A Thermal-Cycling Method for Disaggregating Monoclonal Antibody Oligomers

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Received 21 October 2013; revised 7 December 2013; accepted 20 December 2013

Published online 22 January 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23863

ABSTRACT: Non-native oligomeric forms of biopharmaceutical proteins are therapeutically inactive, and potentially toxic and immunogenic, and therefore undesirable in pharmaceutical formulations. Immunoglobulin G class of antibodies are known to form stable nonnative oligomers through Fab–Fab interactions. In this paper, we investigate thermal-cycling as a technique for disaggregating antibody oligomers. Aggregate containing monoclonal antibody (mAb) samples were exposed to rapid heating and cooling cycles in a thermal-cycler. The heating phase of the thermal-cycle resulted in partial unfolding of the Fab domain, leading to the release of monomer from the oligomer complexes, whereas the rapid cooling that followed led to refolding and minimized the probability of protein reaggregation. The extent of mAb oligomer disaggregation was determined by size-exclusion chromatography and hydrophobic interaction membrane chromatography, whereas protein refolding was assessed by circular dichroism spectroscopy. The thermal-cycling technique in addition to being suitable for disaggregating protein oligomer samples could also potentially be useful for studying the mechanisms of protein aggregation and disaggregation. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:870–878, 2014

Keywords: protein aggregates; monoclonal antibody; oligomer; disaggregation; refolding; thermal-cycling; chromatography; protein folding/refolding; stability; circular dichroism

INTRODUCTION

Monoclonal antibodies (mAbs) were first reported in the 1970s.1 The current biopharmaceutical protein market is dominated by mAbs belonging to the immunoglobulin G (or IgG) class of antibodies.2 An IgG is a large molecule that is particularly prone to aggregation.3 The formation of oligomers and higher aggregates leads to loss in biological activity of biopharmaceutical products, and the aggregates can themselves be toxic or immunogenic to patients.4 mAbs belonging to the IgG1 subclass have been shown to form stable dimer and higher oligomers by Fab–Fab interactions.5 There is a significant volume of information available in the literature on methods used to detect and remove mAb oligomers and higher aggregates.6–8

A complex protein molecule such as IgG represents a macromolecular system in which each domain contributes independently toward overall stability.9 The native state of a protein is determined by complex events and interactions that tend to minimize the free energy of individual polypeptide chains. Under physiological condition, the native conformation of a protein is only marginally more stable than the other possible forms.10 Therefore, any perturbation in the form of externally added energy or changes in solution conditions such as pH and ionic strength could disrupt the complex array of non-covalent interactions responsible for stabilizing the native structure of a protein, leading to the formation of partially unfolded intermediates.10 These intermediates are unstable and if present in large quantities could form non-native dimer and higher oligomers, and eventually, larger particulate aggregates. Various mechanisms for non-native aggregation of proteins have been proposed.10–14 Many over-expressed recombinant proteins form non-native aggregates called inclusion bodies within their host cells.15

The process of obtaining disaggregated native protein molecules from non-native aggregates such as inclusion bodies is referred to as protein refolding.16–21 The sequence of events involved in protein refolding is the opposite of those in nonnative aggregation. The interactions holding the aggregate complexes together are first broken down through partial or complete protein unfolding. This leads to the formation of unfolded intermediates, which are then refolded back to the native state in a controlled manner. Several strategies for protein refolding have been reported.16–21 A commonly used approach is to unfold and release the protein molecules sequestered in aggregate complexes using high concentrations of chaotrophic agents such as urea or guanidine hydrochloride followed by refolding and renaturation by controlled removal of these agents.16,17 Co-solutes could sometimes be used to improve the refolding yield.18 Alternatively, molecular chaperones, which are protein additives that shield the protein molecules being refolded from unwanted hydrophobic interactions, thereby preventing them from re-aggregating are employed.19

