

# IgG Aggregation Mechanism for CHO Cell Lines Expressing Excess Heavy Chains

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**Abstract** Aggregates in protein therapeutics like IgG monoclonal antibodies (mAb) are detrimental to product safety and efficacy. It has been reported that aggregates form in Chinese hamster ovary (CHO) cell lines expressing greater amount of heavy chain (HC) than light chain (LC). In this study, we observed that aggregates could form within the cells with excess HC and were partially secreted into the supernatant. The aggregates in the supernatant consisted of mainly HC and were partially dissociated under either reducing or denaturing conditions. Mutation of a predicted free cysteine on HC to prevent disulfide bonding did not reduce aggregation. Re-transfecting CHO cells with excess HC with more BiP, an important IgG molecular chaperone, partially reduced unwanted aggregates and fragments possibly by helping retain more incomplete products within the cell for either proper assembly or degradation. A second transfection of LC into CHO cells with excess HC to increase the LC expression to a level greater than the HC expression successfully removed all aggregates and fragments. mAb product aggregation in CHO cells with excess HC occur due to a combination of limited chaperones and LC:HC ratio. These results provide added insights to aggregate formation and would be useful for development of mAb cell lines with reduced aggregates.

**Keywords** Monoclonal antibody · Chinese hamster ovary cells · BiP · IgG aggregation

## Introduction

All proteins including monoclonal antibody (mAb) therapeutics have the tendency to aggregate. Aggregates can be classified by their covalent/non-covalent bonds, reversible/non-reversible nature, size, and conformation [1, 2]. The presence of aggregates in protein therapeutics like IgG mAb can result in immunogenic reactions, complications during product administration, and impair product quality and efficacy [2]. Aggregation can occur during the various steps of the mAb manufacturing process, starting from cell line generation to the scale up culture process followed by purification, formulation all the way to storage [1–3]. The presence of aggregates after filtration and centrifugation of the culture complicates the purification steps as the commonly used protein A affinity mAb purification method does not discriminate between monomers and aggregates as long as the Fc region is intact [4]. Several extra polishing steps like size exclusion and ion-exchange chromatography are required after affinity purification to remove aggregates [4, 5].

It is beneficial to minimize aggregation from the early steps of production during clone selection and culturing. In order to maximize yields, high amounts of recombinant mAb peptides are expressed by each cell. This could lead to intracellular aggregation due to the high amount of unfolded proteins or inefficiencies of the molecular chaperones at controlling proper protein folding [6]. This has been demonstrated in Chinese hamster ovary (CHO) cells where the expression of a simple ATIII glycoprotein above a threshold triggered aggregation and lowered yield [7]. mAb

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expression is further complicated by its multimeric nature where the heavy chain (HC) and light chain (LC) peptides are translated separately before being assembled with the aid of various ER proteins [8, 9]. One study observed that aggregate levels were reversibly correlated to the LC:HC mRNA ratios in stable mAb-producing clones and high level of aggregates formed in clones with the LC:HC mRNA ratio below 1.5 [10]. It was also observed that increased aggregates were associated with decreased LC:HC mRNA ratios in response to a temperature shift from 37 °C to lower temperatures [11]. We had generated two mAb producing stably transfected CHO cell pools, LIHID and HILID, with controlled intracellular LC:HC ratios of 3.4 and 0.3, respectively. We had observed higher mAb productivity for transfected pools with the higher ratio. The mAb produced by HILID stable pools with excess HC comprised over 10 % aggregates, while LIHID stable pools with excess LC had less than 1 % aggregate [12]. Further analysis of the nature and mechanism of mAb aggregation in CHO cells with excess HC was not reported in our previous study.

In this work, we studied where aggregates formed in HILID cells with excess HC, characterized and investigated the nature of aggregates. Cell engineering of molecular chaperones has mostly been performed to improve cell productivity [13–15], with little reports of chaperone engineering to reduce product aggregation [11]. Chaperone engineering is not always a straight-forward experiment due to the complexity of the mammalian protein-folding machinery [13, 16]. We studied how CHO cells with excess HC would respond to the accumulation of intracellular HC polypeptides and the effect of over-expressing a binding immunoglobulin protein (BiP) chaperone on the mAb aggregate formation. An extra transfection of only LC had been suggested as a solution to reduce aggregates arising due to excess HC but was not evaluated as the authors had considered that the mechanisms of aggregation were unknown and simply altering the LC:HC ratio could be insufficient [10]. We designed a strategy to increase LC expression in HILID stable pools using stringent selection markers and demonstrated that a second transfection of LC was effective at reducing aggregates. The results obtained would provide insights to the causes of mAb aggregation and to generate cell lines with reduced aggregates.

