Using hydrogen peroxide to prevent antibody disulfide bond reduction during manufacturing process

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Abstract

During large-scale monoclonal antibody manufacturing, disulfide bond reduction of antibodies, which results in generation of low molecule weight species, is occasionally observed. When this happens, the drug substance does not meet specifications. Many investigations have been conducted across the biopharmaceutical industry to identify the root causes, and multiple strategies have been proposed to mitigate the problem. The reduction is correlated with the release of cellular reducing components and depletion of dissolved oxygen before, during, and after harvest. Consequently, these factors can lead to disulfide reduction over long-duration storage at room temperature prior to Protein A chromatography. Several strategies have been developed to minimize antibody reduction, including chemical inhibition of reducing components, maintaining aeration before and after harvest, and chilling clarified harvest during holding. Here, we explore the use of hydrogen peroxide in clarified harvest bulk or cell culture fluid as a strategy to prevent disulfide reduction. A lab-scale study was performed to demonstrate the effectiveness of hydrogen peroxide in preventing antibody reduction using multiple IgG molecules. Studies were done to define the optimal concentration of hydrogen peroxide needed to avoid unnecessary oxidization of the antibody products. We show that adding a controlled amount of hydrogen peroxide does not change product quality attributes of the protein. Since hydrogen peroxide is soluble in aqueous solutions and decomposes into water and oxygen, there is no additional burden involved in removing it during the downstream purification steps. Due to its ease of use and minimal product impact, we demonstrate that hydrogen peroxide treatment is a powerful, simple tool to quench reducing potential by simply mixing it with harvested cell culture fluid.

Introduction

The clinical use of recombinant monoclonal IgG antibodies (mAbs) is growing over recent years. MABs are commonly produced in mammalian cells, especially in Chinese hamster ovary (CHO) cells, and are secreted extracellularly into culture media. At the end of the culture process, cells are separated from the culture media during the primary recovery step using methods such as centrifugation, depth filtration, or flocculation to clarify the harvest fluid. During this process, the cells may suffer various stresses, including mechanical shear, exposure to a low dissolved oxygen (DO) environment, or temperature and pH shifts. These stresses may cause cell damage, resulting in the release of undesired intracellular components into the clarified fluid. These cytosolic components, such as lipids and enzymes, can potentially affect product quality, and must be carefully monitored or removed. One prominent example is the release of intracellular reducing agents, which can lead to the reduction of antibody disulfide bonds.1–9

Multiple intracellular redox components have been implicated in the reduction of antibody disulfide bonds, such as the thioredoxin/thioredoxin reductase (Trx/TrxR),5,7,8 and the glutathione/glutathione reductase (Glu/GR)8 systems. The thioredoxin system consists of Trx and TrxR and uses the cofactor nicotinamide, adenine dinucleotide phosphate (NADPH) as an electron donor. The glutathione system is made up of glutathione, glutathione reductase, glutaredoxin and NADPH. The two systems share multiple similarities in components and mechanisms, and so either one of them or even a combination of them has been shown to be responsible for disulfide bond reduction during therapeutic antibody production in CHO cells.8 Manufacturing processes can have a direct impact on the extent of reduction. The disulfide reduction has been observed at manufacturing-scale processes, but not typically observed in lab-scale bioreactors.4,6 During the manufacturing process, extensive reduction of antibodies has been observed after harvest operation or Protein A chromatography,4,5 and multiple process parameters correlate to the extent of the reduction. For example, maintaining high levels of DO during harvest is vital to keep antibody molecules intact.1,4 Mechanical shear forces, which cause cell lysis and cellular components to leak into harvest fluids, also significantly contributes to the reduction.1,4–6 Other process parameters, such as harvest hold time1,4–6,10 and media components (such as copper
ions, cysteine/cystine and pH\textsuperscript{4,10} and temperature,\textsuperscript{4,10} also have an effect on the extent of disulfide reduction. Small-scale models for harvest have been developed in order to study disulfide reduction based on these observations. In the small-scale models, cell extract generated by mechanical shearing was added to harvest cell culture fluid to achieve the desired level of cell lysis and incubated for 0–3 days with controlled DO level by nitrogen gas flushing.\textsuperscript{1,4,6} These studies demonstrated that the reduction of antibodies is caused by the release of intracellular components by mechanical shearing and an anaerobic environment.\textsuperscript{1,4,6}

The reduction of antibody products due to the aforementioned enzyme systems may be complete or partial. Human IgG class antibodies contain two heavy and two light chains that are held together by non-covalent interactions as well as inter-chain disulfide bonds. There are a total of 12 intra-chain disulfide bonds and one disulfide bond between light and heavy chains in all four IgG subclasses. The number of inter-chain disulfide bonds between the two heavy chains is 2 for IgG1 and IgG4, 4 for IgG2 and 11 for IgG3.\textsuperscript{11} Additionally, the inter-chain disulfide bonds are more solvent accessible than intra-chain bonds, and the exposed cysteine residues are considered more reactive than non-exposed cysteine residues.\textsuperscript{11} Therefore, the inter-chain disulfide bonds are more susceptible to reduction.\textsuperscript{8}

In order to ensure antibody product quality, manufacturing in-process controls are necessary to control low molecular weight (LMW) species formed from reduction of antibody disulfide bonds. As a result, several strategies have been proposed in recent years to control disulfide reduction. Chemical inhibitors have been tested to prevent antibody reduction, including pre- and post-harvest treatment with anti-reduction agents, such as cupric sulfate,\textsuperscript{12} ethylenediaminetetraacetic acid (EDTA), thioredoxin inhibitors,\textsuperscript{13} cysteine, methyl blue\textsuperscript{14} and coenzyme Q analogs.\textsuperscript{15} Lowering the pH of clarified harvest has also been explored as a non-chemical means of controlling reduction.\textsuperscript{4,5}

Additionally, keeping DO in pre-harvest or clarified harvest above a certain level (usually 20% or more) can minimize reducing potential. Air sparging has been shown to be a robust and universal mitigation strategy to prevent disulfide bond reduction.\textsuperscript{13,14} Chilling the harvest material also generally lowers the enzymatic or chemical reaction rates, as governed by the Arrhenius equation. Combined with other mitigation strategies (aeration and addition of cysteine), chilling can be applied in the manufacturing process to extend clarified harvest holding time with no compromise on final drug substance (DS) stability.\textsuperscript{4,10} Although knowledge and methods surrounding these mitigations has increased over years, implementation of these methods in production is not without difficulties, such as introduction of chemical byproducts that need to be removed by chromatography steps, increased processing time, and the risk of off-target modifications or damage to the antibody product.

