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Alleviating nonlinear behavior of disulfide isoforms in the reversed-phase liquid chromatography of IgG2

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Abstract

Reversed-phase chromatography is an established method for characterizing the disulfide isoforms of IgG2. This work explores the effect of mobile phase gradient profile and sample concentration on the separation of disulfide isoforms. The acidic mobile phase can alter the relative proportions of disulfide isoforms, but only when the level of the reactive A1 isoform is much higher than for typical conditions of separation and typical IgG2 samples. Otherwise, there is minimal disulfide scrambling. A slower gradient and flow rate modestly improve resolution, but the peaks remain heavily overlapped. Resolution is further improved and nonlinear chromatography lessened when injection is performed under non-stacking conditions. Non-stacking conditions also keep the concentration from spiking at the head of the column, reducing noncovalent associations that can promote disulfide scrambling. The higher resolution from non-stacking injection reveals the presence of at least seven species.

Keywords

Monoclonal antibody; IgG2; disulfide scrambling; nonlinear; enriched

1. Introduction

Human monoclonal antibodies and related products have been the fastest growing category of therapeutics in pharmaceutical industry [1]. Monoclonal antibodies are increasingly used for autoimmune diseases by targeting the immune response underlying the disease rather than treating the symptoms [2]. For cancer, whose prevalence is expected to increase significantly over the next fifty years [3,4], monoclonal antibodies allow tumor-specific therapy either by directly targeting specific cells, or by targeting drug delivery to the tumor cells [5].

Pharmaceutical manufacturing of these large proteins induces some structural heterogeneity, such as aggregation [6,7] and intra-molecular disulfide scrambling [8]. Disulfide scrambling

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is an especially severe problem for IgG2, compared to IgG1 and IgG4, because there are more unpaired cysteines in IgG2 and it has two more disulfide bonds in the hinge region [9,10]. Three major structural isoforms (IgG2-A, IgG2-A/B, and IgG2-B) resulting from disulfide scrambling have been reported for IgG2 [11–13]. The A form has the classic Y structure, and it is comprised of two different variants, A1 and A2, which differ in connectivity of disulfides in the hinge region [14]. The B form has a disulfide bond linking each half of the Fab region to the hinge of the Fc region, and the A/B form is a hybrid of A and B, having only one Fab cysteine linked to an Fc cysteine in the hinge. The B and A/B forms also have variants [15]. Analytical characterization of these disulfide isoforms is required at critical points during manufacturing and clinical studies [13,16]. Reversed-phase liquid chromatography (RPLC) is used for characterizing the composition of IgG2 disulfide isoforms due to its high resolution, reproducibility and ease of use, as well as its facile coupling to mass spectrometry [17,18].

Four overlapping peaks have been observed for IgG2 by RPLC at 80 °C, but the peaks were not well resolved [12,19]. RPLC uses conditions that are known to denature antibodies and promote aggregation [20–23], which means that it is possible that the analysis itself causes a change in very sample composition being tested [10]. In this study, the concentration dependence of the chromatography of IgG2 and its redox-enriched isoforms is studied, and new gradient conditions are explored to alleviate nonlinear chromatography, improve resolution and avoid changes in sample composition.

2. Experimental section

2.1 Materials

Acetonitrile, sodium azide, histidine, cysteine, cystamine, guanidinium chloride (GuHCl) formic acid and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isopropyl alcohol (IPA) and tris(hydroxymethyl)aminomethane (Tris) were purchased from Macron Fine Chemicals (Center Valley, PA, USA). All chemicals and reagents were of analytical grade. Recombinant monoclonal IgG2 antibodies were shipped from Pfizer Inc. (St. Louis, MO, USA) to Purdue University for the chromatographic studies.

