Discrimination Among IgG1- κ Monoclonal Antibodies Produced by Two Cell Lines Using Charge State Distributions in nanoESI-TOF Mass Spectra

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Charge state distributions (CSDs) of proteins in nanoESI mass spectra are affected by the instrumental settings and experimental conditions, in addition to the conformations of the proteins in the analyzed solutions. In the presented study, instrumental and experimental parameters—the desolvation gas flow rate, temperature, pH, buffer (ammonium acetate), and organic modifier (methanol) concentrations—were optimized according to a reduced central composite face experimental design to maximize the separation of CSDs of monoclonal IgG1- κ antibodies produced by two production systems (CHO and GS-NS0 cell lines). Principal component analysis and Fisher linear discriminant analysis were then used to reduce the dimensions of the acquired dataset and quantify the separation of the protein classes, respectively. The results show that the IgG1- κ molecules produced by the two production systems can be clearly distinguished using the described approach, which could be readily applied to other proteins and production systems. (*J Am Soc Mass Spectrom* 2009, 20, 1030–1036) © 2009 American Society for Mass Spectrometry

Electrospray ionization mass spectrometry (ESI-MS) is a highly suitable technique for structural analysis of various macromolecules, partly because of the possibility of analyzing multiply charged ions from biological molecules, e.g., proteins in solution [1, 2]. Further advantages for such analyses are offered by nanoESI-MS [3] (compared with conventional ESI), including reductions in sample consumption, greater tolerance of nonvolatile salts, and the softness of the technique. However, although nanoESI is a preferable ionization method for structural MS analyses of biological macromolecules, nanoESI has been shown to be highly dependent upon the geometry of the nanoflow needle; thus some needle-to-needle irreproducibility is to be expected [4].

Charge-state distributions (CSD) of biological macromolecules in ESI-MS are being increasingly employed for exploring their conformations in solutions [4, 5]. Changes in protein conformation can result in changes in the CSD in an ESI mass spectrum [6, 7]. The mechanism whereby (nearly) desolvated ions are generated from a charged droplet is not completely understood [8]. Proteins acquire more charges in solutions that promote unfolding than the same proteins in solutions that promote folded conformations [7], but it should be noted that other factors than protein compactness may

also contribute to differences in CSD patterns [9–11]. For instance, in addition to altering the conformation of proteins, changes in the solvent also affect the ionization process, i.e., proton-transfer reactions. Thus, the solvent and the operating conditions have different conformational effects on proteins with differing stability during the electrospray process. Hence, understanding the influence of experimental conditions on protein CSDs is important to extract the structural information they may contain.

In this study, the effects of selected instrumental and experimental parameters on the CSDs of IgG1- κ monoclonal antibodies produced by two production systems [Chinese hamster ovary (CHO) and glutamine synthetase-negative secreting (GS-NS0) cell lines] were investigated, using statistical design of experiment, DoE [12], and response surface methodology (RSM) to maximize the cluster separation based on their nanoESI-MS spectra. Humanized IgG1 monoclonal antibodies, which have promising therapeutic applications, are frequently subject to post-translational modifications, e.g., glycosylation, which may vary substantially depending on the cell line and cell culture protocols used to produce them, with consequent effects on their conformation, stability, and solubility [13]. Therefore, a major objective was to identify conditions in which the instrumental settings are not strong determinants of the conformational stability of the proteins during ESI, and thus the resulting CSDs are related to their compactness in solution before their transfer to the gas phase.

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DoE is a structured approach for determining mathematical relationships between multiple parameters affecting one or more variables of interest, often used to optimize production processes. The principal purposes of RSM are to provide a description of responses (a “response surface”) to varied parameters in the region of the studied observations, and to facilitate identification of the area in which the response is optimal. Principal component analysis [14] (PCA) is a useful statistical method for reducing the dimensionality of large datasets and for identifying and visualizing major trends, groups, and features in them. In addition to these techniques, the supervised pattern recognition technique Fisher linear discriminant analysis (Fisher-LDA) [15] was used here to evaluate the separation of the investigated proteins’ CSDs. The objective of Fisher-LDA is to project data obtained from samples of interest into a space of low dimensions in which classes of objects (here proteins) are separated by maximizing the distance between the means of the classes while minimizing the variance within each class, i.e., extracting characteristics that only contain information pertinent to the classification.