## Materials and Methods

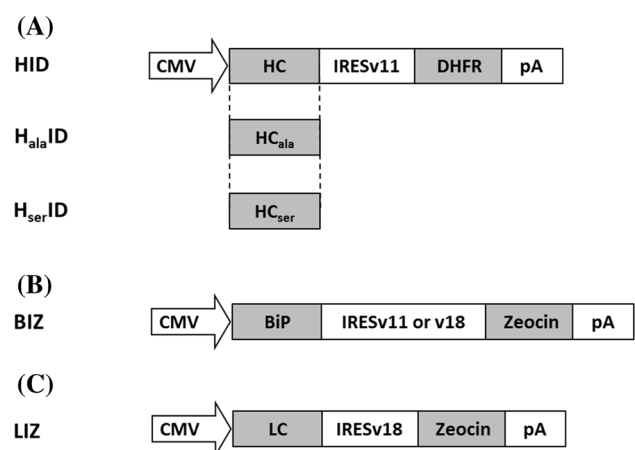
### Vector Construction

Vectors used were modified from a previously described LIHID vector which LC, HC, and dihydrofolate reductase (DHFR) genes were linked by internal ribosome entry site

elements (IRES) [12]. The HID vector was constructed by replacing LC–IRES–HC with HC (Fig. 1a). The IRES upstream of the DHFR selection marker was replaced with a mutant IRES variant we had generated, IRESv11 [17]. Variants of the HID vector with mutations to the cysteine on the HC which normally forms a disulfide bond with a LC to either alanine (nucleotide substitutions of T725G and G725C) or serine (substitution of G725C) were obtained by using QuikChange multisite-directed mutagenesis kits (Agilent, Santa Clara, CA). The variants were labeled as H<sub>ala</sub>ID and H<sub>ser</sub>ID. To generate LIZ and BIZ vectors (Fig. 1b, c), the DHFR selection marker on the LIHID vector was first replaced by a Zeocin antibiotics selection marker cloned from pCDNA3.1/Zeo (Life Technologies, Carlsbad, CA). Following which, LC-IRES-HC was replaced with either just LC or CHO BiP cDNA (genbank: GCA\_000223135.1). Finally, the IRES sequence upstream of Zeocin for LIZ vector was replaced with a mutant IRESv18 with an estimated 1.4 % strength of the wild-type IRES, and the IRES sequence upstream of Zeocin for BIZ was replaced with either IRESv18 or IRESv11 with a relative 25 % strength of the wild-type IRES. HID, H<sub>ala</sub>ID, and H<sub>ser</sub>ID were transfected to DG44 cells, while LIZ and BIZ were used to re-transfect earlier generated HILID stable pools with excess HC.

### Cell Culture and Transfections

DHFR-deficient CHO DG44 cells (Life Technologies) were grown in protein-free media (PFM) supplemented with 0.1 mM sodium hypoxanthine and 0.016 mM thymine (HT; Life Technologies). PFM was prepared from a 1:1 mixture of HyQ PF (Hyclone, Logan, UT) and CD CHO (Life Technologies), supplemented with 1 g/L sodium



**Fig. 1** Plasmid vectors constructed. **a** Vectors for expression of HC and HC mutants, **b** vector re-transfected into HILID pool to increase BiP expression, **c** vector re-transfected into HILID pool to increase LC expression

bicarbonate (Sigma-Aldrich, St-Louis, MO), 6 mM Glutamine (Sigma-Aldrich), and 0.05 % Pluronic F-68 (Life Technologies). Cell lines LIHID and HILID were generated in an earlier report [12] and cultured in PFM supplemented with 50 mM methotrexate (MTX, Sigma-Aldrich). Cells were passaged every 3–4 days by diluting the cultures to  $2 \times 10^5$  cells/mL in fresh media. Cell viability and density were determined by trypan blue exclusion method using a Vi-Cell XR cell viability analyzer (Beckman Coulter, CA).

Transfections were performed using SG solution and program FF-137 on a 4D-Nucleofector system (Lonza, Cologne, Germany).  $1 \times 10^7$  cells and 5  $\mu$ g of plasmid were used for each experiment. Transfected DG44 cells with HID vector series were transferred to selection media of PFM media without HT followed by gene amplification using 50 mM MTX. HILID pools that were re-transfected with LIZ or BIZ vectors underwent selection using 200  $\mu$ g/mL Zeocin<sup>TM</sup> (Invivogen, San Diego, CA). Selection was deemed completed when cell viability reached above 95 %. Recovered cultures were seeded at  $2 \times 10^5$  cells/mL and supernatant was collected after 10–11 days when viability dropped to 50 % for further experiments. All transfections were performed in duplicates.

#### ELISA and Western Blotting

Cell lysis was performed using CellLytic<sup>TM</sup> M with added protease inhibitor cocktail (both from Sigma-Aldrich). Intracellular protein from  $1 \times 10^7$  cells was extracted using 200  $\mu$ L of the mix. Cell lysates were used to determine the intracellular polypeptides of LC:HC ratio by enzyme-linked immunosorbent assay (ELISA) and identify intracellular mAb components by Western blotting. ELISA and Western blotting were performed as previously described [12]. Precision Plus dual color protein pre-stained standards (Bio-Rad, Hercules, CA) were added to each blot to identify species sizes. Undiluted cell lysates and 10 ng of mAb in the supernatant were used for Western blotting.

#### Purifying of mAb Products

mAb was purified using a Tricorn 5/150 protein A column packed with MabSelect SuRe (GE Healthcare, Uppsala, Sweden) loaded on a GE ÄKTA Explorer 100 (GE Healthcare). Sample was injected at a flow rate of 3 mL/min. A terminator wash buffer of 2 M sodium chloride (Merck, Darmstadt, Germany), 250 mM imidazole (Merck), 10 mM EDTA (Sigma-Aldrich), and 4 M urea (Sigma-Aldrich) adjusted to pH 7.0 was passed through the column followed by an elution buffer of 100 mM acetate (Sigma-Aldrich) and 100 mM arginine (Sigma-Aldrich) at pH 3.5. Eluted samples were neutralized using 1 M Tris (Sigma-

Aldrich) and the column was regenerated using 0.1 M glycine (Merck) adjusted to pH 2.5.