Another category of refolding techniques is based on the application of very high pressures, typically around 2000 atmospheres, in the presence of chaotropes.20,21 High pressure favors the dissociation of aggregates and protein refolding, and interferes with intermolecular hydrophobic interactions and thereby potentially prevents re-aggregation. An important finding of these high-pressure-based protein disaggregation and refolding studies is that the formation of non-native aggregates such as amyloid structures proceeds through an aging process, and only the early stage fibrils are dissociated under high pressures. Late-stage fibrils remain unaltered indicating their greater stability upon maturation.22 Disaggregation of non-native aggregates under high pressure is only observed if
there is an overall reduction in system volume.\textsuperscript{22,23} In spite of the vast amount of literature covering different aspects of the pressure-driven aggregation and disaggregation processes, very little is known about the effect of high pressure on immunoglobulin aggregation/disaggregation. In fact, a study on bovine milk immunoglobulins reported insignificant change in IgG structure at high pressure.\textsuperscript{24} Also, unlike inclusion bodies, which consists primarily of aggregated proteins, typical mAb samples contain mainly monomer and smaller amounts of oligomers.\textsuperscript{5} Therefore, an ideal technique for processing such antibody samples would have to disaggregate oligomers in a targeted manner, while leaving the monomer already present unaffected.

It has been reported that non-native amyloid aggregates reversibly dissociate when heated and this process inhibited further amyloid growth.\textsuperscript{22} Pasteurization at 60°C has also been reported to be effective at reducing dimer content in human serum antibody samples.\textsuperscript{25,26} However, circular dichroism (CD) results reported in one of these studies\textsuperscript{25} indicated that the secondary structure of the antibody molecule was altered.\textsuperscript{25} Moreover, analysis of pasteurized samples also showed that although the dimer content was reduced, the higher oligomer content was significantly increased.\textsuperscript{26} Unfolding of proteins by heating has been studied by several researchers.\textsuperscript{27–29} As the unfolded form is unstable, continued heating typically leads to the formation of non-native oligomers by re-aggregation of unfolded species. Some experimental studies have shown that the detrimental effects of heating could be reversed by cooling, which apparently favors protein refolding.\textsuperscript{30,31} Monoclonal antibody oligomers are formed by Fab–Fab interactions\textsuperscript{3} as the Fab domain is relatively less stable than the Fc domain\textsuperscript{9} at neutral pH. The Fc domain is more likely to be involved in antibody aggregation that takes place in acidic conditions.\textsuperscript{22} We hypothesized that disaggregation of non-native protein oligomers along with the refolding of released monomer could potentially be achieved by multiple cycles of rapid heating and cooling, with minimal holding of samples at elevated temperatures. The heating phase would partially unfold and disaggregate the oligomers present in a given sample, whereas the cooling phase would refold these released molecules to their native state (see Fig. 1). The low temperature of the cooling phase would also minimize the chances of partially unfolded monomers colliding and re-aggregating. In order to obtain proof-of-concept for this hypothesis, we thermal-cycled mAb samples containing known amounts of non-native oligomers, and compared these with untreated control samples using different analytical techniques. Disaggregation was confirmed by size-exclusion chromatography (SEC) and hydrophobic interaction membrane chromatography (HIMC), whereas refolding was verified by Circular Dichroism (CD) spectroscopy. The results obtained are discussed.