As the simplest of the peroxides, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) is primarily used as an oxidizing agent. H\textsubscript{2}O\textsubscript{2} spontaneously decomposes and generates one water and one half oxygen molecules and is formed in natural biochemical processes in living cells. It is generally cytotoxic, and can be used to induce apoptosis in high enough concentrations.\textsuperscript{16} One mechanism of cellular defense against hydrogen peroxide is to express the enzyme catalase. The enzyme is remarkably efficient, and one catalase molecule can convert over one million molecules of hydrogen peroxide to oxygen and water, while decreasing the generation of reactive oxygen species.\textsuperscript{17}

Hydrogen peroxide has been used in the formation of disulfide bonds in multiple synthetic peptides of various lengths and structures.\textsuperscript{18} The process is controlled under mild conditions without noticeable side reactions with Trp, Tyr and Met residues. In the biopharmaceutical industry, hydrogen peroxide is frequently used in forced degradation studies to determine possible biologics degradation pathways.\textsuperscript{19}

In this study, we explored the use of hydrogen peroxide to treat pre- and post-harvest culture in order to prevent disulfide reduction. Hydrogen peroxide can oxidize reducing agents and prevent the reduction disulfide bonds. One major concern in using hydrogen peroxide is the potential for oxidation of the mAb, so a lab-scale study was conducted to optimize the hydrogen peroxide concentration range that can prevent LMW generation while not oxidizing the mAb. Our study demonstrates that introduction of a controlled concentration of peroxide can prevent disulfide reduction in a number of mAb products with minimal impact to product quality. Finally, we show that due to the lack of chemical impurities, ease of use, and minimal risk to product, peroxide can be used as a mitigation tool to prevent disulfide reduction during mAb production.

Results

Disulfide bond reduction during manufacturing

The reduction of mAbs has been observed in process development for several IgG1 and IgG4 antibody molecules in either the clarified bulk (CB) or at Protein A elute (PAE).\textsuperscript{14,22} The difference in free thiol concentrations between samples of mAb 1 CB that showed disulfide reduction versus samples that did not was significant (Figure 1A). In the case of mAb 1, when the free thiol level in the CB was below 100 μM, there was low risk of LMW generation; a free thiol level of 100–200 μM posed a potential risk; and free thiol level above 200 μM was a high risk for LMW generation. It is worth noting that Ellman’s reagent (2,2′-dinitro-5,5′-dithiobenzoic acid; DTNB) reacts with sulfhydryl groups both from small sulfhydryl-containing molecules, like reduced glutathione, cysteine and lipoic acid, in addition to the free sulfhydryl groups in proteins. Thus the baseline level of free thiol level depends on cell lines, culture media composition (small sulfhydryl-containing molecules) and cell viability. Nevertheless, when the reduction of disulfide bonds occurred, the free thiol level tended to be much higher than baseline.

The level of free thiol in a CB is very dynamic and pertains to the compositions and handling process. In addition to air exposure, other factors such as storage temperature and pH also contribute to the dynamics. Adjusting the pH of mAb 1 CB from 7 to 4.8, followed by the removal of cells by depth filtration led to a small decrease in free thiol level and percentage
of LMW that was observed (Figure 1B and 1C). When the cell harvest was treated with a combination of pH 4.8 and dextran sulfate (0.05 g/L and mixing for 60 minutes followed by centrifugation and filtration), the free thiol level dropped significantly, consistent with the observation that flocculation removes host cell proteins, including Trx/TrxR and Glu/GR and may also remove some small sulfhydryl-containing molecules. The level of free thiol also varied with temperature. Incubation of the control CB at 37°C for 75 min caused a 60% drop of free thiol content. However, if 1 mM of NADPH, a necessary component for the TrxR and GR reactions, was added to the solutions before 37°C incubation, an increase of free thiol level was seen in the control and pH adjusted CB (Figure 1A). These dynamic changes suggest that the free thiol level should be monitored in the pre- to post-harvest steps in order to better forecast the risk of LMW generation.

**Using redox indicators to predict disulfide bond reduction**

Since the disulfide bond reduction is a redox reaction, it is possible to use redox indicators as a simple, rapid and robust way to replace the DTNB test and to forecast the potential risk of LMW formation during harvest and recovery. A redox indicator undergoes a definite color change at a specific electrode potential in a similar way as pH indicators undergo a color change at a specific pH. To find the appropriate redox indicator, an array of commercially available redox indicators were tested, and several were identified as the best potential candidates that can be used in biologics manufacturing process.

An example of a redox indicator is 2,6-dichlorophenolindophenol (DCPIP, Figure 2A). Its color changed in mAb 2 purified DS, which had high percentage of LMW (Figure 2B), and its color can be completely or partially or not changed in CB with high, medium and low level of free thiols, respectively (Figure 2C). To determine the DCPIP color change range, the DCPIP stock solution was added to a series dilution of mAb 2 cell lysates with known free thiol concentrations (Figure 2D). DCPIP underwent a color change in mAb 2 CB with 3–5% cell lysate with known free thiol concentrations of 80–100 μM range. Therefore, when a CB sample had a free thiol concentration higher than 100 μM, the DCPIP changed color and this feature makes DCPIP a rapid and easy-to-use method to forecast the risk of disulfide bond reduction during manufacturing.

A study was designed to examine if the color change in DCPIP correlated with the free thiol levels and was able to correctly predict the generation of LMW (Figure 2E). The mAb 3 CB was generated by depth filtration with air or with nitrogen gas flushing of the filter train prior to the processing. The nitrogen gas flushing was used to generate an “airless” condition where there was no oxygen exposed to the fluid during the harvest process. The resulting CB was aliquoted into small containers with 0, 20, 50 and 100% air overlay headspace volume to liquid volume and
incubated at 4°C, 24°C and 37°C for one day. As shown in Table 1, the presence of air during harvest and recovery was critical for prevention of LMW generation. In this case, the starting CB had a lower free thiol concentration (51 μM) compared to the airless condition (207 μM) and the DO level was sufficient (>37%) to suppress LMW generation even during holding conditions of percent air overlay and at different temperatures. In contrast, the % air overlay during holding became important for the CB generated in the airless condition. The worst case was an airless harvest and airless holding, where the intact mAb monomer decreased from 96.5% to 81.6%. In addition, when the air overlay was 20% or more, disulfide reduction did not occur.

Incubation temperature had a different effect on free thiol concentration. The highest free thiol concentrations and maximal reduction occurred at 24°C holding. The free thiol amount after 4°C holding was close to the amount before holding. Holding at 37°C was presumed to be the optimal temperature for TrxR and GR enzymatic activity. However it universally caused a decrease in the free thiol amounts across all conditions, and the data was consistent with mAb1 results as shown in Figure 1.

At the end of the 1-day holding study, each sample was mixed with DCPPIP stock solution. The color change was assessed by visual observation (Figure 2F). Table 1 showed that the sequence of color change had strong correlation with free thiol concentrations; the higher the free thiol concentration, the faster the DCPPIP color change occurred. All the samples that were harvested in aerated conditions did not show any color change.