#### Aggregation Analysis of Protein A Purified mAb

The aggregation of protein A purified mAb was determined using size exclusion chromatography (SEC). The instrument setup consisted of a HPLC system (Shimadzu, Kyoto, Japan), with a binary pump, an auto injector, a thermostated column oven, and a UV-Visible detector. The chromatography columns used were TSK Guard column SWXL,  $6 \times 40$  mm and TSK gel G3000 SWXL,  $7.8 \times 300$  mm (Tosoh Corporation, Tokyo, Japan). Column oven temperature was set at 25 °C and the regular mobile phase included 0.2 M sodium phosphate (Merck) and 0.1 M potassium sulfate buffer at pH 6.0 (Merck). Flow rate was 0.5 mL/min. All samples were analyzed immediately after protein A purification to avoid particulate formation during storage.

Subsequent SEC analysis of aggregates was performed using methods with modified mobile phases similar to that previously reported by Gomez et al. [11]. One experiment was performed using mobile phase with added 6 M guanidine (Sigma-Aldrich). Another was performed with 30 min incubation at 37 °C of the product with 10 mM DTT (Promega) prior to injection and running with mobile phase containing 10 mM DTT. The final experiment was performed with doubling  $K_2SO_4$  salt concentration from 0.1 to 0.2 M. Duplicate measurements were performed for two separately collected samples for each experiment.

#### Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from LIHID and HILID pools using a RNeasy<sup>®</sup> Mini Kit (Qiagen, Valencia, CA). mRNA levels for BiP were then analyzed using a two-step qRT-PCR protocol.  $\beta$ -actin was used as the internal control. Briefly, 100 ng of RNA was reverse transcribed to cDNA using the ImProm II<sup>TM</sup> reverse transcription system (Promega) in a 40  $\mu$ L reaction. These cDNA samples were analyzed on an iQ-5 Real-time PCR System (Bio-Rad) using a recipe of 10.0  $\mu$ L of SsoFast<sup>TM</sup> Evagreen<sup>®</sup> Supermix (Bio-Rad), 500 nM (final concentration) of forward and reverse primers (Research Biolabs, Singapore), 2.0  $\mu$ L of above synthesized cDNA, and topped up with HPLC water (Merck, San Diego, CA, USA) for a 20  $\mu$ L reaction. Primers used are shown in Table 1. mRNA was extracted from two independently transfected cultures and analyzed with duplicate measurements for each sample. The collected threshold cycle (Ct) values were analyzed using a  $2^{-\Delta\Delta Ct}$  method [18].

**Table 1** Primers used for qRT-PCR

Primer	Sequence
BiP-forw	5'-CGGTGGAACCTTCGATGTG-3'
BiP-rev	5'-TCCATAACCCGCTGATCAAAG-3'
Actin-forw	5'-AGCTGAGAGGGAAATTGTGCG-3'
Actin-rev	5'-GCAACGGAACCGCTCATT-3'

## Results

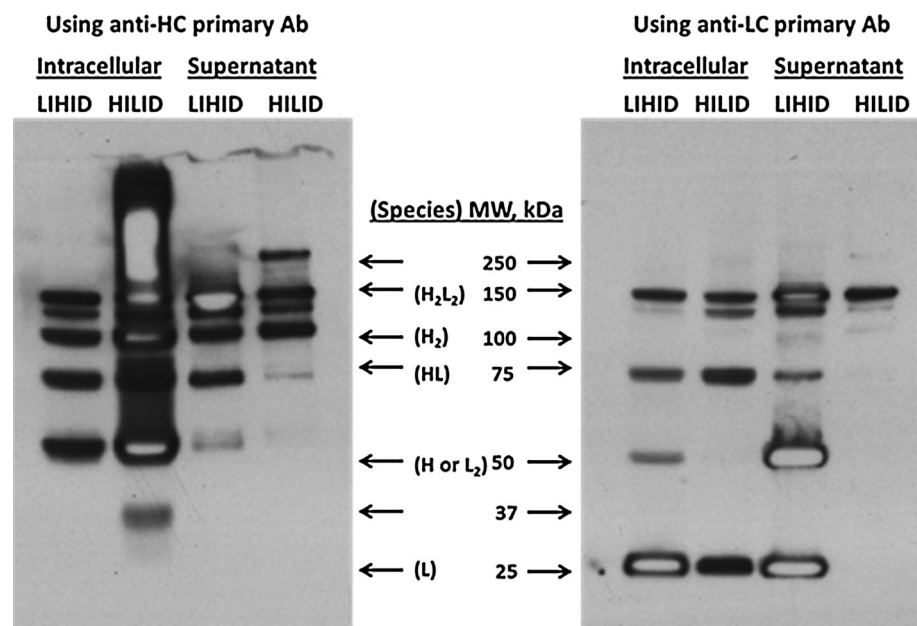
### Analysis of Aggregate Formation

We had previously generated pools of mAb-producing CHO cells with controlled LC:HC ratio using an IRES-based vector [12, 19]. HILID pools with excess HC exhibited lower productivity, slower growth, and higher aggregates compared to LIHID pools with excess LC [12, 20]. In this study, we were interested in understanding the causes of the aggregate formation and to reduce the aggregate levels for the HILID pools. We first performed Western blotting analysis of the cell lysate and supernatant to determine where aggregates formed in HILID stable pools with excess HC (LC:HC ratio of 0.3) and to identify the possible components within the aggregates. Cell lysate and supernatant for LIHID cell pools with excess LC and low mAb aggregates (LC:HC ratio of 3.4) were also analyzed for comparison. We had previously also observed excess HC in the supernatant of the HILID pools and excess LC in the LIHID pools in Western blots which corresponded to the LC:HC ratios [12]. The samples were analyzed separately using anti-HC and anti-LC detection antibodies (Fig. 2). Over-exposure during development of