**Figure 1.** Proposed disaggregation and refolding of mAb by thermal cycling.

### MATERIALS AND METHODS

#### Materials

Purified mAb hlgG1-CD4 monomer (batch 12, 23rd March 1999) and hlgG1-CD4 “dimers” (batch D6, 22nd July 1993) were kindly donated by the Therapeutic Antibody Centre (Oxford, United Kingdom). The hlgG1-CD4 monomer, as received, contained almost 100% unaggregated mAb, whereas the hlgG1-CD4 “dimers” contained almost equal amounts of hlgG1-CD4 monomer and dimer and smaller amounts of other oligomers. Prolonged storage of the monomer led to the formation of small amounts of dimer. This material is referred to as monomer-rich hlgG1-CD4 in this paper. There was some vial to vial variation in composition but on an average the monomer-rich hlgG1-CD4 contained about 94% monomer and 5% dimer (as measured by SEC). The composition of the “dimers” also changed on storage and was found to contain on an average about 37% monomer, 28% dimer, and 35% higher oligomers (i.e., trimer, tetramer, and pentamer). This material is referred to as aggregate-rich hlgG1-CD4 in this paper. Human mAb TZM of the IgG1 subclass expressed in HEK-293 cell line was kindly donated by the Biotechnology Research Institute, NRC Canada, Ottawa, Canada. The samples provided were known to contain mAb aggregates (mainly dimer) and are referred to as HEK mAb in this paper. Sodium phosphate monobasic (S0751), sodium phosphate dibasic (S0876), and ammonium sulfate (A4418) were purchased from Sigma–Aldrich (St. Louis, Missouri). Sodium chloride (SOD 002.205) was purchased from Bioshop (Burlington, Ontario, Canada). Purified water (18.2 MΩ cm) used in this study was obtained from a Diamond\textsuperscript{24} NANOpure (Barnstead, Dubuque, Iowa) water purification unit. Amicon Ultra-4 Centrifugal Filters (fitted with Ultrace-50 membrane) purchased from Millipore (Billerica, Massachusetts) were used for buffer exchange and concentration of mAb samples. Genuine Axygen Quality PCR tubes (PCR-05-C; 0.5 mL thin wall) used for thermal-cycling experiments were purchased from Axygen Inc. (Union City, California).

#### Thermal-Cycling and Heating Experiments

The mAb samples were buffer exchanged with 20 mM sodium phosphate buffer (pH 7.0) and concentrated using Amicon Ultra-4 centrifugal filters. Thermal-cycling was carried out using a PCR thermal cycler (Eppendorf Mastercycler* personal; Applied Biosystems, Carlsbad, California). Based on the specifications of the thermal cycler, PCR tubes with 500 and 200 μL capacities could only be used with the corresponding volume limitation in these tubes being 100 and 20 μL, respectively. The mAb concentration and volume used in the thermal-cycling experiments was primarily dictated by the requirements of the analytical methods carried out after thermal cycling, that is, SEC, HIMC, and CD spectroscopy. For this reason, 500 μL PCR tubes were used. Preliminary thermal-cycling experiments were carried out with different combinations of low- and high-end temperatures, and heating and cooling rates. On the basis of the disaggregation profiles thus obtained, the combination of 10°C (low-end) and 60°C (high-end) and heating/cooling rate of 3°C/s was chosen. It must be stressed that this is not the optimum condition but the best amongst the sets of conditions examined in our preliminary studies. The disaggregated mAb samples obtained by this procedure are referred to as thermal-cycled samples. These were compared with control
mAb samples maintained at room temperature for the duration of the thermal-cycling experiments. These control samples are referred to as untreated samples. Constant temperature heating experiments were also carried out for comparison where the mAb samples were maintained at 60 °C in a water bath for the duration corresponding to the thermal-cycling experiments. These samples are referred to as heated samples. The thermal-cycled and heated samples were equilibrated to room temperature (22 ± 1 °C) and diluted to the appropriate concentration prior to analysis by SEC, HIMC, and CD spectroscopy.

Size-Exclusion Chromatography
The aggregate content of the different mAb samples was determined by SEC using a Varian Star HPLC system (Varian, Palo Alto, California) fitted with a WTC-030S5 Wyatt SEC column (Wyatt Technology Corporation, Santa Barbara, California), using phosphate-buffered saline (PBS) as the mobile phase at a flow rate of 0.3 mL/min. PBS contained 20 mM sodium phosphate and 250 mM sodium chloride (pH 7.0). The volume of sample injected for SEC analysis was 50 μL. The eluate from the column was monitored using a UV detector set at 280 nm wavelength.