Additional redox indicators with different standard reduction potentials were also tested in a similar way, thionine and methylene blue were found to change color at 600–800 and >1,000 μM free thiol levels (data not shown), respectively. These dyes can be put together to comprise a ladder of indicators to rapidly forecast the level of risk during manufacturing.

### A scale-down model for recapitulating disulfide bond reduction during harvest and primary recovery

A large body of evidence suggests that disulfide bond reduction is correlated with cell lysis during the harvest operation and with the CB being held in closed containers (e.g. disposable bags) without a sufficient amount of air at room temperature over time. Accordingly, a small scale model was developed to mimic this phenomenon by filling a small amount of CB (10 to 300 mL) in a sealable container and generating airless conditions by flushing with nitrogen gas and holding at room temperature followed by NR_Caliper analysis for LMW (Figure 3).

Since CB from manufacturing operation may vary from lot to lot in term of cell damage and in holding time at various steps from pre-harvest to post-harvest, the LMW content can vary over a wide range from a few percent to near 100%. To simulate small-scale studies, a worst case scenario (described in Materials & Methods) was generated in which cellular lysate from CHO cells was mixed with the supernatant from the

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**Figure 2.** Using redox indicator DCPPIP treatment and observation of color change as a forecasting marker for LMW generation. (A) Structure and reaction of DCPPIP. (B) mAb2 purified DS samples on the left with > 95% LMW (3.1 sulfhydryl groups per antibody protein), showed colorless when treated with DCPPIP. mAb2 samples on the right with over 98% intact antibody (0.4 sulphydryl per antibody protein) showed purple color (in pH 5.5) in the presence of DCPPIP. (C) Color change of DCPPIP in CB of mAb 2 from different culture conditions. DCPPIP on the left two tubes became completely colorless, indicating higher reducing potential existed the CB; DCPPIP in the middle two tubes had blue color, indicating no reducing events; and DCPPIP on the right tube became partially reduced. (D) Free thiol concentrations as a function of mAb 2 percentage of cell lysis. The 100% cell lysate of mAb 2 was mixed with regular CB to generate a dilution curve of cell lysates and free thiol concentrations were measured and reported as the average of duplicate samples. Linear regression equation and R square were calculated with Excel. (E) Study design to test the order of color change of DCPPIP and free thiol amount. (F) Image of DCPPIP test results of the study in Table 1. Samples on top panel received air before and during primary recovery. Samples at bottom had nitrogen gas flushing before and during primary recovery. The order of test tubes from left to right were in the same order as in Table 1 from top to bottom.
original volume of cell culture to simulate 100% cell lysis and resulted in generation of LMW even in the presence of air (Figure 4A), filled into a sealable container, flushed with nitrogen gas and incubated at room temperature for 1 day. Under these conditions, all the intact mAb 2 antibody molecules in CB became completely fragmented (Figures 4B and

Table 1. Free thiol concentrations and DCPIP color change in mAb 3 CB generated and held at various air and airless conditions.

| Air exposure during harvest (Y/N) | Air overlay amount (% of liquid) | Storage temperature | % Air saturation | % CO2 saturation | Free SH group concentration (µM) | DCPIP color change | NR Caliper % monomer |
|-----------------------------------|----------------------------------|---------------------|-----------------|-----------------|---------------------------------|-------------------|-------------------|
| Y                                 | NA                              | 0                   | NA              | NA              | 51                              | NA                | 96.5              |
| N                                 | NA                              | 0                   | NA              | NA              | 247                             | NA                | 96.5              |
| Y                                 | 0                               | 37                  | 37              | 6.4             | 36                              | Blue              | 95.1              |
| Y                                 | 0                               | 24                  | 91.3            | 7.1             | 42                              | Blue              | 96.3              |
| Y                                 | 0                               | 4                   | 100             | 7.5             | 46                              | Blue              | 96.3              |
| Y                                 | 20                              | 37                  | 77.8            | 6.1             | 36                              | Blue              | 94.9              |
| Y                                 | 20                              | 24                  | 100             | 6.4             | 46                              | Blue              | 96.4              |
| Y                                 | 20                              | 4                   | 100             | 6.9             | 47                              | Blue              | 96.6              |
| Y                                 | 50                              | 37                  | 84.1            | 5.3             | 38                              | Blue              | 95.3              |
| Y                                 | 50                              | 24                  | 100             | 6.3             | 47                              | Blue              | 96.3              |
| Y                                 | 50                              | 4                   | 100             | 6.9             | 49                              | Blue              | 96.5              |
| Y                                 | 100                             | 37                  | 96.1            | 4.7             | 34                              | Blue              | 94.9              |
| Y                                 | 100                             | 24                  | 100             | 5.4             | 70                              | Blue              | 95.8              |
| Y                                 | 100                             | 4                   | 100             | 6.2             | 49                              | Blue              | 96.2              |
| N                                 | 0                               | 37                  | 38.2            | 6.4             | 38                              | Blue              | 96.2              |
| N                                 | 0                               | 24                  | 14.7            | 9.7             | 135                             | Colorless          | 81.6              |
| N                                 | 0                               | 4                   | 36              | 7.3             | 171                             | Colorless          | 96.3              |
| N                                 | 20                              | 37                  | 70.1            | 5.5             | 34                              | Blue              | 95.4              |
| N                                 | 20                              | 24                  | 62.9            | 7.5             | 84                              | Partial change     | 96.7              |
| N                                 | 20                              | 4                   | 92.9            | 6.9             | 121                             | Colorless          | 96.6              |
| N                                 | 50                              | 37                  | 85.5            | 4.7             | 33                              | Blue              | 95.7              |
| N                                 | 50                              | 24                  | 86.3            | 6.6             | 83                              | Partial change     | 96.1              |
| N                                 | 50                              | 4                   | 100             | 6.2             | 133                             | Colorless          | 96.8              |
| N                                 | 100                             | 37                  | 95.4            | 4               | 34                              | Blue              | 95.6              |
| N                                 | 100                             | 24                  | 96.6            | 5.4             | 81                              | Partial change     | 96.5              |
| N                                 | 100                             | 4                   | 100             | 5.3             | 138                             | Colorless          | 96.2              |

NA: Not applied.

Figure 3. Using hydrogen peroxide to prevent disulfide bond reduction. The mAb 2 lab scale generated CB was aliquoted into containers with various concentrations of hydrogen peroxide. Airless condition inside containers was generated by nitrogen flushing and was held at room temperature for one day. The resulting samples were analyzed directly by non-reduced (A, C, E, G, I) and reduced (B, D, F, H, J) Caliper without Protein A purification. (A and B) CB with no addition of hydrogen peroxide was exposed to air as control. CB with 0 mM (C and D), 0.33 mM (E and F), 1 mM (G and H), and 3 mM (I and J) hydrogen peroxide was held under airless condition. (K) Summary of non-reduced Caliper results of mAb fragmentation. Since there was excess light chain of this mAb secreted from host cells. The amount of light chain was not included as a fragment. Abbreviations used for LMW species: LC, light chain; HC, heavy chain; HL, half antibody with one light chain and one heavy chain; HHL, partial antibody with one light chain and two heavy chains.
The free thiol content in the 100% cell lysis CB was measured at 1 to 3 mM, which was at least 10 times higher than those from normal operation.