In summary, the overall aim was to develop an approach based on CSD and multivariate data analysis for extracting sufficient, reliable structural information to discriminate between proteins produced by the two cell lines that could also be applied to other proteins produced by other systems.

Experimental

Proteins and Materials

Humanized IgG1- κ monoclonal antibodies (mAbs) were produced in two cultivation systems, CHO [16] and GS-NS0 [17] cells. A four-step purification platform involving solubility, size separation, charge separation, and filtering were used. The proteins have the same amino acid sequence. No significant differences between the CHO- and GS-NS0-derived mAbs were detected in their binding ability in animal or human in vitro assays, but the former were found to have significantly higher binding ability than the latter in in vivo animal assays.

The CHO-derived and GS-NS0 derived mAbs, designated Ab1 and Ab2, were dissolved in 10 mM citrate buffer at concentrations of 5 and 8 mg/mL, respectively. Portions of the solutions for MS analysis were buffer-exchanged, using P-30 Bio-Rad micro bio-spin columns (Bio-Rad, Hercules, CA), into solutions with permutations (listed in Table 2) of three concentrations of ammonium acetate (0.2, 0.6, and 1 M), three pH values (3, 5, and 7; adjusted by formic acid), and organic modifier (methanol; 0, 15, and 30%) with final protein concentrations of 2 mg/mL. These test solutions were prepared in randomized order, and stored at 8 °C before analysis, in each case for less than 8 h.

NanoESI Mass Spectrometry

Portions of each of the test Ab1 and Ab2 protein solutions, with the selected permutations of ammonium acetate concentration, pH, and proportion of methanol were introduced by direct-infusion into a Q-TOF 1.5 mass spectrometer (Waters, Manchester, UK), modified for high mass operation (MSVision, Allerød, Denmark), equipped with a Z-spray nanoflow source and a metal-coated borosilicate capillary (Proxeon Biosystems, Odense, Denmark) of “medium” length with an i.d. at the emitter tip of $\sim 1 \mu\text{m}$. Mass spectra were recorded in positive ion mode with desolvation-gas flow rate varied (as listed in Table 1) and the following settings constant for each analysis: spray-tip potential 1500 V, nozzle-to-skimmer potential 40 V, nozzle temperature 25 °C, and collision energy 80 eV. Data were acquired at 1 s per spectrum, in continuum mode between 1000 and 10,000 m/z and compiled by MassLynx version 4.1 software (Waters). The traces presented here are sums of at least 60 spectra. To increase the pressure in the ion transfer stage and collision cell and thus improve the focusing of the protein ions during their passage through the quadrupoles and TOF system by collisional cooling, gas was pumped in [18]. The total pressure in the analyzer varied between 8.0×10^{-2} and 1.4×10^{-1} mbar. A 15 mg/mL cesium iodide solution was used for external mass calibration.

Experimental Design

A reduced central composite face (CCF) experimental design was employed to identify optimal experimental conditions for maximizing the separation between Ab1 and Ab2 proteins. In this design, obtained using MODDE software ver. 6.0 (Umetrics AB, Umea, Sweden), four variables (X_1 = pH, X_2 = ammonium acetate concentration, X_3 = methanol concentration, and X_4 = desolvation gas flow rate) were varied over three levels (low, medium, and high), as summarized in Table 1. Spectra were acquired from analyses of five replicates of the Ab1 and Ab2 test solutions, at least, at each experimental point, and Matlab ver. 7.0 (MathWorks, Inc., Natick, MA) was used to analyze the resulting multivariate data. Test solutions that yielded weak MS signals, due to Ab1 or Ab2 precipitation, were run three times. Fischer-LDA was then employed to evaluate the separation between Ab1 and Ab2 at each experimental point, yielding Fisher criterion (F) values (the higher the better), which were used as the response for the respective experimental points in the optimization process. To validate the model, replicates of selected test solutions