Hydrophobic Interaction Membrane Chromatography
Hydrophobic interaction membrane chromatography has been demonstrated to be an efficient technique for analyzing aggregate content in mAb samples.\(^8,3^3\) The monomer-rich hlgG1-CD4 sample was analyzed using a custom-designed membrane module\(^3^4\) containing five hydrophilized polyvinylidene fluoride (0.22 μm pore size; GVWP14250; Millipore) membrane discs (18 mm diameter), integrated with an AKTA prime plus liquid chromatography system (GE Healthcare Biosciences, Quebec, Canada). Aggregate-rich hlgG1-CD4 samples were analyzed using a similar module containing 10 membrane discs. The HIMC experiments were carried out at a flow rate of 2 mL/min. The binding buffer consisted of 1.5 M ammonium sulfate prepared in 20 mM sodium phosphate buffer (pH 7.0), whereas the elution was carried out with ammonium sulfate-free buffer, i.e. 20 mM sodium phosphate buffer (pH 7.0), using a linear gradient (0%–100% eluting buffer) of 40 mL. The gradient was started 2 mL after sample injection. The volume of sample injected for HIMC analysis was 500 μL, the total protein concentration in the monomer-rich samples being about 0.3 mg/mL, whereas that in the aggregate-rich samples being 0.1 mg/mL.

Circular Dichroism
Circular dichroism experiments were carried out to analyze the changes in secondary structure to verify protein refolding. These were carried out using a Model No.416 CD spectrometer (AVIV Biomedical Inc., Lakewood, New Jersey). Quartz cuvettes having path length of 1 mm were used for the far UV (190–260 nm) measurements. Samples were scanned at a rate of 1 nm per 3 s with constant nitrogen purging at 25 °C, and data obtained were processed and reported in terms of mean residual ellipticity expressed in deg cm\(^2\) dmol\(^{-1}\).

RESULTS AND DISCUSSION
Thermal cycling of the monomer-rich hlgG1-CD4 was carried out for 2 h between 10 °C and 60 °C with identical heating and cooling rates of 3 °C/s. The total protein concentration in each sample was at least 0.63 mg/mL. Figure 2 shows the SEC chromatograms obtained with the untreated and thermal-cycled monomer-rich hlgG1-CD4 samples. The untreated sample contained mostly monomer (peak 2, 26.5 min retention time) and a small amount of dimer (peak 1, 22.9 min retention time). Comparison of the two chromatograms shows that thermal cycling resulted in an increase in the proportion of monomer and a corresponding decrease in the proportion of dimer. Table 1 summarizes the percentage of monomer and dimer in these samples, calculated by peak area integration. On the basis of the data shown in table, the percentage of hlgG1-CD4 dimer disaggregated to monomer by thermal cycling, as measured by SEC was 52.7%.

In order to assess the effect of constant temperature heating, monomer-rich hlgG1-CD4 samples were heated at 60 °C for 2 h. Figure 3 shows the HIMC chromatograms obtained with the untreated, thermal-cycled (10 °C and 60 °C, with identical heating and cooling rates of 3 °C/s), and heated monomer-rich hlgG1-CD4 samples. In HIMC, which separates proteins based on hydrophobicity difference, the monomer appears first followed by aggregates in order of increasing degree of aggregation.\(^8,3^3\) The untreated monomer-rich hlgG1-CD4 contained mainly monomer (14.2 mL) and some dimer (~18 mL).

Table 1. Comparison of composition of untreated and thermal-cycled monomer-rich hlgG1-CD4 as determined using size exclusion chromatography

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Thermal cycled</th>
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<tr>
<td>Monomer</td>
<td>94.12 ± (2.30)</td>
<td>97.22 ± (3.65)</td>
</tr>
<tr>
<td>Dimer</td>
<td>5.88 ± (1.32)</td>
<td>2.78 ± (1.58)</td>
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Note: Numbers in brackets indicate maximum error in peak area measurement.
A comparison of the chromatograms show that thermal cycling resulted in a decrease in dimer content (peak 2) and a corresponding increase in the monomer content (peak 1). This is consistent with the SEC results discussed in the previous paragraph, i.e., thermal-cycling resulted in dimer disaggregation. The chromatogram obtained with the heated sample showed a broadened monomer peak with the monomer–dimer ratio being almost the same as the untreated sample. The broadening of the monomer peak suggests that heating caused some damage or structural change to a proportion of the monomer present in the sample. Whether this actually happened was verified by far-UV CD experiments discussed later on in the paper. The monomer peak for the thermal-cycled sample was however identical in shape to that in the untreated sample, implying that no structural changes took place. Therefore, thermal-cycling not only disaggregated the dimer, but the intermittent exposure to high temperature reduced the chances of damage to the hIgG1-CD4 monomer present in the sample.