**Hydrogen peroxide can inhibit the reduction of disulfide bonds**

One way to control the reduction of disulfide bonds in mAbs is to eliminate the reducing potential contained in the CB before mAbs are reduced. The use of H$_2$O$_2$ for this purpose was tested. As shown in Figure 3, the control mAb 2 CB generated about 20% LMW after being held in airless conditions at room temperature for 1 day (Figure 3C). Hydrogen peroxide was added to the same CB at concentrations of 0.33, 1 and 3 mM prior to airless holding. Results showed that 3 mM (about 0.01%) hydrogen peroxide can completely prevent the generation of LMW (Figure 3E, 3G and 3I). These results strongly suggest that hydrogen peroxide effectively prevents disulfide bond reduction.

To determine the minimum concentration of H$_2$O$_2$ needed to inhibit LMW generation in the worst case, the above described 100% lysed cell culture model was used. In this study, the 100% lysed cell culture was held in airless conditions in the presence of 0, 5 or 10 mM H$_2$O$_2$. After 1 day holding, no intact mAb2 antibody molecules remained and the LMW species were dominated by light and heavy chains with a minor fraction of halfmer, suggesting that the reduction was almost complete as compared to the control sample that was exposed to air (Figures 4A and 4B). Adding 5 mM and 10 mM of H$_2$O$_2$ can partially (Figure 4C) and completely (Figure 4D) inhibit LMW generation, respectively. These NR_Caliper results from unpurified CB were summarized in Figure 4E. After Protein A purification, the samples with 0, 5 and 10 mM H$_2$O$_2$ had 0, 10.1 and 98.3% pure antibody monomer, respectively, from NR_caliper results (Figure 4F). The sample that was exposed in air had 83% pure antibody monomer. These results confirm that 10 mM H$_2$O$_2$ can effectively prevent mAb reduction in the worst case of CB processing.

There are many peroxide-containing compounds, inorganic or organic, that can be substituted for hydrogen peroxide. Two inorganic forms, sodium percarbonate and sodium perborate, were tested as a substitute for hydrogen peroxide. The results show that both peroxides can effectively suppress the reduction of disulfide bonds in a concentration-dependent manner (Figure 5).

**Hydrogen peroxide treatment and oxidation of antibody molecules**

One major concern of using hydrogen peroxide is its potential to oxidize the mAbs. Methionine residues are most susceptible to hydrogen peroxide oxidation.\(^23\), \(^24\) The mAbs 2 and 3 (belonging to IgG4 and IgG1 subclasses, respectively) were analyzed by mass spectrometry for methionine oxidation after various hydrogen peroxide treatments. For both molecules, after addition of H$_2$O$_2$ (1 mM to 10 mM) and incubation at room

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**Figure 4.** Evaluation of using hydrogen peroxide to prevent disulfide bond reduction in worst case. The tested mAb 2 CB had 100% cell lysis as described in Materials and Methods. The holding conditions with or without air were the same as in Figure 3. (A) CB sample without hydrogen peroxide treatment and held with air serves as a control. (B) CB sample without hydrogen peroxide treatment and held in airless conditions. (C) 5 mM hydrogen peroxide was added to CB sample before holding in airless condition. (C) 10 mM hydrogen peroxide was added to CB sample before holding in airless condition. The resulting CB samples (A to D) were analyzed directly by non-reduced Caliper without Protein A purification. (E) Summary of non-reduced Caliper results of mAb fragmentation from unpurified CB. (F) Summary intact antibody purities from samples A to D purified by Protein A chromatography and analyzed by non-reduced Caliper.
temperature for 1 day, a small increase in oxidation that was
dependent on H$_2$O$_2$ concentration was observed in the same
lot of samples that were under the described hydrogen peroxide
treatments (Tables 2 and 3). However, the H$_2$O$_2$-induced oxi-
dation changes were insignifi-
cant for both mAb 2 and 3, as the
tryptic peptide mapping and LC–MS/MS method has a 1% var-
iation. These results suggest that, under optimal peroxide con-
centrations, mAb oxidation mediated by hydrogen peroxide or
other peroxides shows no significant increase, and the level of
oxidation can be controlled.

**Effects of hydrogen peroxide treatment on product quality attributes**

In addition to oxidation, the potential effects of hydrogen per-
oxide on other DS quality attributes and downstream purifi-
cation process were also evaluated in a lab-scale study. The
experimental design included both mAb 2 (an IgG4) and mAb 4 (an IgG1). For mAb 4, the hydrogen peroxide was added to
pre-harvest cell culture fluid and post-harvest CB (without
cells). After various hydrogen peroxide treatments (see Material
and Methods section), the resulting CBs were processed
through Protein A chromatography, followed by low pH viral
inactivation and neutralization steps using center process point
conditions before analysis. The analytical results for mAb 4,
shown in Table 4, indicated there were no noticeable changes
in all the attributes that were tested between hydrogen peroxide
treated and untreated samples. Process impurities (host cell
proteins and residual DNA) were also comparable to the con-
trol and acceptable within the process range (data not shown).
Similar results were also obtained for mAb 2 that was treated
by 0, 3, 5 and 10 mM H$_2$O$_2$ at room temperature for 1 day
(Table 4). Therefore, our results suggest that hydrogen peroxide
treatments (up to 10 mM) have no noticeable changes in all the

![Figure 5. Using alternative peroxides to prevent antibody disulphide bond reduction. Sodium percarbonate and sodium perborate were used in worst case of mAb 2 CB with 100% cell lysis. (A) CB sample held with air without any peroxides. (B) CB sample held in airless condition without any peroxides. (C) A representative result of 5 mM sodium percarbonate-treated CB sample. The sodium percarbonate was added before holding in airless condition. Results came from non-reduced Caliper on unpurified CB (A to C). (D) Summary sodium percarbonate and sodium perborate treatment. The results came from non-reduced Caliper on unpurified CB.](image-url)