2.2 Reversed-phase chromatography

The RPLC column, Poroshell 300SB-C8 column, 5 μ m, 300 Å, 75 mm $\times$ 2.1 mm i.d., was purchased from Agilent Technologies (Wilmington Delaware), is the same column used in previously reported work on analysis of disulfide scrambling [17]. A Waters Acquity UPLC I-Class system (Milford, MA, USA) with a binary pump was used to perform all RPLC experiments. The IgG2 sample was prepared in 10 mM histidine buffer (pH 5.6). The chromatographic conditions were the same as those of previous reports [17,19]: mobile phase A contained 2% IPA, 98% H₂O with 0.1% TFA, and mobile phase B contained 70% IPA, 20% acetonitrile, 10% H₂O and 0.1% TFA. The gradient was 25–40% B in 30 min, with a flow rate of 500 μ L/min. For the slower gradient separation, the gradient was 28–31% B over 40 min, with a flow rate of 100 μ L/min. For the separation with nonstacking injection, the sample was injected at 29.2% B and maintained for 0.1 min, then a gradient

from 29.2% B to 30.7% B was run over 28 min. The flow rate was also 100 $\mu\text{L}/\text{min}$. In all cases, the column temperature was set to 80 $^{\circ}\text{C}$, and UV absorbance at 210 nm was used for detection. Chromatograms were normalized by dividing by the area after baseline subtraction.

2.3 Size exclusion chromatography

The size-exclusion column, TSKgel UltraSW Aggregate, 3 μm , 300 $\text{\AA}$, 300 mm $\times$ 7.8 mm i.d.), was purchased from Tosoh Bioscience (King of Prussia, PA, USA). A Thermo Accela UHPLC system (Thermo Scientific, Waltham, MA, USA) with an in-line PDA detector was used for the size-exclusion chromatography (SEC) analyses. The mobile phase was 0.1 M sodium sulfate and 0.05% sodium azide in 0.1 M PBS buffer at pH 6.7 and the column was at room temperature. The flow rate was at 500 $\mu\text{L}/\text{min}$. UV absorbance at 280 nm was used for detection.

2.4 Redox enrichment of disulfide isoforms

Disulfide isoforms IgG2-A1 and IgG2-B were enriched using procedures from literature [9]. Briefly, two buffers were prepared. One buffer consisted of 6 mM cysteine and 1 mM cystamine in 200 mM Tris buffer at pH 8.0, which shifts the composition toward the B form. The other buffer was the same except that it also contained 1.0 M GuHCl to shift the composition to the A1 form. Each enrichment was done by incubating the IgG2 at 3 mg/mL in the appropriate buffer system at 4 $^{\circ}\text{C}$ for approximately 48 h. The enriched samples were diluted to different concentrations in 10 mM histidine buffer (pH 5.6) before separation by RPLC.

2. Results and discussion

Figure 1a shows chromatograms for four different concentrations using the published gradient. The chromatogram for the highest concentration, 1 mg/mL, agrees with those in the literature for the same concentration, gradient and column [9,17]. The peak assignments from the literature for A1, A2, A/B, and B, are indicated. In this work, chromatograms for progressively lower concentrations of 0.5, 0.2 and 0.1 mg/mL were also obtained and these are included in Figure 1a as well. The data show that decreasing concentration shifts the chromatograms to later retention times, which is the hallmark of nonlinear chromatography. For silica based stationary phases, nonlinear chromatography is typically due to a saturation of a small number of strong silanol sites [24,25]. The data also exhibit a change in the relative peak heights in the chromatogram, which is potentially an important problem in applying this method to analysis of isoform composition. To explore this issue in more detail, the same chromatograms are displayed in Figure 1b on a scale in which their areas are normalized. These data reveal that the relative peak heights are indeed concentration dependent. One possibility for such behavior is nonlinear chromatography, where the unresolved peaks within the chromatogram shift in time relative to one another. One can see that the resolution is lower for the lower concentrations, indicating that the later peaks do shift toward the first peak, which could explain the increase in the height of the first peak for the lower concentrations. Another possibility is that the isoform composition of the sample is changed by the conditions of the chromatography. Since acidity promotes aggregation and

disulfide scrambling [20], this is a concern. A fit of the chromatogram to multiple peaks could distinguish these possibilities but this is not feasible because the resolution is too low. These two possibilities are addressed by increasing resolution.

To increase resolution, since a longer column is not offered, a six-fold slower gradient and a five-fold slower flow rate were used. The results are presented in Figure 1c. The resolution is shown to be somewhat higher, with the first peak newly exhibiting a shoulder, but there is still extensive peak overlap. The chromatograms normalized for area are given in Figure 1d, where it is easier to see that resolution has improved, particularly for the lower concentrations. The chromatograms still exhibit nonlinearity and the resolution is still too low to allow a firm conclusion as to whether isoform composition changes with concentration.