On the basis of the above-mentioned results (i.e., Figs. 2 and 3; Table 1) that clearly show that the decrease in dimer content because of thermal-cycling was matched by a corresponding increase in monomer content in the sample, it may be concluded that the hIgG1-CD4 dimer did disaggregate into its constituent monomers. It is known that the formation of non-native IgG1 dimer during storage takes place because of Fab–Fab interaction.5,7,33,35–38 Also, calorimetry-based experiments have shown that significant unfolding followed by irreversible denaturation of the Fab region of an IgG molecule takes place above 61°C, whereas significant changes in the Fc region is observed at much higher temperatures.9 Short exposures to temperatures as high as 60°C as experienced in the thermal-cycling experiments could therefore be expected to lead to partial unfolding of Fab region of the mAb molecules. In a dimer, such unfolding would lead to the release of the constituent monomer molecules and the rapid cooling phase following the short exposure to high temperature would ensure refolding of the partially unfolded Fab domain. The lower temperatures in the cooling phase would also reduce the frequency of molecule to molecule collision, thereby minimizing the chances of antibody re-aggregation.

Whether the monomer obtained by dimer dissociation was properly refolded and if this represented the correct native conformation was verified by CD spectroscopy. The CD spectrum of a multidomain protein such as an antibody is the net effect of the summation of contributions of the α-helices, β-sheets, β-turns, and randomly coiled component domains. When a protein unfolds, aggregates, denatures, or misfolds, major changes occur at the level of the secondary structure and these changes can be identified in the far-UV region (240–190 nm) of the spectrum.39 The α-helices in proteins show two negative peaks at 222 and 208 nm, respectively, and a single positive peak at 193 nm, whereas the presence of β-sheets is confirmed from negative and positive peaks at 218 and 198 nm, respectively.39,40 IgG has a predominantly β-sheet structure and its residual ellipticity is known to be reduced to zero around 206 nm because of the contribution of the β-sheets.9,41 Figure 4 shows the far-UV CD spectra obtained with untreated, thermal-cycled, and heated monomer-rich hIgG1-CD4. The presence of negative peak around 218 nm and positive peak close to 200 nm in all three profiles confirm the predominance of β-sheet secondary structure. The main difference between the profiles

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**Figure 3.** Hydrophobic interaction membrane chromatography chromatograms for untreated thermal-cycled and heated monomer-rich hIgG1-CD4 (solid line, untreated; large dashed line, thermal-cycled for 2 h, small dashed line, heated for 2 h; peak 1, monomer; and peak 2, dimer).

**Figure 4.** Far-UV CD spectra for monomer-rich hIgG1-CD4 samples (solid line, untreated; dashed line, thermal cycled; and dotted line, heated).
was with regards to the wavelength corresponding to the zero residual ellipticity, this being around 208 nm for the untreated sample. Thermal-cycling led to a slight decrease in this wavelength, moving it closer to 206 nm, which is ideally expected for native IgG. On the contrary, constant temperature heating resulted in a slight increase in ellipticity at this wavelength, presumably indicating some protein denaturation or degradation, consistent with the HIMC results shown in Figure 3. The magnitudes of these downward and upward shifts in the zero-ellipticity wavelength because of thermal-cycling and heating, respectively, were not significant enough to be independently used to draw any major conclusion. These results could however be used to support the SEC results shown in Figure 2 and Table 1 and the HIMC results shown in Figure 3. Overall, it may be reasonable to conclude that thermal-cycling did result in disaggregation of hIgG1-CD4 dimer, and the refolding of the released monomer, whereas constant temperature heating resulted in some structural damage of the hIgG1-CD4 monomer.