| Bioreactor | CB generation | holding | H$_2$O$_2$ (mM) | %H18ox (M252) | %H30ox (M358) | %H36ox (M428) |
|------------|---------------|---------|----------------|---------------|---------------|---------------|
| 1          | centrifuge    | No holding | 0              | 5.8           | 2.4           | 2.3           |
| 1          | centrifuge    | airless  | 0              | 6.1           | 2.7           | 2.3           |
| 1          | centrifuge    | airless  | 3              | 6.3           | 2.7           | 2.2           |
| 1          | centrifuge    | airless  | 5              | 6.3           | 2.8           | 2.3           |
| 1          | centrifuge    | airless  | 10             | 6.4           | 2.8           | 2.5           |
| 1          | depth filtration | air         | 0              | 5.4           | 2.5           | 2             |
| 1          | depth filtration | airless   | 0              | 5.6           | 2.6           | 2.1           |
| 1          | depth filtration | airless   | 5              | 5.8           | 2.7           | 2.2           |
| 1          | depth filtration | airless   | 10             | 5.9           | 2.7           | 2.2           |
| 2          | centrifuge    | air       | 0              | 3.7           | 2.7           | 4.3           |
| 2          | centrifuge    | airless   | 0              | 3.7           | 2.7           | 4.3           |
| 2          | centrifuge    | airless   | 5              | 3.7           | 2.8           | 4.7           |
| 2          | centrifuge    | airless   | 10             | 3.9           | 2.8           | 4.9           |
| 3          | centrifuge    | air       | 0              | 3.8           | 3.1           | 4.9           |
| 3          | centrifuge    | airless   | 0              | 3.9           | 3             | 5.3           |
attributes that were tested in samples treated with hydrogen peroxide before and after cell removal.

**Discussion**

The reduction of disulfide bonds during mAb manufacturing is an enzymatic redox reaction. The root cause of LMW formation can be traced to reducing potential changes that occur during the mAb manufacturing process, which are currently not well understood. Therefore, the appearance of LMW results from a combinatorial effect of cell culture conditions (temperature, DO, pH, viability, cell density, lysis percentage, etc.) that creates a reducing environment for the mAb. This is further exacerbated by the CB holding conditions (e.g., temperature, duration, aeration) that retain the mAb in the reducing environment. The detection of LMW is usually visualized in CB or Protein A eluate pool steps. Therefore, it would be ideal to have parameters that can rapidly predict the level of risk of LMW appearance at or after primary recovery process. Determination of the free thiol level with Ellman’s reagent (DTNB) is an ideal assay that correlates to the occurrence of LMW (Figure 1A). Additionally, the use of redox indicators as a replacement for the DTNB assay can be a simple and rapid method to forecast the risk level for LMW generation. It should be noted that the correlation between free thiol assay and NR Caliper results are non-linear, i.e., the percentage of LMW is not proportional to the free thiol concentrations in all ranges. The percentage of LMW shows relatively no change until the free thiol concentrations pass a threshold. Based on our observations, we propose that there is a low risk of LMW generation if free thiol level is under 100 μM; a free thiol level of 100–200 μM is a warning sign; and free thiol level above 200 μM is a high risk for LMW generation.

Many mitigation strategies for LMW generation have been proposed to either inhibit the enzymes involved in the redox reaction or to oxidize and deplete critical enzyme cofactors, such as NADPH. Air sparging has been shown as a robust way to prevent mAb reduction, and it is proposed to maintain a minimum of 30% DO in harvest cell culture fluid. However, the total free thiol level can range from micromolar level during manufacturing to millimolar level after holding the CB without air (Table 1 and Ref. 5). In our worst case CB condition, exposure to air cannot completely suppress LMW (Figure 4).

Complete decomposition of a 10 mM solution of H₂O₂ to water and oxygen can theoretically generate 5 mM oxygen, but this process is slow in the absence of catalysts. Therefore, the hydrogen peroxide added to CB is not expected to fully decompose. However, CHO cells express catalase, and the enzyme may help to accelerate the H₂O₂ decomposition process. Furthermore, hydrogen peroxide can be mixed with water in any ratio, and removal of excess hydrogen peroxide should not be a burden for downstream purification. In this study, we evaluated

| Table 3. Oxidation of mAb 3 methionine residues after hydrogen peroxide treatment. |
|---|---|---|---|---|---|---|---|
| Bioreactor | CB generation | holding | H₂O₂ (mM) | %H₃ox | %H₁₀ox | %H₁₂ox | %H₁₃ox | %H₂₂ox | %H₃₆ox | %H₄₂ox |
| 1 | Depth filtration | no holding | 0 | 0.5 | 1.2 | 0.9 | 1.2 | 1.3 | 1.9 | 0.7 |
| 2 | Centrifuge | airless | 0 | 0.5 | 1.3 | 1.1 | 1.1 | 1.4 | 2 | 0.7 |
| 3 | Depth filtration | airless | 2 | 0.5 | 1.3 | 1 | 1.2 | 1.4 | 2 | 0.7 |
| 4 | Depth filtration | airless | 3 | 0.5 | 1.3 | 1 | 1.2 | 1.5 | 2 | 0.7 |
| 5 | Depth filtration | airless | 5 | 0.5 | 1.4 | 1.1 | 1 | 1.5 | 2 | 0.7 |
| 6 | Centrifuge | airless | 10 | 2.9 | 0.7 | ND | 0.9 | 1.5 | 1.7 | 0.5 |
| 7 | Centrifuge | airless | 5 | 2.8 | 0.7 | ND | 1 | 1.9 | 1.7 | 0.5 |
| 8 | Centrifuge | airless | 3 | 2.9 | 0.8 | ND | 0.9 | 1.9 | 1.7 | 0.5 |
| 9 | Centrifuge | airless | 5 | 3.2 | 0.9 | ND | 1.3 | 1.6 | 1.8 | 0.5 |
| 10 | Centrifuge | airless | 10 | 3.6 | 0.9 | ND | 1.2 | 1.7 | 1.9 | 0.6 |

ND: Not determined.

For the mAb 4, the treatments were at either pre-harvest cell culture fluid or CB steps, the resulting CBs were gone through Protein A chromatography and low pH viral inactivation and neutralization (PAVIB) steps using process center point conditions. The analytical results came from PAVIB samples. For mAb 2, the hydrogen peroxide treated and control CB samples were purified by high-throughput Protein A columns before analysis. ND, not determined.