Simplifying the chromatogram can be used to test for a possible change in sample composition. This is accomplished by using redox enrichment, which is normally employed as a tool to identify the disulfide isoforms in the chromatogram [9]. Redox enrichment shifts the relative proportions toward a single disulfide isoform to simplify the chromatogram. Figure 2a shows the normalized chromatograms obtained for a 1 mg/mL sample of IgG2 that was enriched in isoform B, after which the chromatogram was run with varying injected concentration. All injected concentrations show extreme enrichment of the B isoform, with negligible concentration dependence in the shape of the chromatogram. The results establish that the B isoform is quite stable under the chromatographic conditions. Figure 2b shows the chromatograms for the case where the A1 isoform was enriched, again on a scale normalized for area. These chromatograms exhibit a change in isoform composition with concentration. Specifically, the highest peak increases 30% in height from the highest concentration of 1 mg/mL to the lowest concentration of 0.1 mg/mL. Correspondingly, there is a decrease in the peak heights for the B and A/B isoforms. These changes can no longer be possibly due to nonlinear chromatography because it is a later peak being elevated in height at lower concentration. The chromatogram indicates that the highest concentration of sample enriched in the A1 form, i.e., 1 mg/mL, exhibits a change in isoform composition by partially re-converting to the B form. The same is true but to a much lesser extent for the 0.2 mg/mL sample, which gives almost the same chromatogram as the 0.1 mg/mL sample. These results reveal that the A1 form is not stable under the conditions of the chromatographic separation. This is consistent with a recent report that the A1 form is the least stable IgG2 isoform due to less protection of the disulfide bond [26]. These data in Figure 2a and 2b support a conclusion that the conditions of reversed phase chromatography are capable of changing the sample composition for a sufficiently high concentration of the A1 isoform, but not for IgG2 concentrations typically used. For the slower gradient and flow rate, the chromatograms of Figure 2c and 2d, respectively, show that the B form is slightly reactive and the A1 form is significantly more reactive. A likely reason is that the longer time in the acidic mobile phase, due to the slower flow rate, allows more disulfide scrambling.

The fact that high concentration promotes the change in isoform composition suggests aggregation is a factor. Size-exclusion chromatography was used here to explore aggregation in aqueous acidic solution. IgG2 was exposed to low pH for 30 s, then returned

to neutral pH and analyzed by SEC. The results are presented in Figure 3. No aggregation was detected when a small amount of TFA was added, 0.02%, for IgG2 at 1 mg/mL, as shown in Figure 3a. By contrast, when the level of TFA was the same as that used in RPLC, 0.1%, Figure 3b shows that there is a significant amount of dimer formed, and the shoulder to the left of the dimer peak indicates trimers, as well. Also, there is low recovery, as evidenced by the plateau at late elution times, consistent with aggregates diffusing more slowly out of the pores [27]. For a ten-fold lower IgG2 sample exposed to 0.1% TFA, Figure 3b shows that there is less aggregation. Further study (not shown) establishes that formic acid is just as effective as TFA in promoting aggregation, and that intermediate pH levels between the two presented in Figure 3 give intermediate levels of aggregation.

It is interesting that the size-exclusion results clearly show that acidic pH promotes aggregation but there is no evidence of aggregation in the reversed-phase chromatograms. This suggests that the aggregates evident in SEC are non-covalent. This was confirmed by injecting the IgG2 sample in the RPLC column after treatment in aqueous solution and 0.1% TFA, and observing that the chromatogram was exactly the same as that for the untreated sample injected at neutral pH. Since the redox-enriched samples show scrambling for high levels of the A1 isoform, a possible interpretation is that non-covalent aggregates involving A1 isoforms cause the disulfide scrambling. Again, the concentration of A1 in the 1 mg/mL IgG2 sample is much less than that for the sample redox-enriched in A1, therefore, any disulfide scrambling caused by the RPLC is negligible.