Monomer-rich hIgG1-CD4 samples were then thermal-cycled for 8 h to examine the long-term effects of the heating and the cooling cycles on protein stability. As a control experiment, a monomer-rich sample was also heated at a constant temperature of 60°C for 8 h. Figure 5 shows the SEC chromatograms obtained with the untreated (a), thermal-cycled (b), and heated (c) mAb samples. Consistent with the CD results shown in Figure 4, constant temperature heating resulted in significant protein degradation, as evident from the low-molecular-weight peaks observed in chromatogram C (peaks 3 and 4). Prolonged thermal cycling resulted in slight degadation of the mAb, as can be inferred from the tiny peak 3 in chromatogram B. Mazur et al. have reported that thermal degradation products of IgG consist mostly of light chain dimers, Fab fragments, light chain polypeptides, and heavy chain hydrolysis products. However, such degradation peak was absent in monomer-rich hIgG1-CD4 thermal-cycled for 2 h (see Fig. 2).

To demonstrate that dimer disaggregation by thermal-cycling was not unique to hIgG1-CD4, experiments were carried out using HEK mAb samples known to contain significant amounts of dimer. The thermal-cycling protocol used was identical to that used for monomer-rich hIgG1-CD4, i.e., between 10°C and 60°C for 2 h with identical heating and cooling rates of 3°C/s. The total protein concentration in each sample was 0.63 mg/mL. The SEC chromatograms obtained with the untreated and thermal-cycled HEK mAb samples are shown.
Table 2. Comparison of composition of untreated and thermal cycled HEK monoclonal antibody as determined using size exclusion chromatography.

<table>
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<tr>
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<th>Untreated</th>
<th>Thermal cycled</th>
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<tr>
<td>Monomer</td>
<td>84.75 ± (2.93)</td>
<td>94.28 ± (2.25)</td>
</tr>
<tr>
<td>Dimer</td>
<td>15.25 ± (7.46)</td>
<td>5.72 ± (4.10)</td>
</tr>
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</table>

Note: Numbers in brackets indicate maximum error in peak area measurement.

in Figure 6. As observed with hIgG1-CD4, thermal cycling resulted in a significant reduction in the dimer content (peak 1) and a corresponding increase in the monomer content (peak 2). Table 2 shows the composition of the untreated and thermal-cycled samples as measured by SEC. The percentage of dimer disaggregated to monomer by thermal-cycling was 62.5%.

To assess whether the disaggregated HEK mAb monomers obtained by thermal cycling were properly refolded, CD experiments were carried out using thermal-cycled HEK mAb samples. The protein concentrations used in these thermal-cycling experiments were higher than those used in the experiments reported for hIgG1-CD4 in Figure 3, that is, 1 and 7 mg/mL as opposed to 0.63 mg/mL. Figure 7 shows the far-UV CD spectra obtained with untreated and thermal-cycled samples (for 2 and 4 h) from experiments carried out using 1 mg/mL HEK mAb. These results are consistent with those shown in Figure 4. A comparison of the profiles obtained with the two thermal-cycled samples indicates that a slightly higher proportion of β-sheets were obtained after 4 h of thermal-cycling. Figure 8 shows the far-UV CD spectra obtained with untreated and thermal-cycled samples (for 2 and 4 h) from experiments carried out using 7 mg/mL HEK mAb. Although the trends observed in the profiles are largely similar as those in the experiments carried out using 1 mg/mL HEK mAb, the higher protein concentration produced some distortions in these profiles, particularly at lower wavelengths.

To investigate the effect of thermal-cycling on higher oligomers, aggregate-rich hIgG1-CD4 samples were thermal-cycled for 4 h between 10°C and 60°C with identical heating and cooling rates of 3°C/s. The total protein concentration in each sample was 0.63 mg/mL. The SEC results obtained with the untreated and thermal-cycled aggregate-rich mAb samples are shown in Figure 9. The untreated aggregate-rich sample contained in addition to monomer and dimer, significant quantities of higher oligomers, mainly trimer and tetramer (<20 min retention time). Table 3 summarizes the composition of the analyzed samples. Thermal-cycling resulted in significant reduction in the oligomer content (peaks 1 and 2) but the dimer content (peak 3) increased slightly. Overall, there was a significant increase in the monomer content (peak 4). The slight increase in dimer content suggests that it was an intermediate in the oligomer to monomer disaggregation process. The formation of non-native dimer has been identified as one of the key steps in protein aggregation. The above results clearly indicate that oligomers disaggregate to the dimer form before further disaggregation to monomer. Overall, the percentage of oligomers (including dimer) disaggregated to monomer was 34.6%, whereas the percentage of higher oligomers disaggregated to dimer and monomer was 77.7%.