| Table 4. Hydrogen peroxide treatment on product quality attributes of mAbs 2 and 4. |
| --- | --- | --- | --- | --- | --- | --- |
| Molecule | Conditions | Purity (%) | Charge variants (%) | Intact mAb (%) | Protein A Step yield (%) |
| mAb4 | Centrifuged CB, 4°C, 4 days | Reduced | 100 | 96.3 | 50.5 | 46.5 | 2.9 | 95.5 | 4.4 | 0.1 | 94.2 |
| 10 mM H₂O₂ added to centrifuged CB, 4°C, 4 days | Non-Reduced | 100 | 96.5 | 49.6 | 47.4 | 3.0 | 95.7 | 4.2 | 0.1 | 94.4 |
| 10 mM H₂O₂ added to pre-harvest culture fluid, centrifuged, 4°C, 4 days | Main | 100 | 96.7 | 50.3 | 46.4 | 3.2 | 95.5 | 4.4 | 0.1 | 92.6 |
| 10 mM H₂O₂ added to pre-harvest culture fluid, centrifuged, 4°C, 3 days; N₂ flushing, RT for 1 day | Acidic | 100 | 96.5 | 49.5 | 47.4 | 3.1 | 95.4 | 4.5 | 0.1 | 98.4 |
| mAb2 | Centrifuged CB, N₂ flushing, RT, 1 day | Basic | ND | 99.2 | 49.0 | 18.0 | 33.1 | 98.8 | 1.2 | 0 | ND |
| 3 mM H₂O₂, N₂ flushing, RT, 1 day | Monomer | ND | 99.2 | 50.1 | 16.8 | 33.1 | 98.8 | 1.2 | 0 | ND |
| 5 mM H₂O₂, N₂ flushing, RT, 1 day | HMW | ND | 99.2 | 46.4 | 19.1 | 34.5 | 98.7 | 1.2 | 0 | ND |
| 10 mM H₂O₂, N₂ flushing, RT, 1 day | LMW | ND | 99.2 | 49.6 | 47.4 | 3.0 | 95.7 | 4.2 | 0.1 | 94.4 |

For the mAb 4, the treatments were at either pre-harvest cell culture fluid or CB steps, the resulting CBs were gone through Protein A chromatography and low pH viral inactivation and neutralization (PAVIB) steps using process center point conditions. The analytical results came from PAVIB samples. For mAb 2, the hydrogen peroxide treated and control CB samples were purified by high-throughput Protein A columns before analysis. ND, not determined.
the use of hydrogen peroxide to prevent antibody disulfide bond reduction during harvest and primary recovery. Our results strongly suggest that hydrogen peroxide, or alternative peroxides inorganic or organic such as sodium percarbonate or sodium perborate, can effectively prevent antibody disulfide bond reduction, potentially due to depletion of the reducing agents that cause antibody reduction.

Hydrogen peroxide is a form of reactive oxygen species. Indeed, hydrogen peroxide can oxidize small molecule reducing agents as well as DS if excess peroxide exists. The redox potential of a protein or a redox-active small molecule is often expressed as an electrochemical potential (Ep) using the Nernst equation. By comparing the relative redox potentials of proteins and other redox-active molecules, it is possible to predict the likelihood of redox reaction order for hydrogen peroxide with different reducing agents that may be present in the CB harvest. The order is expected to be NADPH (Ep = -0.32 Volts), lipoic acid (Ep = -0.29 Volts), thioredoxin (Ep = -0.28 Volts) glutathione (Ep = -0.23 Volts) before the oxidation of protein cysteine and methionine (Ep = -0.16 Volts) residues. The oxidation mechanism of glutathione (GSH) and cysteine (CSH) by hydrogen peroxide indicates that glutathione disulfide (GSSG) is the main product in neutral pH and at a low molar ratio of hydrogen peroxide to GSH. At increased concentration ratios of hydrogen peroxide to CSH from 1:1000 to 1:1, the oxidized product changes from cystine (CSSC) to cysteine sulfonic acid (CSO2H) to cysteine sulfonic acid (CSO3H). These findings suggest that optimization of the hydrogen peroxide concentration is critical to control the final outcome of the redox reaction.

In this study, we optimize the range of hydrogen peroxide concentrations to prevent antibody disulfide bond reduction and to minimize oxidation. Depending on the conditions of the clarified harvest bulk, 3 mM concentration of hydrogen peroxide is sufficient to keep antibody intact in a CB harvested in airless conditions (Figure 3), but higher concentrations of hydrogen peroxide (up to 10 mM) may be needed to prevent the mAb reduction from low cell viability/high cell density lots. In the two antibody molecules that we tested, one IgG1 and one IgG4, no significant oxidation of methionine residues was found under test conditions. It should be noted that the oxidation of methionine residues is molecule-specific based on the location of the methionine residues in the protein structure, medium compositions, culture conditions, and final concentration of hydrogen peroxide.

Materials And Methods

Protein stocks

Two IgG4 molecules (1 and 2) and two IgG1 molecules (3 and 4) were used in this study. The IgG4 molecules had a single point mutation of serine to proline in the hinge region motif CPSC of original IgG4 to resemble the IgG1 inter-chain disulfide bond structure CPCC. All the mAbs were produced in CHO cell culture with cell viabilities ranging from 50 to 90%. The CB was usually generated with depth filtration unless otherwise specified, in which case it was generated by centrifugation. The mAb 1 cell culture in some cases was treated with low pH plus dextran sulfate before depth filtration. Downstream purification was achieved by Protein A chromatography, additional polishing chromatographic steps and a final ultrafiltration/diafiltration step into a histidine-sugar-based buffer. Protein concentrations were determined by absorption at 280 nm (A280) by a Drosense-96 spectrometer (Trinean NV, 9050 Gent, Belgium).

Free sulfhydryl content (thiol) assay

The method used DTNB with slight modification from the literature. Briefly, 10 μL sample of CB or DS was mixed with 15 μL of TE buffer (50 mM Tris-HCl, 20 mM EDTA, pH 7.6) in a 96-well plate. DTNB solution (5 mg/ml in ethanol; Sigma-Aldrich, D8130) was freshly mixed with 8 M guanidine-HCl in 0.2 M Tris (pH 8) at volume ratio of 1:9 before use. 100 μL of the DTNB/guanidine solution was added to each well and mixed. Duplicate samples were tested. Spectral absorption at 412 nm was measured and a molar extinction coefficient value of 13,600 M⁻¹ cm⁻¹ was used for the calculation of free thiol content.

Redox Indicator Assays

2, 6-dichlorophenolindophenol (D2932, Spectrum Chemical MFG Corp, Gardena, CA) was dissolved in water to make a fresh 1% stock solution. The oxidized form of 2, 6-dichlorophenolindophenol was blue at neutral pH and became colorless when reduced. In acidic pH, the oxidized form of 2, 6-dichlorophenolindophenol was purple and changed to colorless when reduced. In order to determine the DCPIP color range for reduced and non-reduced samples, 100% mAb 2 cell lysate (see below) was mixed with regular CB produced by centrifugation to generate a series of 0, 3, 5, 10, 20, 30, 40, 50, 60 and 70% dilutions of cell lysate. The free thiol content of each dilution was measured immediately and plotted as a function of the dilution (Figure 2D). DCPIP (10 μL) was added into the serial cell lysate dilutions (1 mL at room temperature) to determine which dilution showed color change by visual inspection within a duration of a few seconds to minutes.

Non-reduced and reduced Caliper

The LabChip GXII Touch HT System (PerkinElmer) was used for mAb purity analysis under both reducing (R_Caliper) and non-reducing (NR_Caliper) conditions according to the manufacturer’s recommendation. NR_Caliper was the most convenient, high throughput tool to separate and quantify the antibody fragments, including 2 heavy 1 light chains (HHL), one heavy one light chains (HL), heavy chain (HC) and light chain (LC). Size-exclusion chromatography was used to monitor intact mAb and high molecular weight (HMW) species.