the blot ensured that even small amounts of aggregates present would be visualized. LIHID lysate and supernatant samples had similar results for both detection antibodies and no aggregates were observed. HILID lysates exhibited a myriad of species with HC which appeared as a smear starting from 50 kDa, the size of a single HC to the start of the gel. Appearance of these unwanted products was likely exacerbated by the excess HC as the smearing was not visible using the anti-LC primary antibody. While this might not represent the actual species present within the cell due to differences between the cellular environment and the blot, this shows that HC species highly prone to aggregation are abundant within HILID pools. This smearing was not as evident in the HILID supernatant with slight smearing above the 150 kDa IgG monomer band and a high molecular weight band above. The high molecular weight species (above 150 kDa) comprised mostly of HC as the band was significantly darker using the anti-HC antibody compared to the faint band using anti-LC even with the over-exposure. Subsequent Western blots of the reduced products confirmed that only correct HC and LC subunits were present (data not shown), similar to our previous report [12].

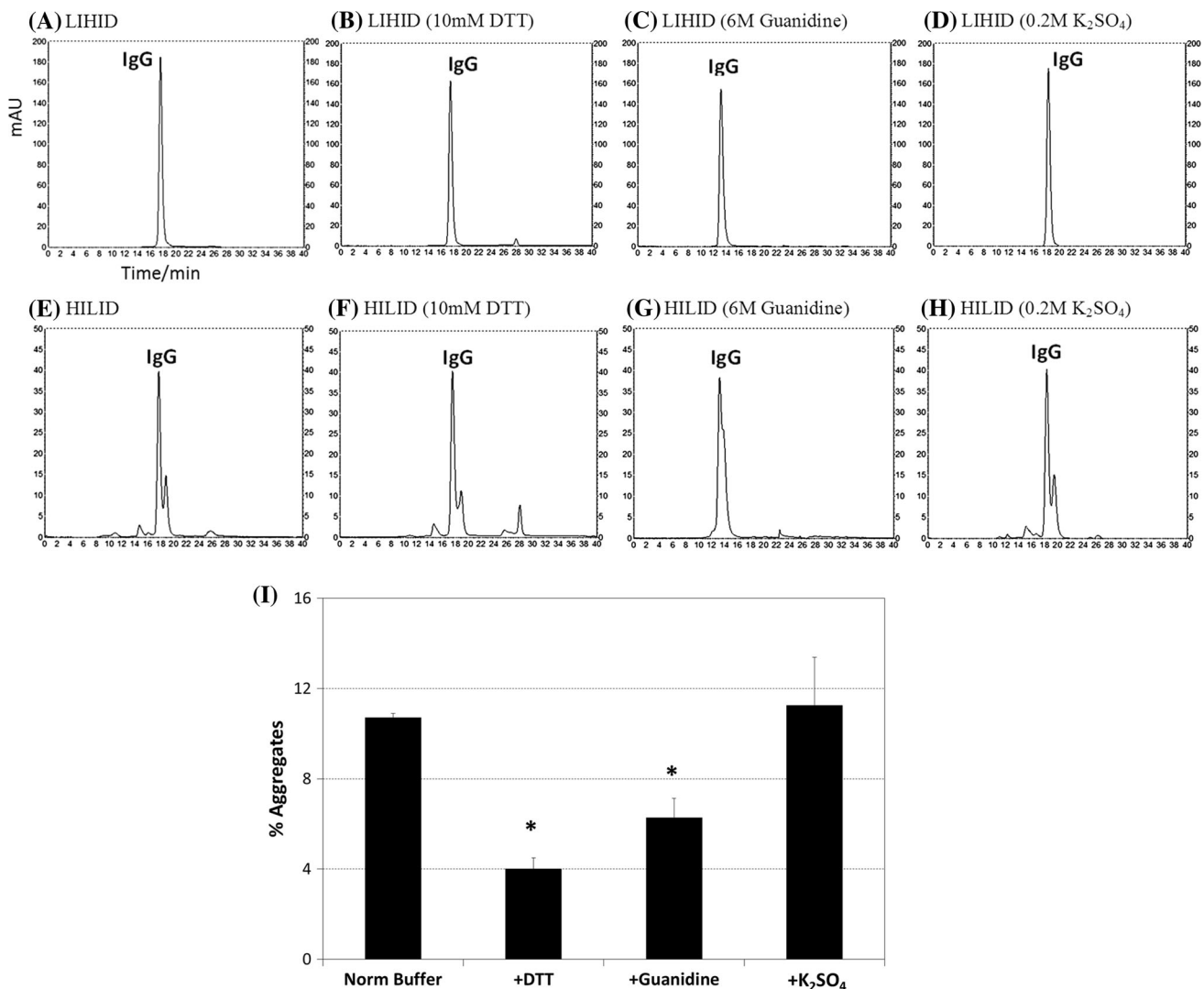
SEC analysis was performed on the protein A purified anti-HER2 IgG from HILID pools to identify the mechanisms causing the aggregates to form. Three conditions were tested: reducing condition with 10 mM DTT, denaturing condition with 6 M guanidine, and increased salt concentration to 0.4 M K<sub>2</sub>SO<sub>4</sub>. The conditions were determined based on a previous report [11]. The reducing condition would test for disulfide bonding, the denaturing conditions for hydrophobic interactions, and the change to salt concentration for charge interactions. Purified samples