Since the A1 isoform is the unstable form, there is a need for even better resolution to quantitate this minor component in IgG2 samples because it reaches much higher levels in drug products. It is the nonlinear chromatography that is limiting the resolution; therefore, the use of nonstacking injection followed by gradient elution is explored. In gradient elution of proteins, the protein is normally loaded onto the head of the column, thereby greatly elevating the concentration of IgG2 on the hydrophobic surface, inviting nonlinearity. This pre-concentration is needlessly high since protein separations are not limited by injected width under typical conditions. Nonstacking injection at a more moderate retention factor would maintain a narrow injected zone but would be expected to give more linear chromatography and higher resolution.

The idea of nonstacking injection is tested by first devising a mobile phase that would give a moderate retention factor. Figure 4a shows the conventional gradient elution chromatogram for the 0.2 mg/mL sample, which was selected because the results presented thus far indicate that this concentration will give a reliable chromatogram. The chromatogram is the same as that in Figure 1c where the slower gradient was used, and the figure points out that the first peak elutes at 29.2% acetonitrile. One can anticipate that this acetonitrile percentage might be a reasonable starting point for choosing the isocratic mobile phase for injection. Figure 4b shows chromatograms for various isocratic mobile phase percentages, which are followed by the same slow gradient. These exhibit the expected behavior that small changes in the amount of water affect retention time more than they affect resolution. The results reveal that the optimal resolution is the very same 29.2% mobile phase B of Figure 4a. This simplifies rational design of the analysis: choose the mobile phase at which the first peak elutes. The chromatograms also show that the overall resolution is considerably higher than

for even the slow gradient. This is expected because nonstacking elution is capable of higher resolution than gradient elution [28], and at least some of the separation is occurring under isocratic conditions.

The chromatograms and their concentration dependence are shown in Figure 5a for this nonstacking gradient, and also shown on a scale normalized for peak area in Figure 5b. The chromatograms normalized for area show that the nonlinear chromatography has all but disappeared as a result of avoiding the pre-concentration onto the head of the column. All concentrations have virtually identical chromatograms, and virtually identical ratios of peak heights for the first and third peaks, which are presumably still the A1 and B isoforms. The sensitivity is also approximately two-fold higher for the new gradient compared to the original gradient, which can be seen by comparing the AU axes in Figures 1a and 5a. In short, the new gradient provides higher resolution, greatly reduces nonlinearity, and provides higher sensitivity.

To test the effect of the nonstacking injection on disulfide scrambling, the chromatography is again simplified by using redox enrichment to generate predominantly one form. Figure 5c and 5d show the concentration dependence of the chromatograms for the enriched B and A1 isoforms using the nonstacking injection. These results indicate that the on-column disulfide scrambling of the A1 form is slightly worse for the highest concentration, although it remains negligible for the two lower concentrations. Nonetheless, disulfide scrambling does not affect the chromatography of the original 1 mg/mL IgG2 sample because it has a sufficiently low concentration of A1 compared to the enriched samples. The non-stacking gradient is not significantly different with respect to disulfide scrambling than is the original gradient.

The peak assignments need to be confirmed. While one would expect the same elution order, the redox enrichment data are used to confirm identities of the peaks. The data for the slower gradient elution and flow rate are shown in Figure 6a for an IgG2 concentration of 0.2 mg/mL. The data show that the first and largest peak is the B form, as was known before, but the shoulder is new. It is tentatively attributed to a variant of the B isoform, based on its similar retention behavior, which suggests a similar shape. The order of elution of the A1 form agrees with the previous assignments. Based on peak height, the peak between the B and A1 forms is assigned to the A/B form. The peak in the enriched case does not align exactly with the chromatogram of the original IgG2 solution for the conventional gradient, and this is because of peak positions shifting from nonlinear chromatography. For the new gradient, the results are shown in Figure 6b. The A1 peak height was much lower since it is better resolved from the higher A/B peak, and the enriched sample was diluted to approximately match that in the sample. The data for the new gradient are thus consistent with the elution order being the same as for the conventional gradient.

