concentration, pH, buffer concentation, sugar, salt, surfactant)	2	27	FTIR, SEC	Main effect of single factor and interaction effects of combined factors	This work

DLS, dynamic light scattering; DSC, differential scanning calorimetry; SDS-PAGE, SDS-polyaclylamidegel electrophoresis; CD, circular dichroism; FFF, field flow fractionation; DSF, differential scanning fluorimetry; DSLS, differential static light scattering; HPSEC, high performance size exclusion chromatography.

on its concentration, the nature and concentration of the protein and the influence of other excipients. Thus, it is difficult to predict the effect of salt on protein aggregation. Therefore, systematic investigations by a heuristic approach are indispensable.

No significant change in protein storage stability was found in all IgG formulations by Tween 80 (Fig. 6) in this study. Nonionic surfactants are extensively used to prevent protein aggregation under various processing conditions, such as refolding, mixing, freeze–thawing and drying, and reconstitution [8]. However, nonionic surfactants have also been reported to have an adverse effect on protein stability during storage [10,24]. The adverse effects are strongly temperature and formulation-dependent. Addition of 0.1% Tween 80 promoted aggregation of IL-2 during storage at 40 °C, but the tendency of IL-2 aggregation was different in different formulations [10]. Tween 20 has been shown to enhance the aggregation of pegylated granulocyte colony stimulating factor (PEG-G-CSF) at 1 mg/ml in solution in a concentration-dependent manner during storage at 29 °C [24]. Tween 80 at 0.1% has no effect on the aggregation of IL-1β at 100 μg ml^{-1} at pH values from 3 to 7 [18]. Our FTIR results revealed that Tween 80 did not show a significant effect on T_{agg}, but the combination of Tween 80 and buffer concentration, and Tween 80 and pH had a significant positive effect. These results suggested that the effect of surfactant depends on other formulating conditions as well as the type of protein.

The stress and accelerated testings showed that the effect of each excipient on conformational stability of proteins was identical to that on storage stability. Sugar at a concentration of more than 300 mM had a significant positive effect on both conformational and storage stability while NaCl had a negative effect. Tween 80 did not show a significant effect on either type of stability. These results suggested that the T_{agg} values using FTIR correlates to the storage stability at 40 °C analyzed by SEC. Therefore, it would be expected that the short-term stress testing using FTIR is available for predicting the influence of excipients on long-term storage stability.

The highest T_{agg} (78.8 °C) was obtained in formulation No. 17, which gave the third smallest value in fraction of aggregate (1.8% at 8 weeks storage). The smallest value in fraction of aggregate (1.5% at 8 weeks storage) was found in formulation No. 3, while T_{agg} in No. 3 is the second highest (78.2 °C). The difference between these two formulations (Nos. 17 and 3) is pH value alone (Table 2). As described in the above section, aggregation factor analysis by stress and accelerated testings in this study revealed that protein concentration, buffer concentration, sugar, Tween 80 and NaCl had similar effects on both conformational and storage stabilities. However, only pH had an inconsistent effect. Therefore, with the exception of pH, it is not surprising that all the aggregation factors gave consistent results between the condition giving the highest T_{agg} by FTIR analysis and the condition giving the smallest fraction of aggregate in the SEC analysis.

These results suggest that, with the exception of pH, short-term stress testing using FTIR is capable of replacing long-term accelerated testing for analyzing aggregation factors in protein formulations. Concerning the formulation pH, it is decisive factor for the long-term storage stability as well as temperature [4]. pH plays an important and complex role in long-term storage stability. For example, pH has a significant effect on the preferred chemical degradation pathways. Deamidation and disulfide bond scrambling occur at neutral and basic pH, while peptide bond hydrolysis occurs at either low or high pH. Therefore, the optimum pH value cannot be predicted based on short-term stress testing, and should be determined by long-term accelerated testing. Based on these results, we propose a simple strategy using FTIR, SEC and DOE techniques to analyze aggregation factors and screen the suitable condition of protein formulation. This strategy consists of the following three steps. First, points of measurements are determined using DOE to carry out an effective search in a vast parameter space. Secondly, the effects of aggregation factors, except pH, are examined by short-term stress testing using FTIR. Statistical analysis of the obtained data using an interaction model of combined factors will predict the ideal protein storage conditions. Thirdly, long-term accelerated testing is performed using SEC by fixing the various factors, except pH, according to the findings determined in the second step. This three-step strategy is expected to produce a suitable condition for the protein formulation without having to perform extensive and time-consuming investigations.