**Fig. 2** Western blotting of intracellular proteins and supernatant from LIHID and HILID using separate anti-HC and anti-LC detection antibodies. Samples were non-reduced and the increased exposure was performed to enable visualization of products present in small amounts



from LIHID and HILID were first analyzed using the regular mobile phase buffer as a control (Fig. 3a, e). LIHID only had a single monomer peak corresponding to about 145 kDa as determined by light scattering. HILID had 13 % aggregates, 60 % monomers, and 27 % of IgG fragments. As the purification process was optimized for IgG monomers, some incomplete product or aggregates could have been washed off earlier or remain bound to the protein A column after elution, resulting in loss of some products as seen in Fig. 2. Testing the various processes on IgG monomers from a LIHID pool ensured that the treatments did not affect IgG monomers. Only a single monomer peak was observed during SEC analysis for all three

conditions (Fig. 3b, c, d). Treatment of HILID samples resulted in a drop in the amount of aggregates from 13 to 4 and 6 % in the reducing and denaturing conditions, respectively (Fig. 3f, g). The disulfide bonds on the IgG monomers remained intact likely due to their more stable conformation compared to aggregates and the weak denaturing conditions of low DTT concentration and heating. Peaks on the chromatogram profile for HILID in the mobile phase with 6 M guanidine were less distinct compared to the regular mobile phase and the aggregate and fragment peaks became a shoulder off the main IgG peak (Fig. 3g). The aggregate peak in this case was identified by comparing the LIHID and HILID chromatograms obtained



**Fig. 3** Chromatograms of protein A purified mAb produced by LIHID (a–d) and HILID (e–h). Samples analyzed with addition of either 10 mM DTT, 6 M guanidine or 0.2 M K<sub>2</sub>SO<sub>4</sub> to the liquid phase during SEC analysis. The main peaks labeled IgG are peaks for full IgG monomers. Peaks or peak shoulders appearing before the IgG were considered higher molecular weight aggregates. Peaks and

shoulders after the main peak are low molecular weight IgG fragments. **i** Amount of aggregates detected in the normal SEC mobile phase and with the various additions. Each experiment was performed in replicates. \*denotes the change was statistically significant using a two-tailed student *t* test ( $p < 0.05$ )

using the same buffer. There was no change in the amount of aggregates in the increased salt buffer (Fig. 3h). Analysis was done for samples from two sets of LIHID and HILID cell pools and similar results were observed. The average amount of aggregates measured after each treatment of duplicate cell pools is shown in Fig. 3i. Based on these observations, the aggregates in HILID CHO cell pools with excess HC likely formed due to a combination of disulfide bonding and hydrophobic interactions between free extra HC polypeptides.

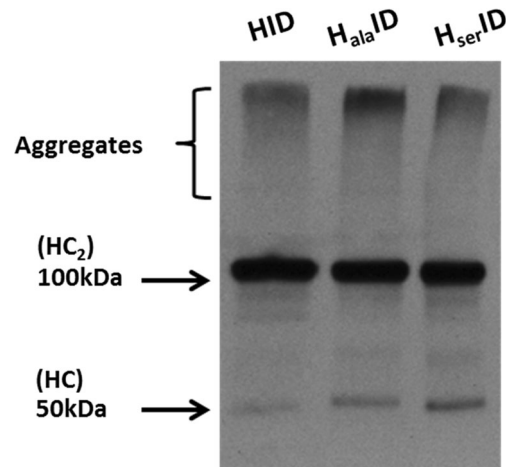
mAb synthesis involves formation of HC<sub>2</sub> dimers before folded LC are added [8, 21]. HC polypeptides are highly hydrophobic and inclined to aggregate. BIP binds to exposed HC hydrophobic region to prevent aggregation. BIP is released when a folded LC becomes available. HC dimer pairs with LC through disulfide bonding at fifth cysteine on HC (C223) and the last cysteine on LC (C236) [22]. Under the condition of excess HC and limited BIP, hydrophobic regions of variable region and first constant domains would be exposed. We hypothesized that incomplete mAb fragments, HC<sub>2</sub>, and HC<sub>2</sub>LC would aggregate through disulfide bonding at free cysteine 223 and hydrophobic interactions. We designed three experiments to test this hypothesis: (1) Mutation of cysteine on HC (C223A, C223S), (2) over-expression of BIP, and (3) a second transfection of only LC.