Table 1. Levels of parameters varied in the CCF design

Level	X1 pH	X2 buff. (M)	X3 org. (%)	X4 gas (L/h)
–1	3	0.2	0	0
0	5	0.6	15	200
1	7	1	30	400

Table 2. Reduced CCF design matrix for optimizing the separation of Ab1 and Ab2

Exp. point	Run order	No. of replicates prot. Ab1/Ab2	X ₁ pH	X ₂ buff. (M)	X ₃ Org. (%)	X ₄ gas (L/h)	Fisher criterion
N1	2	5/5	3	0.2	0	0	0.30
N2	5	3/3	3	1	0	0	0
N3	3	3/3	3	1	30	0	0
N4	1	5/4	3	0.2	0	400	2.01
N5	6	3/3	3	1	0	400	0
N6	7	3/3	3	0.2	30	400	0
N7	4	3/3	3	0.6	15	200	0
N8	13	5/6	5	0.2	15	200	0.55
N9	11	9/8	5	1	15	200	0.06
N10	14	5/5	5	0.6	0	200	2.30
N11	12	3/3	5	0.6	30	200	0
N12	8	3/3	5	0.6	15	0	0
N13	9	3/3	5	0.6	15	400	0
N14	10	3/3	5	0.6	15	200	0
N15	17	6/6	7	0.2	0	0	0.57
N16	20	3/3	7	0.2	30	0	0
N17	16	5/5	7	1	30	0	3.57
N18	19	8/8	7	1	0	400	3.33
N19	21	3/3	7	0.2	30	400	0
N20	15	5/5	7	1	30	400	3.92
N21	18	6/5	7	0.6	15	200	2.21
N22	22	6/6	3	0.2	0	0	0.15
N23	23	5/5	7	1	0	400	6.28
N24	24	5/5	7	1	30	0	2.59
N25	25	9/6	7	1	30	400	5.13
N26	26	3/3	3	1	0	400	0
N27	27	3/3	5	0.6	15	0	0
N28	28	3/3	5	0.6	30	200	0
N29	29	3/3	3	1	0	0	0

were also run (experimental points N22–N29 in Table 2). The response ($Y = F$ value, a measure of class separation) can be expressed by the following second degree polynomial equation:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_4 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{14}X_1X_4 + A_{23}X_2X_3 + A_{24}X_2X_4 + A_{34}X_3X_4 + A_{11}X_1X_1 + A_{22}X_2X_2 + A_{33}X_3X_3 + A_{44}X_4X_4 \quad (1)$$

in which the regression variance is partitioned into independent (A_0), linear (A_1, A_2, A_3, A_4), quadratic ($A_{11}, A_{22}, A_{33}, A_{44}$), and cross-product ($A_{12}, A_{13}, A_{14}, A_{23}, A_{24}, A_{34}$) components to determine the fit of the function and the relative significance of each component, as shown in Table 2. The response monitored (Y) was the F value expressing the separation of the Ab1 and Ab2 protein clusters. In total, 21 permutations of experimental conditions were applied, and replicates of eight of these permutations (Table 2).

Results and Discussion

The pH, buffer (ammonium acetate) concentration, organic modifier (methanol) concentration, and desolvation gas flow rate were varied to identify the optimal settings for maximizing the separation of Ab1 and Ab2 proteins in terms of their CSDs in nanoESI-MS spectra.

A response surface model (describing the relationships between the controlled experimental factors and the response, the F value) was then obtained by fitting the acquired data by partial least-squares regression [19], with R^2_{adj} and Q^2 values of 0.798 and 0.688, respectively. These values summarize the fit of the model, being the proportions of variation of the response that can be explained by the model (adjusted for degrees of freedom) and predicted by the model, respectively. Equation 2 expresses the separation of the Ab1 and Ab2 proteins in terms of F values, as functions of four main factors (pH, buffer concentration, % organic modifier, and desolvation gas flow rate), a two-factor interaction effect (pH * buffer concentration) and quadratic effect (pH * pH). The terms not included were found to be insignificant at a confidence level of 95%.

