A Thermal-Cycling Method for Disaggregating Monoclonal Antibody Oligomers

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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 chaotropic 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

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there is an overall reduction in system volume.[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.[24] Also, unlike inclusion bodies, which consists primarily of aggregated proteins, typical mAb samples contain mainly monomer and smaller amounts of oligomers.[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.[22] Pasteurization at 60°C has also been reported to be effective at reducing dimer content in human serum antibody samples.[25,26] However, circular dichroism (CD) results reported in one of these studies[25] indicated that the secondary structure of the antibody molecule was altered.[25] Moreover, analysis of pasteurized samples also showed that although the dimer content was reduced, the higher oligomer content was significantly increased.[26] Unfolding of proteins by heating has been studied by several researchers.[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.[30,31]

Monoclonal antibody oligomers are formed by Fab–Fab interactions[5] as the Fab domain is relatively less stable than the Fc domain[9] at neutral pH. The Fc domain is more likely to be involved in antibody aggregation that takes place in acidic conditions.[32] 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.

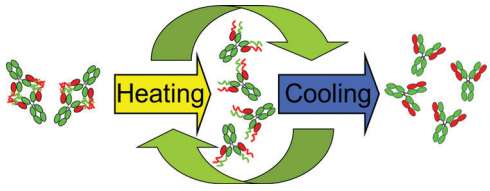

Figure 1. Proposed disaggregation and refolding of mAb by thermal cycling.

MATERIALS AND METHODS

Materials

Purified mAb hIgG1-CD4 monomer (batch 12, 23rd March 1999) and hIgG1-CD4 "dimers" (batch D6, 22nd July 1993) were kindly donated by the Therapeutic Antibody Centre (Oxford, United Kingdom). The hIgG1-CD4 monomer, as received, contained almost 100% unaggregated mAb, whereas the hIgG1-CD4 "dimers" contained almost equal amounts of hIgG1-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 hIgG1-CD4 in this paper. There was some vial to vial variation in composition but on an average the monomer-rich hIgG1-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 hIgG1-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™ NANOpure (Barnstead, Dubuque, Iowa) water purification unit. Amicon Ultra-4 Centrifugal Filters (fitted with Ultracel-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