Figure 10 shows the HIMC chromatograms obtained with untreated and thermal-cycled (between 10°C and 60°C for 4 h with identical heating and cooling rates of 3°C/s) aggregate-rich hIgG1-CD4. These results indicate that thermal cycling resulted in a significant decrease in higher aggregate content (peak 3) and a big increase in the monomer content (peak 1). The dimer content (peak 2) remained more or less unchanged. These results are consistent with those discussed in the previous paragraph in the context of SEC analysis (see Fig. 9 and Table 3).

Figure 11 shows the CD spectra obtained with untreated, thermal-cycled, and heated aggregate rich hIgG1-CD4. All three spectra indicate β-sheet-dominant structure with negative peak at 218 nm. The wavelength corresponding to zero residual ellipticity for the untreated aggregate-rich hIgG1-CD4 was around 211 nm. This was significantly higher than the corresponding wavelength of untreated monomer-rich hIgG1-CD4 (208 nm). Several papers on thermally induced protein aggregation have reported that the wavelength for zero residual ellipticity shifts to a lower value when IgG is denatured or aggregated. Explanation for this difference presumably lies in the nature of the non-native aggregates formed during prolonged storage and those formed by heating. A comparison of the profiles shown in Figures 4, 7 and 8, and 11 with those in the papers discussing thermal IgG aggregation would suggest that aggregates obtained by heating contained significant random coiled structures, whereas aggregates present in the hIgG1-CD4 samples used in the current study appear more ordered with high β-sheet content. Examination of the three
profiles shown in Figure 11 indicates a slight downward shift in the ellipticity value corresponding to 218 nm wavelength because of heating, and a slight upward shift at this wavelength because of heating. However, the main difference between the three profiles was with regards to their shape, particularly the difference in magnitude of residual ellipticity on the negative side around the 218 nm wavelength corresponding to the $\beta$-sheets. With the thermal-cycled samples, the profile shifted downwards on the negative side, looking somewhat similar to that obtained with monomer-rich hIgG1-CD4 (see Fig. 4). On the contrary, the negative peak on the spectrum obtained with the heated sample was flattened. Such flattening of the negative peak could be because of a combination of further aggregation and protein degradation that has been shown to occur during heating (see Fig. 5). With constant temperature heating, the probability of the partially folded intermediates colliding to re-aggregate is significantly increased when compared with thermal cycling where the rapid cooling phase minimizes protein mobility.

The experimental results discussed in this paper clearly demonstrate that thermal cycling can be used for disaggregating nonnative mAb oligomers and simultaneously refolding of released monomer to its native conformation. The rapid heating phase of a thermal cycle unfolds the aggregated proteins, whereas the rapid cooling phase refolds the released proteins to their native state while ensuring that re-aggregation is kept at a minimum. The thermal-cycling parameters used in this study were not selected based on precise optimization. The choice of 60°C as the high-end temperature of the thermal-cycle used in this study was however deliberate, given that significant unfolding of the Fab region of an IgG molecule that is typically involved in aggregate formation takes place around and above 61°C. The heating and cooling rates used in the initial experiments for this study were chosen arbitrarily, and the best condition among a relatively small set of experimental conditions was chosen. Therefore, the opportunity for further optimization of the thermal-cycling technique for better mAb oligomer disaggregation definitely exists. Moreover, the conditions suitable for disaggregation and refolding oligomers of different proteins could be quite different. The current study relied on far-UV CD

![Figure 8](image) Far-UV CD spectra for aggregate containing concentrated HEK samples (7.0 mg/mL). (Solid line, untreated; dotted line, thermal cycled for 2 h; and dashed line, thermal cycled for 4 h).

![Figure 9](image) Size-exclusion chromatography chromatograms for aggregate-rich hIgG1-CD4 samples (thick line, untreated; thin line, thermal cycled; peaks 1 and 2, higher oligomers; peak 3, dimer; and peak 4, monomer).