Mass spectrometric measurement of antibody oxidation for mAb

Samples from mAbs 2 and 3 were reduced by dithiothreitol, alkylated by iodoacetamide and digested with trypsin. The tryptic digest was chromatographically separated using a Waters
ACQUITY UPLC system (Milford, MA U.S.A.) before being analyzed by Thermo Scientific Orbitrap Q-EXACTIVE™ PLUS mass spectrometer (Bremen, Germany). A Waters Acquity BEH C18 column (1.7 μm, 2.1 × 150 mm) was used for separation (at 40°C). A linear gradient of 2% to 80% mobile phase B over 110 mins was used to elute the peptides (mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile) at a flow rate of 0.2 mL/min.

The Q Exactive Plus mass spectrometer was operating in data-dependent mode to switch between MS and MS/MS acquisition. Ions were generated using a sheath gas flow rate of 40, an auxiliary gas flow rate of 10, a spray voltage of 3 kV, a capillary temperature of 275°C, and an S-Lens RF level of 60. Resolution was set at 70,000 (AGC target 3e6) and 17,500 (AGC target 1e5) for survey scans and MS/MS events, respectively. The dynamic exclusion duration of 10 s was used with a single repeat count. Relative quantitation was achieved by dividing the peak areas of oxidized peptides by the sum of native peptides and oxidized peptides in selected ion chromatograms of the survey scans.

Hydrogen peroxide and other peroxide treatment
A 30% stock of hydrogen peroxide (Sigma-Aldrich cat. 216763, density of 1.11 g/mL) was freshly diluted with water to 3% stock (v/v, equal to 980 mM) before use. The 3% stock was added to cell culture or CB to final concentrations of 0.1 to 20 mM as desired. To test the effect of hydrogen peroxide on antibody disulfide reduction, hydrogen peroxide stock was added to the CB at final concentrations of 0, 0.1 0.33, 1, 3, 5 10 or 20 mM.

Two inorganic chemicals containing hydrogen peroxide, sodium percarbonate (Sigma-Aldrich, Cat. 371432, 2Na₂CO₃·3H₂O₂) and sodium perborate (Sigma-Aldrich, Cat. 372862, NaBO₃·H₂O) were also tested. Both sodium percarbonate and sodium perborate undergo hydrolysis upon contact with water, producing hydrogen peroxide, and carbonate or borate, respectively.

Small-scale model of disulfide bond reduction
A 500 mL bottle with an aspirated cap (FlexBioSys, Cat FXB-500M-0005) was filled with 300 mL CB. Alternatively, a 50 mL BioProcess bag (ThermoFisher, Cat SH30658.11) was filled with 30 mL with CB. The container was connected to a pure nitrogen gas supply line and flushed with nitrogen gas (<5 psi) at room temperature for 1 to 2 hours, and then kept enclosed in an airless condition for 1 day. After the hold, the sample was analyzed by the free thiol assay immediately or mixed with iodoacetamide (final concentration 20 mM) before being analyzed by NR_Caliper.

To generate the worst case for antibody disulfide bond reduction, 100% cell disruption was artificially induced by mechanical disruption. The cell culture was centrifuged (500 g for 20 min), the cell pellet was resuspended in 1/10 of original volume in RIPA buffer (ThermoFisher, Cat 8990, 25 mM Tris, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% NP-40, pH 7.6) and lysed at room temperature for 30 minutes with shaking, then chilled on ice and passed through a tissue homogenizer at high speed for 3 min. It was assumed that these steps achieved total cell lysis. The lysed material was then added back to the centrifuged supernatant. This cell lysate was filled into 50 mL bags with or without hydrogen peroxide, then flushed with nitrogen gas and held in an airless condition at room temperature for 1 day as described above. After incubation, the cell lysate was centrifuged twice at 3,000 g for 60 minute at 4°C and passed a 0.2 micron filter and analyzed as described above.

Hydrogen peroxide treatment and its effect on product quality attributes
For mAb 4, the cell culture fluid from lab-scale bioreactors was used for the following two experiments. In the first experiment, CB was generated by centrifugation and filtration using a 0.2 micron filter. The resulting CB was either (a) stored at 4°C for 4 days as a control or (b) combined with hydrogen peroxide to a final concentration of 10 mM peroxide and stored at 4°C for 4 days. In the second experiment, hydrogen peroxide was added to the cell culture fluid (with cells), incubated at room temperature for 1 hour before centrifugation. The resulting CB was either (a) stored at 4°C for 4 days as a control or (b) placed at room temperature on the third day and flushed with nitrogen gas, and held at room temperature for another day.

All the resulting samples were processed through Protein A chromatography followed by low pH viral inactivation and neutralization steps using process center point conditions. Product quality attributes were evaluated by size-exclusion ultra-performance liquid chromatography to analyze intact antibody and aggregate levels, by imaged capillary isoelectric focusing for charge variants distribution, and by Caliper under both reduced and non-reduced conditions for purity. Process impurities were estimated by ELISA for host cell proteins and by qPCR for residual DNA.

For mAb 2, hydrogen peroxide was added to CB to a final concentration of 0, 3, 5 and 10 mM, then held at room temperature for 1 day under nitrogen gas flushing. The resulting samples were processed through high throughput Protein A chromatography before analysis.

Abbreviations

| Abbreviation | Description                  |
|--------------|------------------------------|
| CB           | clarified bulk               |
| DCPIP        | 2,6-dichlorophenolindophenol |
| DO           | dissolved oxygen             |
| DS           | drug substance               |
| DTNB         | 2,2'-dinitro-5,5'-dithiobenzoic acid |
| LMW          | low molecular weight         |
| mAbs         | monoclonal antibodies        |

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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References

1. Mun M, Khoo S, Do Minh A, Drnovick J, Trexler-Schmidt M, Kao YH, Laird MW. Air sparging for prevention of antibody disulfide bond reduction in harvested CHO cell culture fluid. Biotechnol Bioeng. 2015;112:734–742. doi:10.1002/bit.25495

2. Ruaudel J, Bertschinger M, Letestu S, Giovannini R, Wassmann P, Moretti P. Antibody disulfide bond reduction during process development: Insights using a scale-down model process. BMC Proceedings. 2015;9(Suppl 9):P24. doi:10.1186/1753-6561-9-S9-P24

3. Mullan B, Dravis B, Lim Á, Clarke A, Janes S, Lambooy P, Lambooy P, Olson D, O’Riordan T, Ricart, B, et al. Disulphide bond reduction of a therapeutic monoclonal antibody during cell culture manufacturing operations. BMC Proc. 2011;5(Suppl 8):P110. doi:10.1186/1753-6561-5-S8-P110

4. Trexler-Schmidt M, Sargis S, Chiu J, Sze-Khoo S, Mun M, Kao YH, Laird MW. Identification and prevention of antibody disulfide bond reduction during cell culture manufacturing. Biotechnol Bioeng. 2010;106:452–461.