#### Effect of Mutating Cysteine 223 on HC on Aggregate Formation

As aggregates mainly consisted of HC polypeptides, we had hypothesized that one reason for the aggregates forming in the HILID pools was due to excess HC with unpaired free cysteines forming unwanted disulfide bonding. To test this hypothesis, a series of plasmids expressing only HC were generated (Fig. 1a). HID has the same HC sequence as used on the HILID plasmid, and another two with the cysteine which was to pair with a cysteine on a LC mutated to either alanine or serine, H<sub>ala</sub>ID, and H<sub>ser</sub>ID. HC expressed from H<sub>ala</sub>ID and H<sub>ser</sub>ID vectors should not form high molecular weight aggregates due to disulfide bonding. Aggregates were visible in HC with mutation of the free cysteine to either alanine or serine, suggesting that mechanisms other than disulfide bonds were contributing to the aggregates (Fig. 4). This result complemented the earlier observations that the aggregates comprised by a majority of HC and formed with a combination of different bonds like disulfide bonding and hydrophobic interactions.

#### Increased Expression of BIP to Reduce Aggregates

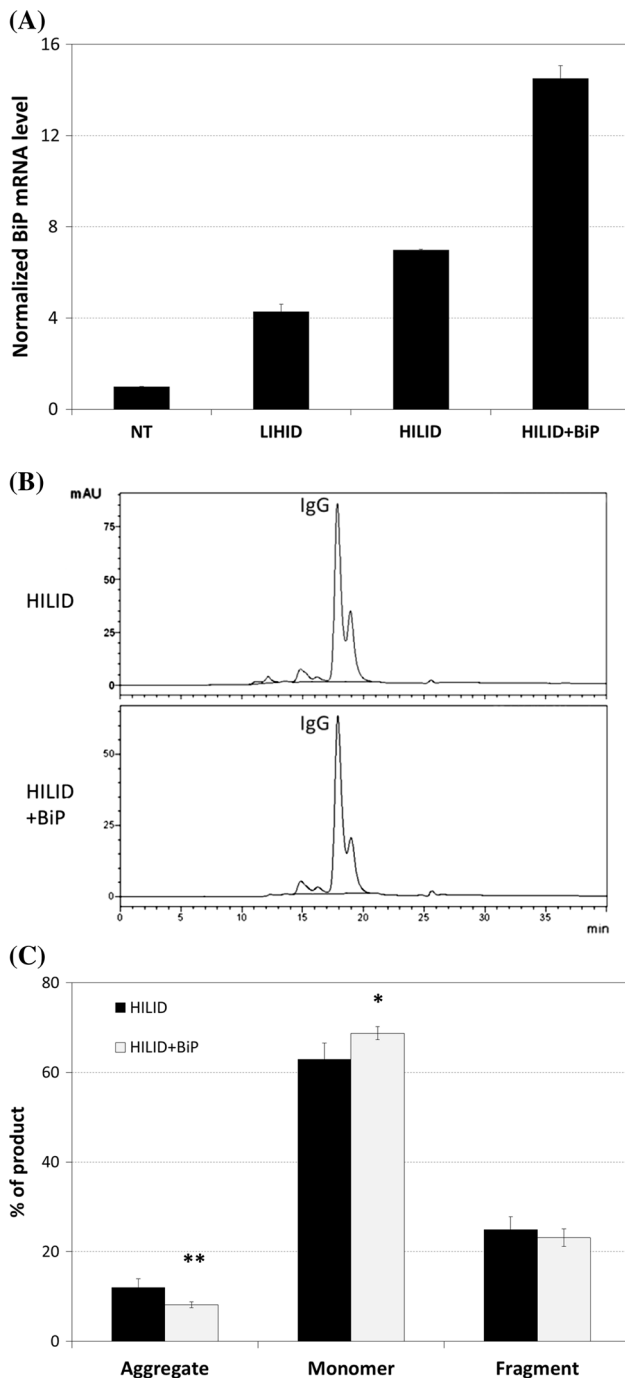
BiP expression levels were examined for non-transfected CHO DG44 cells, LIHID and HILID pools by qRT-PCR



**Fig. 4** HC aggregates after cysteine mutation. Expression of only IgG HC (HID) and HC mutants with the cysteine for disulfide pairing with LC mutated to alanine (H<sub>ala</sub>ID) and serine (H<sub>ser</sub>ID). Samples were probed with anti-FC detection antibody

(Fig. 5a). BiP was upregulated at 4.3 and 7-fold higher for LIHID and HILID, respectively, compared to non-transfected CHO DG44 cells (NT). This could be due to the cell having to cope with the increased IgG expression for LIHID and large amounts of excess HC for HILID. BiP over-expression is linked to the unfolded protein response (UPR) that cells activate to handle the protein overload. Although BiP binds directly to incorrectly folded HC to aid in retaining them in the ER, we observed incomplete products being secreted. It is possible that the cell's own BiP upregulation could be still insufficient, leading to a breakdown in the cell's quality control mechanism and elevated product aggregation.