We believe the strategy proposed here will be practically useful for the evaluation of aggregation factors of therapeutic proteins and facilitate the quick determination of their suitable formulation. The suitable condition determined by this strategy should be confirmed by real-time stability tests in chilled environment for further development.

Currently, the approaches to managing protein aggregation during long-term storage can be divided into two categories. One is a traditional stability screening, in which the impact of a particular factor is examined in detail. Another is a high-throughput formulation screening (HTS), in which the combination of multiple factors is also taken into account. Several recent reports for therapeutic protein formulations are summarized in Table 3. Traditional stability screening [5–7,25,26] is performed by changing one factor at a time. This methodology can certainly generate clear results and easily interpret the effects of the factor at many different levels. However, it is difficult to detect interactions among different factors. HTS [27–30] is based on the use of multiwall-microplates. Such microplates can be placed in automated liquid-processing systems for sample preparation, dispensing, and handling. The use of multiwall-microplates and automated systems enables the simultaneous screening of numerous excipients and experimental conditions to find the optimal formulation. However, this methodology is time-consuming and requires large amounts of protein and expensive instruments [30]. In this work, we used DOE technique to determine the number of conditions of measurements, because it can reduce the amount of protein. As demonstrated above, the combination of FTIR, SEC and DOE explored the main effect of single factor as well as the interaction effect of combined factors without spending large amount of samples. Considering the advantageous, our approach using FTIR, SEC and DOE techniques could be regarded as a third category (statistical screening). Very recently, He et al. [30] reported that DOE approach can be successfully applied to the screening of antibody formulations early in development lifecycle. Their analysis also seems to be in the third category. Statistical screening using DOE technique is quite effective. However, we do not intend to argue that it is always superior to other categories. Since each category has own analytical advantages, it is important to choose appropriate one that meets a purpose. Otherwise, a proper combination may provide synergy. For example, the conditions obtained by statistical screening can serve as initial parameters of further HTS evaluation, which may reduce time and sample remarkably.

4. Conclusions

D-optimal design, a DOE technique, was used to devise experiments to analyze parameters affecting protein stability. Six aggregation factors at two levels for each parameter were examined. The analysis of FTIR measurements used as stress testing showed that pH and sugar significantly increased T_{agg}, but NaCl significantly decreased $T_{agg.}$ Although surfactant and buffer concentrations did not display a statistically significant impact on T_{agg} as a single factor, the interaction effects of NaCl and pH, buffer concentration and surfactant, pH and surfactant significantly increased T_{agg}. These results indicate that analysis of interaction effects is important for evaluating the stability of protein formulations.

The levels of pH, salt and sugar significantly affected T_{agg} in low concentration-IgG formulations, but only sugar displayed a significant positive effect on T_{agg} in high concentration-IgG formulations. The effects of aggregation factors found in low concentration-formulations do not always correspond to those in high concentration-formulations. Thus, examining high concentration-testing is necessary in order to design appropriate protein formulations.

The results from stress and accelerated testings revealed that pH showed contradictory effects on protein conformational and storage stabilities. However, with the exception of pH, all the aggregation factors gave consistent results between the condition giving the highest T_{agg} by FTIR analysis and the condition giving the smallest fraction of aggregate in the SEC analysis.

Taking into consideration all the results presented in this study, we propose a three-step strategy for analyzing the aggregation factors and screening the suitable condition of protein formulation using FTIR, SEC and DOE techniques. Specifically, determination of points of measurements by DOE to perform an effective search, a short-time stress test using FTIR to examine the effect of aggregation factors, except pH, and a long-term accelerated testing using SEC with fixed factors to fine tune pH values. This simple strategy is effective to analyze the aggregation factors of proteins and determine the suitable conditions of protein formulations.

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