5. Kao YH, Hewitt DP, Trexler-Schmidt M, Laird MW. Mechanism of antibody reduction in cell culture production processes. Biotechnol Bioeng. 2010;107:622–632. doi:10.1002/bit.22848

6. Hutterer KM, Hong RW, Lull J, Zhao X, Wang T, Le M E, Borisov O, Piper R, Liu, YD, et al. Monoclonal antibody disulfide reduction during manufacturing: Untangling process effects from product effects. Mabs. 2013;5:608–613. doi:10.4161/mabs.24725

7. Koterba, KL, Borgschulte T Laird MW. Thioredoxin I is responsible for antibody disulfide reduction in CHO cell culture. J Biotechnol. 2012;157:261–267. doi:10.1016/j.jbiotec.2011.11.009

8. Handlogten MW, Zhu M, Ahuja S. Glutathione and thioredoxin systems contribute to recombinant monoclonal antibody interchain disulfide bond reduction during bioprocessing. Biotechnol Bioeng. 2017;114:1469–1477. doi:10.1002/bit.26278

9. Hutchinson N, Bingham N, Murrell N, Farid S, Hoare M. Shear stress analysis of mammalian cell suspensions for prediction of industrial centrifugation and its verification. Biotechnol Bioeng. 2006;95:483–491. doi:10.1002/bit.21029

10. Chung WK, Russell B, Yang Y, Hudak S, Cao M, Wang J, Robbins D, Ahuja S, Zhu M. Effects of antibody disulfide bond reduction on purification process performance and final drug substance stability. Biotechnol Bioeng. 2017;114:1264–1274. doi:10.1002/bit.26265

11. Liu H, May K. Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function. Mabs. 2012;4:17–23. doi:10.4161/mabs.1.4.18374

12. Chaderjian WB, Chin ET, Harris RJ, Echeverry TM. Effect of copper sulfate on performance of a serum-free CHO cell culture process and the level of free thiol in the recombinant antibody expressed. Biotechnol Prog. 2005;21:500–503. doi:10.1021/bp0407920

13. Yung-Hsiang Kao, Michael W. Laird, Melody Trexler Schmidt, Rita L. Wong, Daniel P. Hewitt. Prevention of disulfide bond reduction during recombinant production of polypeptides. U.S. Patent. No. 8,574,869 B2, 2013.

14. Singh et al. Compositions and methods for antibody production. World International Property Organization. WO 2015/085003 A1. 2015.

15. Li WW, Heinzle J, Hachnel W. Site-specific binding of quinones to proteins through thiol addition and addition-elimination reactions. J Am Chem Soc. 2005;127:6140–6141. doi:10.1021/ja050974x

16. Hampton MB, Orrenius S. Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. FEBS Lett. 1997;414:552–556. doi:10.1016/S0014-5793(97)01068-5

17. Aebi H. Catalase in vitro. Methods Enzymol. 1984;105:121–126. doi:10.1016/0076-6879(84)50116-3

18. Sidorova MV, Molokoedov AS, Az’muko AA, Kudriavtseva EV, Krause E, Ovchinnikov MV, Bespalova ZhD. [Use of hydrogen peroxide for closing disulfide bridges in peptides]. Bioorg Khim. 2004;30:115–125.

19. Nowak C, Cheung KJ, Dellatore MS, Katiyar A, Bhat R, Sun J, Ponniah G, Neill A, Mason B, Beck A et al. Forced degradation of recombinant monoclonal antibodies: A practical guide. Mabs. 2017;9:1217–1230. doi:10.1080/19420862.2017.1368602

20. Aalberse RC, Schuurman J. IgG4 breaking the rules. Immunology. 2002;105:9–19. doi:10.1046/j.0019-2805.2001.01341.x

21. Arner ES, Zhong L, Holmgren A. Preparation and assay of mammalian thioredoxin and thioredoxin reductase. Methods Enzymol. 1999;300:226–239. doi:10.1016/S0076-6879(99)90012-9

22. Ruaudel J, Bertschinger M, Letestu S, Giovannini R, Wassmann P, Moretti P. Antibody disulfide bond reduction during process development: Insights using a scale-down model process. BMC Proceedings. 2015;9(9):24. doi:10.1186/1753-6561-9-S9-P24

23. Luo Q, Joubert MK, Stevenson R, Ketchum RR, Narhi LO, Wyppy J. Chemical modifications in therapeutic protein aggregates generated under different stress conditions. J Biol Chem. 2011;286:25134–25144. doi:10.1074/jbc.M111.160440

24. Stracke J, Emrich T, Rüger P, Schloothauer T, Kling L, Knaupp A, Hertenberger H, Wolfert A, Spick C, Lau W, et al. A novel approach to investigate the effect of methionine oxidation on pharmacokinetic properties of therapeutic antibodies. Mabs. 2014;6:1229–1242. doi:10.4161/mabs.29601

25. Keizer HG, van Rijn J, Pinedo HM, Joenje H. Effect of endogenous glutathione, superoxide dismutases, catalase, and glutathione peroxide on adriamycin tolerance of Chinese hamster ovary cells. Cancer Res. 1998;48:4493–4497.

26. Sevier CS, Kaiser CA. Formation and transfer of disulfide bonds in living cells. Nat Rev Mol Cell Biol. 2002;3:836–847. doi:10.1038/nrm9347

27. Miller KK, Weaver KH, Rabenstein DL. Oxidation/reduction potential of glutathione. J. Org. Chem. 1993;58:4144–4146. doi:10.1021/jo00067a060

28. Aslund F, Berndt KD, Holmgren A. Redox potentials of glutaredoxins and other thiol-disulfide oxidoreductases of the thioredoxin superfamily determined by direct protein–protein redox equilibria. J Biol Chem. 1997;272:30780–30786. doi:10.1074/jbc.272.49.30780

29. Kim G, Weiss SJ, Levine RL. Methionine oxidation and reduction in proteins. Biochim Biophys Acta. 2014;1840:901–905. doi:10.1016/j.bbadis.2013.04.038

30. Abedinzadeh ZG, Gardes-Albert M, Ferradin C. Kinetic study of the oxidation mechanism of glutathione by hydrogen peroxide in neutral aqueous medium. Can. J. Chem. 1989;67:1247–1255. doi:10.1139/v89-190

31. Luo D, Smith SW, Anderson BD. Kinetics and mechanism of the reaction of cysteine and hydrogen peroxide in aqueous solution. J Pharm Sci. 2005;94:304–316. doi:10.1021/jp020253