In order to test if an increase in BiP expression could reduce aggregates, a HILID pool was re-transfected in duplicates with the BIZ vector carrying the CHO BiP cDNA and a Zeocin selection marker (Fig. 1b). The transfected HILID pools underwent a second selection using Zeocin antibiotics to generate new stable pools referred to as HILID + BiP. Transfected cells failed to survive selection using the weaker IRESv18 on Zeocin selection marker. HILID + BiP pools generated using IRESv11, with greater translation efficiency than IRESv18, to drive Zeocin selection marker expression survived selection. Cells were collected for qRT-PCR analysis of chaperone expression levels and the supernatant was collected to check product aggregate levels. Analysis of mRNA expression levels for the re-transfected HILID + BiP cells showed that BiP expression increased 2.7-fold compared to the original HILID pool (Fig. 5a). The amount of aggregates decreased to 8.2 % for HILID + BiP compared to the initial 12 % of the HILID pool and an increase in monomers from 62.9 to 68.7 % (Fig. 5b, c). IgG titer detected increased from 70 to 80 mg/



**Fig. 5** Analysis of BiP expression. **a** mRNA expression level of BiP for non-transfected DG44 cells (NT), LIHID, HILID, and HILID + BiP were checked using qRT-PCR with  $\beta$ -actin used as the internal control. Samples were normalized to NT. **b** SEC chromatograms for HILID before (top) and after increasing BiP expression (HILID + BiP, bottom). Monomer peak is labeled as IgG. Peaks appearing before the main peak were considered high molecular weight aggregates and peaks after considered fragments. **c** Product distribution comparison of HILID (black bars) and HILID + BiP (white bars). Each bar and standard deviations are obtained from two measurements each of biological replicates. \* and \*\* denote that the differences were statistically significant using a two-tailed student *t* test at  $p < 0.1$  and  $p < 0.05$ , respectively

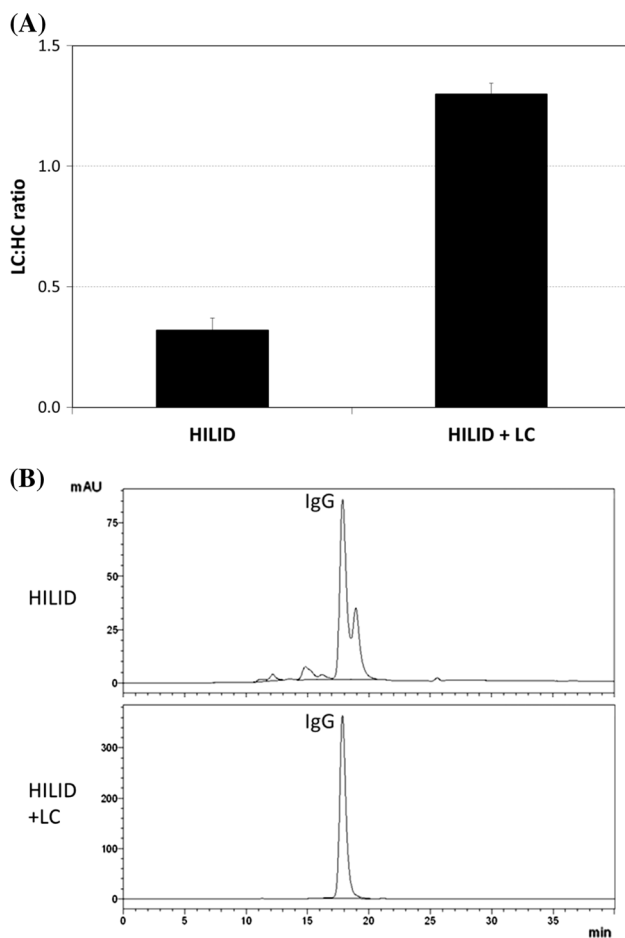
L. There are currently no reports of how BiP chaperone over-expression could affect IgG product quality. It is possible that the increased BiP levels helped to retain more incomplete and aggregation prone IgG within the cell for reassembly or degradation.

#### A Second Transfection of LC to Reduce Aggregates

Over-expression of BiP chaperone did not completely remove the aggregates. We next explored an alternative method to reduce product aggregation in the HILID pools by re-transfecting the cells with more LC cDNA to increase the intracellular LC peptide amount and thus LC:HC ratio. A HILID pool was re-transfected with the LIZ vector carrying the LC cDNA and a Zeocin selection marker (Fig. 1c) and underwent antibiotics selection similar to how HILID + BiP pools were generated. IRESv18 was applied onto Zeocin to enhance selection stringency and maximize LC expression. The newly generated stable cell pools were labeled as HILID + LC. Intracellular LC:HC ratio was verified to have increased from the initial 0.3 for HILID to 1.3 in HILID + LC pools (Fig. 6a). Titer for HILID + LC increased to 160 mg/L as compared to 70 mg/L for HILID. Only a single IgG monomer peak was observed for purified HILID + LC product during SEC analysis (Fig. 6b). Cells producing mAb with poor product quality due to low LC:HC ratio would thus likely benefit from a second transfection of only LC.

#### Discussion

In this study, we used a previously generated set of mAb-producing CHO cell lines with LC:HC ratio controlled at either 3.4 (LIHID) or 0.3 (HILID) to understand the mechanism of aggregation formed under the condition of excess HC. Analytical studies of product from the HILID pool revealed that the aggregates consisted a majority of HC polypeptides and were partially dissociated under reducing and denaturing conditions. We hypothesized that incomplete mAb fragments containing excess HC, such as HC<sub>2</sub> and HC<sub>2</sub>LC were inclined to aggregate due to free cysteines and exposed hydrophobic regions on HC. HC was secreted when expressed in the absence of LC and aggregates still formed between HC polypeptides when the free cysteine was mutated to alanine and serine. The level of aggregate was not significantly reduced compared to the wild-type HC as determined by Western blotting. In a study using COS-7 cells expressing murine IgG, HC was only secreted in the absence of LC when cysteines on the first HC constant region (C<sub>H1</sub>) were removed [23]. Unmodified HC and HC<sub>2</sub> were still being secreted in our CHO cells likely due to its high expression level under a DHFR/MTX