$$Y = 0.40 + 0.97X_1 + 0.60X_2 - 0.35X_3 + 0.27X_4 + 0.69X_1X_2 + 0.78X_1X_1 \quad (2)$$

For each experimental point, the protein Ab1 and Ab2 test solutions were analyzed on the same day and at least three different capillaries were used. In total, 11 charge states (ranging from +20 to +30) associated with the molecular weight of the IgG1- κ were recorded, and 4 to 6 charge states were observed for every tested permutation of protein and conditions. The relative intensities (peak heights) of the charge states were used

Figure 1. Nano-ESI mass spectra of the IgG1 Ab1 in (a) 0.2 M, (b) 0.5 M, and (c) 1 M ammonium acetate buffer, pH 7.

to normalize the data by defining the intensity of the most intense peak as 100%. Fisher-LDA considers not only the means of the classes, but also their spread, hence at least five replicates of each of the Ab1 and Ab2 test solutions were run to evaluate the variance within each group. The more replicates within the classes, the more reproducible F values are expected to be.

The buffer concentration was included as a factor since it strongly affects the desolvation process; insufficient desolvation leads to broadening of the peaks in the spectrum and associated mass shifts, due to adduct formation. The results (Figure 1) indicate that the desolvation process was enhanced by high concentrations (up to 1 M) of the volatile buffer used here (ammonium acetate), and at least 0.2 M was needed to obtain distinguishable CSD spectra. However, some combinations of buffer and methanol concentration, pH, and desolvation gas flow rate appeared to promote some form of aggregation or precipitation, since no ions were detected under these conditions (the responses, F val-

ues, are set to zero in cases where aggregation or precipitation was detected; see Table 1). The maximum and minimum pH values (identified in the preliminary study) were governed by the requirements for detecting the protein in positive ion mode and preventing precipitation, respectively.

The desolvation gas temperature was held constant at 25 °C, since higher temperatures led to precipitation of the protein. The upper limit for the proportion of organic modifier was also limited by protein precipitation. For the low and high levels of the factors see Table 2.

The results of the optimization study are summarized in Table 3, and response surface plots showing the F values indicating the separation of the protein conformations with respect to pH and the proportion of methanol at three buffer concentrations and two levels of desolvation gas flow rate are shown in Figure 2a–d (data for only two levels of desolvation gas flow rate are shown due to its weak impact on the model; see eq 2 for the impact coefficient of each variable).

Table 3. Summary of results of the regression analysis of the effects of the varied parameters on the separation of Ab1 and Ab2

	Degree of freedom	Sum of squares	Mean square (variance)	F	P
Total	29	126.50	4.36		
Constant	1	37.46	37.46		
Total corrected	28	89.05	3.18		
Regression	6	74.93	12.49	19.47	0
Residual	22	14.11	0.64		
Lack of fit (model error)	14	8.55	0.61	0.88	0.60
Pure error (replicate error)	8	5.56	0.69		

Quadratic response surface model fitting. n 29 PLS comp 2. Q_2 0.69, R_2 0.84, R^2_{adj} 0.8. RSD 0.80.

Figure 2. Response contour plots of the measure of the class separation response with respect to the pH and organic modifier concentration (methanol, %) at three levels of buffer concentration (M) and one level of desolvation gas flow rate. (a) buffer concentration 0.2 M, gas flow 200 Lh^{-1} , (b) buffer concentration 0.6 M, gas flow 200 Lh^{-1} , (c) buffer concentration 1 M, gas flow 200 Lh^{-1} , and (d) buffer concentration 1 M, gas flow 400 Lh^{-1} .

Results from the response surface optimization show that high levels of pH, buffer concentration, and desolvation gas flow rate all enhanced the F values, while high levels of methanol reduced them, according to the optimization model. Hence, the best separation of the proteins was achieved with a combination of pH 7, 1 M ammonium acetate buffer, 0% methanol, and 400 L/h desolvation gas flow rate. The interaction factors pH * buffer concentration can be explained by when combining the low pH with high salt concentration, precipitation was observed.