### Table 3. Comparison of composition of untreated and thermal-cycled aggregate-rich hIgG1-CD4 as determined using size exclusion chromatography.

<table>
<thead>
<tr>
<th></th>
<th>Untreated (Peak area)</th>
<th>thermal cycled (Peak area)</th>
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<tbody>
<tr>
<td>Monomer</td>
<td>38.99 ± (2.20)</td>
<td>60.14 ± (3.20)</td>
</tr>
<tr>
<td>Dimer</td>
<td>29.29 ± (1.47)</td>
<td>32.80 ± (3.16)</td>
</tr>
<tr>
<td>Higher oligomers</td>
<td>31.72 ± (3.03)</td>
<td>7.06 ± (3.26)</td>
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Note: Numbers in brackets indicate maximum error in peak area measurement.
Figure 10. Hydrophobic interaction membrane chromatography chromatograms for aggregate-rich hIgG1-CD4 samples (thick line, untreated; thin line, thermal cycled; peak 1, monomer; peak 2, dimer; and peak 3, higher oligomers).

for assessing protein structural changes at the secondary structure level. It would also be very useful and important to study the effects of thermal cycling on changes to tertiary structure of proteins. Also, use of techniques such as differential scanning calorimetry (DSC) would reveal more information on domain-specific structural changes in proteins that are typically linked with aggregation and intermolecular interactions.

The thermal-cycling technique discussed in this paper could, in addition to being suitable for reducing oligomer content in protein samples, be a potentially useful tool for studying the mechanisms of protein aggregation and disaggregation. The thermal-cycling technique could also potentially be used to disaggregate inclusion bodies and refold the released proteins. Several serious ailments such as Alzheimer’s disease and Parkinson’s disease involve protein aggregate formation and some of the therapeutic strategies for treating these diseases involve the use of drugs that inhibit aggregate formation. The thermal-cycling technique discussed in this paper could therefore potentially be used to evaluate the efficacy of such aggregation-inhibiting drugs.

CONCLUSIONS

Thermal cycling resulted in the disaggregation of mAb oligomers and the simultaneous refolding of the released monomer. The heating phase of the thermal cycle resulted in partial unfolding in the Fab domain known to be involved in aggregate formation, resulting in the release of monomer from oligomer complexes. The rapid cooling phase that followed the heating phase facilitated the refolding of the Fab domain of released monomer and minimized the chances of protein re-aggregation through molecule–molecule collision. The CD results seem to suggest that the storage-induced non-native mAb oligomers studied in this work could be quite different from antibody aggregates obtained by heating. The product profile obtained by disaggregation of aggregate-rich hIgG1-CD4 strongly suggests that the dimer was one of the key intermediate forms in the higher oligomer to monomer disaggregation pathway. The thermal-cycling technique reported in this paper, in addition to being suitable for disaggregating non-native oligomers present in protein samples, could potentially be used for refolding inclusion bodies and for testing aggregate inhibiting drugs.

ACKNOWLEDGMENTS

This work is supported by the NSERC Strategic Network for the Production of Single-type Glycoform Monoclonal Antibodies (MabNet). Raja Ghosh holds the Canada Research Chair in Bioseparations Engineering. We thank Drs. Geoff Hale, Pru Bird, Mark Frewin, and other members of the Therapeutic Antibody Center, Oxford University, UK for donating the hIgG1-CD4 mAb samples. We thank Drs. Richard and Raquel Epand (both from McMaster University) for access to the CD instrument and for their help with analyzing CD data. We acknowledge Dr. Yves Durocher from National Research Council, Ottawa, Canada for providing us aggregate-containing samples of IgG1 mAb from HEK293 cell line. We thank Dr. Carlos Filipe for access to the PCR machine used in this study and SeungMi Yoo for helping with the SEC analysis.

Figure 11. Far-UV CD spectra for aggregate-rich hIgG1-CD4 samples (solid line, untreated; dashed line, thermal cycled for 4 h; and dotted line, heated for 4 h).
REFERENCES