The inset in Figure 6b provides an expanded view of the chromatogram for the A1 form to give a view of the isoforms and their variants without the large B peak dominating. These data show a total of seven peaks evident in the chromatogram, which is more than the four or five peaks evident from conventional gradient elution. The existence of additional isoform variants is not surprising since it had previously been shown that the B and A/B

forms have variants [15], as mentioned earlier. A plateau between A1 and A2 was evident in the conventional gradient elution and this is now resolved as a peak, which is visible in the inset of panel b, where it is labeled as unk. This peak has not been assigned to our knowledge. There could still be more peaks since these are not fully resolved, and higher resolution columns are needed for full analysis of the isoform composition of IgG2.

3. Conclusions

RPLC has been the tool of choice to characterize IgG2 disulfide isoform composition, and the results presented here detail a means of obtaining higher resolution chromatograms with far less dependence on concentration. Most significantly, the peak for the reactive A1 form is better resolved. The peaks continue to be overlapped despite the higher resolution because even more peaks are observed with the new gradient. The total number of isoforms and their variants in this typical IgG2 sample remains unknown; therefore, columns of even higher resolution are needed for RPLC analysis of the disulfide isoform composition in IgG2.

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Highlights

- Reversed-phase liquid chromatography has been used as a tool for characterizing disulfide isoforms of IgG2 monoclonal antibody drugs.
- At high injected concentration, the A1 isoform partially reconverts to the B isoform by the time it elutes.
- Nonlinear chromatography and low resolution impede the analysis.
- Injecting under isocratic conditions and then eluting with a slow gradient alleviates nonlinear chromatography and improves resolution.

Figure 1.

Chromatograms using gradient elution for varying IgG2 concentration. a) published conditions of injection at 5% B, a gradient of 25% to 40% B over 30 min, with conditions of 80 °C and a flow rate of 500 µL/min, b) same as panel a but with chromatograms normalized for area to facilitate comparison, where concentration decreases from left to right, c) same as in panel a, but slower gradient of 28% to 31% over 40 min and slower flow rate of 100 µL/min, d) same as panel c but with chromatograms normalized for area to facilitate comparison. In each panel, concentration decreases from left to right. Color figures are available in the on-line version.

Figure 2.

Chromatograms for redox-enriched IgG2 samples at varying concentration, normalized by area. Published gradient for IgG2 enriched in a) B form and b) A1 form. Slower gradient and flow rate for IgG2 enriched in c) B form and d) A1 form. Concentration decreases in each panel from left to right, and color figures are available in the on-line version.

Figure 3.

SEC chromatograms for IgG2 exposed for 30 s to acidic aqueous solution, then neutralized with phosphate buffer. b) 1 mg/mL IgG2 after exposure to 0.02% TFA, which matches that for no exposure (not shown). b) 1 mg/mL IgG2 (—) and 0.1 mg/mL IgG2 (---) after exposure to 0.1% TFA, which is the same %TFA used in RPLC. Note 2× scale difference between the two panels.

Figure 4.

Optimization of mobile phase for nonstacking injection. a) Conventional gradient elution chromatogram for the 0.2 mg/mL IgG2 sample using the same gradient and conditions as in Figure 1c, and the first peaks is marked to show its elution at 29.2% of mobile phase B. b) Chromatograms using nonstacking injection followed by the same gradient, for varying the mobile phase used for nonstacking injection. The percentage of mobile phase B increases from left to right, and color figures are provided in the on-line version.

Figure 5.

Chromatography using nonstacking injection followed by gradient elution. a) Concentration dependence of chromatograms. b) Same as panel a but with scales normalized by area. The dotted lines are to facilitate comparing normalized peak heights. c) Chromatograms for IgG2 sample redox-enriched in isoform B for varying concentration, normalized by area. d) Same as panel c but for enrichment of A1 isoform. In each panel, concentration decreases from left to right. Color figures are available in the on-line version.

Figure 6.

Peak assignments using redox enrichment with injected concentrations of 0.2 mg/mL. In both panels, the chromatogram for the original IgG2 sample is shown in black, the redox enriched sample for the B isoform in red and the redox enriched sample for the A1 isoform in blue. a) Slow gradient and flow rate with conventional injection, and b) nonstacking injection followed by slow gradient and flow rate. The inset of panel b shows the chromatograms for the A1 form on a more sensitive scale, along with arrows showing evidence for at least seven isoforms. Color figures are provided in the on-line version.