**Fig. 6** Increasing expression of LC to reduce aggregates and fragments in HILID pools. **a** Intracellular LC:HC ratio of HILID and after second transfection of LC only vector (HILID + LC). Data and standard deviation are from two measurements each of biological duplicates. **b** SEC chromatograms for HILID before (*top*) and after increasing LC expression (HILID + LC, *bottom*). Monomer peak is labeled IgG. Species appearing before the peak are aggregates and after the peak are fragments

amplification system exceeding the cell's capacity. Most of the aggregation prone motifs on IgG1 (class of the IgG used in this study) are concentrated around the hinge region of the HC [24]. This is also the region where the free cysteine due to the LC absence resides. This could explain why we did not see increased aggregation in the wild-type HC as the two main aggregating mechanisms, disulfide bonding at free cysteines and hydrophobic interactions, likely occur at the same region. Another possible reason for the similar aggregate levels could be that the HC dimers are incorrectly folded and other cysteine residues are participating in disulfide bond formation and cause aggregates. A previous report conducted a similar study on aggregates but only observed aggregate levels decreasing upon adding guanidine and not in DTT [11]. In that study, LC:HC peptide ratios were not determined but they reported a

greater increase in HC mRNA levels as compared to LC mRNA levels for two of the three cell lines studied. It is possible that at the protein level, the LC:HC ratio was still close to one and the incomplete products composed of more mAbs lacking only one LC. In our study, more HC dimers were formed.

BiP upregulation to handle increased protein expression is part of the cell's UPR [25, 26]. Over-expressing BiP in mAb expressing CHO cell lines had been reported to reduce specific secretion rate and increase HC accumulation [13]. There are still no reports on the effect of over-expressing BiP on mAb aggregation. The result here provides evidence that BiP over-expression could help reduce secretion of mAb aggregates and fragments caused by excess HC. BiP binds to the  $C_{H1}$  domain of the unfolded HC to aid retention within the ER and release is triggered by LC [27, 28]. The increased BiP could help with retention of the excess HC within the ER, reducing the secretion of fragments like the  $HC_2$  which are more prone to aggregation. Weakening the selection marker is effective to increase the stable expression of a gene as only clones with more copies of the integrated vector and/or vectors integrated into transcriptionally active site in chromosomes can survive the selection [29]. We attempted to control BiP expression by using IRES variants with different strengths to control the selection gene [17]. Over-expression of BiP using the BIZ vector with Zeocin under the control of IRESv11 did not prevent aggregates forming possibly due to a combination of the limitations of the cell and insufficient BiP to handle all the excess HC. When we tried to maximize BiP expression by using a weaker IRESv18 with Zeocin on BIZ, the transfected cells failed to survive selection. IRESv18 had been successfully used with Zeocin in this report for LC re-transfection. We speculate that the higher BiP levels, due to greater selection stringency from the weaker IRES, resulted in over-accumulation of unfolded IgG and eventually cell death due to inability to cope with the load. The protein-folding machinery is a complex process involving multiple enzymes and engineering of the pathway would likely require more than one target to successfully remove more of the aggregates. Further work is needed to understand how over-expression of chaperones lowers aggregation and if a combination of BiP with other chaperones would have a greater effect.

Cell lines generated using commonly used vectors, co-transfection, and multipromoter single vector, have varied ratios of LC:HC. Lee et al., had previously found that products from clones with low LC:HC mRNA levels were prone to aggregate and suggested super-transfection of LC into clones exhibiting high levels of mAb aggregation but did not perform the experiment [10]. In our study, we show that re-transfecting of cells with low LC:HC ratio with more LC to increase the ratio was an effective method to



reduce aggregation. The excess LC expression was obtained by using a very weak IRESv18 to drive low levels of Zeocin expression to increase the stringency of selection for high producers in stably transfected pools. LC:HC ratios affect expression level and are a critical parameter for mAb aggregation and other quality parameters, such as glycosylation [12, 30–32]. It is favorable to generate cell lines using vectors with the ability to control LC:HC at optimal ratios for both expression level and quality. For cell lines forming aggregate due to excess HC, re-transfection of LC is the easiest and most effective way to reduce aggregates.

Aggregates in protein therapeutics like IgG mAb are detrimental to product safety and efficacy. We studied the underlying mechanism causing product aggregation in mAb-producing CHO cell lines with excess HC. Product was highly aggregation prone within the cell but the majority of these aggregates were not secreted. The poorly folded mAb which managed to escape the cell's quality control mechanisms and enter the supernatant could be dissociated by reducing and denaturing conditions. Interestingly, mutation of a potential-free cysteine on HC did not reduce the amount of aggregates observed between HC dimers. Aggregates could be reduced by over-expression of BiP chaperone and totally removed by a second transfection of LC. mAb aggregation in CHO cells with excess HC occurs due to a combination of limitations of the chaperone levels and LC:HC ratio. Further experiments using clones with varying levels of intracellular HC and BiP before BiP over-expression could yield even more interesting results. A combination of both over-expressing chaperones and increasing LC amounts could be required for some cell lines with high excess of HC and higher aggregates. Overall, the results here would be useful for designing methods to break up aggregates post-purification or to rescue previously generated mAb cell lines with poor product quality.

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