In the response surface plots the contour lines of the response are also closer to each other at the suboptimal buffer concentration than at the other buffer concentrations, implying that changes in the system have relatively strong effects on the separation of the proteins under these conditions, presumably due to poor desolvation. The buffer concentration also affected the width of individual charge state peaks in the spectra (a variable that has been used to indicate the extent of desolvation [20]). The curtain gas flow has a strong influence on the CSD of each protein (see Figure 3), but not on the measure of the class separation of the proteins. Chang-

ing the experimental and instrumental conditions can result in dramatic changes in the CSD of the proteins, but not necessarily in their class separation. The changes in the obtained CSDs may have been due to changes in protein conformation or the ionization process, or both [20].

Changes in solution variables (e.g., changes in pH or concentrations of salts or organic solvents) can affect both the stability and conformation of proteins. Thus, for instance, a protein with low stability may not retain its conformation to the same extent as a similar protein with higher stability during the ionization process as it is transformed from a soluble state to ions in the gas phase. In our experiments, the separation between the CSDs from Ab1 and Ab2 was maximized when the proteins were not exposed to conditions known to promote protein denaturation, e.g., low pH and high organic solvent concentrations.

Figure 4 shows a direct side-by-side comparison of CSD for Ab1 and Ab2 recorded under the optimal conditions. The figure is an indication on the heterogeneities in the glycoproteins. The theoretical molecular mass excluding glycochains of this mAb is 145172 Da. A

Figure 3. NanoESI mass spectra of Ab2 at pH 7 acquired at desolvation gas flows of (a)–(f): 500, 400, 300, 200, 100, and 0 Lh⁻¹. The charge states (*z*) corresponding to the peaks is indicated in each panel.

visual inspection of the principal component (PC) score plot for Ab1 and Ab2 proteins under optimum conditions in the studied experimental domain (Figure 5a),

reveals that Ab1 are distinctly separated from Ab2 according to the first principal component. To be able to identify the most important variables for the classification in the score plot, a loading plot can be used (Figure 5b). The loading plot shows that the lower charge peaks are placed on the corresponding area of the score plot there Ab1 samples are placed. This observation indicates that Ab1 acquired less charge than Ab2 under these conditions, yielding higher relative intensities at low charge states (+25, +26, and +27) and lower relative intensities at higher charge states (+28, +29, and +30). The presented results were in accordance with expectations based on indications that Ab2 is less stable than Ab1 since it had weaker binding ability in *in vivo* animal assays (possibly due to differences in post-translational modifications in the two cell lines).

Conclusions

Stringently controlled instrumental and experimental conditions are required to distinguish proteins with differing conformations or modifications by analyzing their CSD profiles in ESI-MS spectra, since any factor that affects their conformation, gas-phase ion chemistry may have significant effects on their CSDs. Results presented here demonstrate a methodology for distinguishing forms of an IgG1 produced by two cell lines based on optimizing conditions to maximize differences in their CSD patterns, followed by multivariate data analysis. This method should be suitable for rapid characterization of various biopharmaceuticals (e.g., recombinant monoclonal antibodies), optimization of production processes, and quality control (e.g., analyses

Figure 4. A direct side-by-side comparison of CSD for (a) Ab1 and (b) Ab2 recorded under the optimal conditions. The molecular mass obtained from deconvolution of the measured charge state distribution in A-series for Ab1 and Ab2 were ~147951 Da and ~147794 Da, respectively. The convoluted molecular mass in B-series for Ab1 and Ab2 were ~146294 Da and ~146349 Da, respectively.

Figure 5. Results of principal component analysis of data acquired under optimal conditions. (a) PC1 versus PC2 score plot, the black dots indicate data from protein Ab1 and the grey stars indicate data from protein Ab2. (b) Corresponding loading plot. Ab1 yielded higher relative intensities for peaks corresponding to low charge states (variables) +25, +26, and +27 whereas Ab2 yielded higher relative intensities for peaks corresponding to higher charge states (+28, +29, and +30).

of variations between batches, before and after storage, or batches produced by different production systems).

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