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REPORT

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On-column disulfide bond formation of monoclonal antibodies during Protein A chromatography eliminates low molecular weight species and rescues reduced antibodies

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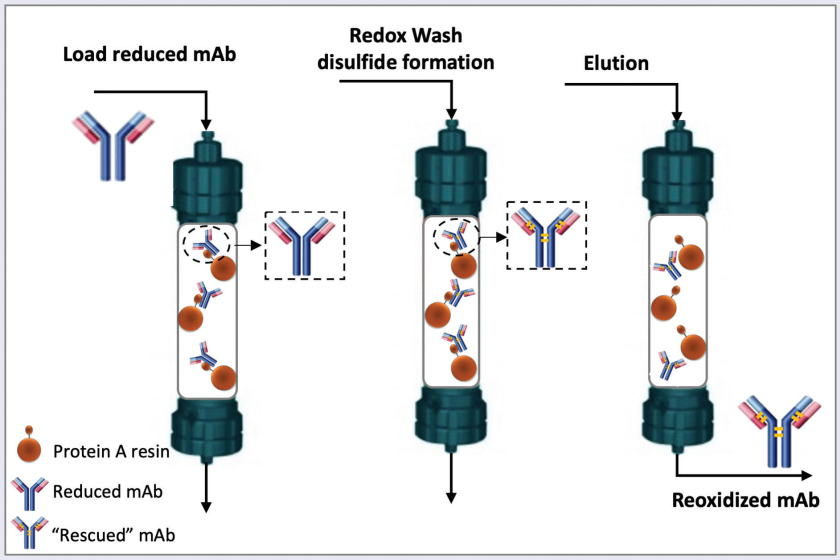
ABSTRACT

Disulfide bond reduction, which commonly occurs during monoclonal antibody (mAb) manufacturing processes, can result in a drug substance with high levels of low molecular weight (LMW) species that may fail release specifications because the drug's safety and the efficiency may be affected by the presence of this material. We previously studied disulfide reoxidation of mAbs and demonstrated that disulfide bonds could be reformed from the reduced antibody via redox reactions under an optimal redox condition on Protein A resin. The study here implements a redox system in a manufacturing setting to rescue the reduced mAb product and to further eliminate LMW issues in downstream processing. As such, we incorporate the optimized redox system as one of the wash buffers in Protein A chromatography to enable an on-column disulfide reoxidation to form intact antibody in vitro. Studies at laboratory scale (1 cm (ID) x 20 cm (Height), MabSelect SuRe LX) and pilot scale (30 cm (ID) x 20 cm (Height), MabSelect SuRe LX) were performed to demonstrate the effectiveness and robustness of disulfide formation with multiple mAbs using redox wash on Protein A columns. By applying this rescue strategy using ≤50 g/L-resin loading, the intact mAb purity was improved from <5% in the Protein A column load to >90% in the Protein A column elution with a product yield of >90%. Studies were also done to confirm that adding the redox wash has no negative impact on process yield or impurity removal or product quality. The rescued mAbs were confirmed to form complete interchain disulfide bonds, exhibiting comparable biophysical properties to the reference material. Furthermore, since the redox wash is followed by a bridging buffer wash before the final elution, no additional burden is involved in removing the redox components during the downstream steps. Due to its ease of implementation, significant product purity improvement, and minimal impact on other product quality attributes, we demonstrate that the on-column reoxidation using a redox system is a powerful, simple, and safe tool to recover reduced mAb during manufacturing. Moreover, the apparent benefits of using a high-pH redox wash may further drive the evolution of Protein A platform processes.



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Introduction

Recombinant monoclonal antibodies (mAbs) are the dominant biotherapeutics currently on the market due to their high specificity and long half-life.[1,2] During mAb process development, aggregates (high molecular weight (HMW)) and fragments (low molecular weight (LMW)) must be minimized due to their increased immunogenic risk and reduced efficacy.[3–5] These product variants also may present a risk to product stability during storage, resulting in shorter shelf life.[3–6] In recent years, with the development of high-titer mammalian cell culture processes, interchain disulfide bond reduction has been observed more often after cell culture harvest, resulting in additional product-related impurities.[6–8] Intracellular components, such as thioredoxin/thioredoxin reductase, have been shown to be largely responsible for disulfide bond reduction.[7–10] To ensure antibody product quality, manufacturing in-process controls are necessary to control LMW species formed from reduction of antibody disulfide bonds. As a result, several strategies across the industry have been proposed to control disulfide reduction in manufacturing, including maintaining harvest dissolved oxygen, chilling the harvested cell culture fluid (HCCF), and limiting the HCCF hold time.[7,8,11–13] Despite implementing active mitigation strategies to prevent disulfide reduction, occasional disulfide reduction can occur during a manufacturing run, resulting in out-of-specification batches and substantial increases in the cost of goods.

Due to the limitations of the above-mentioned mitigation strategies to completely prevent disulfide bond reduction, a method to rescue the "failed" batches (i.e., batches with high levels of LMW species) would be highly valuable. We thus posed the following question: is it possible to eliminate LMW issues through downstream process development? In our previous study, we gained an understanding of *in-vitro* disulfide bond reoxidation and optimized reoxidation conditions to achieve high antibody purity and high reaction rate on Protein A resin (Tang et al., mAbs, in press). A mathematical kinetic model was built to help predict the reoxidation rate and product purity. In fact, a large body of knowledge exists pertaining to *in-vitro* disulfide bond reformation.[14–20] While this prior knowledge provides the basis for use of reoxidation methods in antibody purification processes,[21,22] there are still many questions concerning process performance and product quality. For example, it remains to be seen whether the reoxidation method achieves acceptable product quality attributes, including biophysical, biochemical, and biological properties. Additionally, how implementation of the reoxidation wash might affect the robustness of a given manufacturing process is not known.

In this study, we examined a reoxidation strategy in the downstream process (specifically during the Protein A step) to rescue previously reduced mAbs. Protein A affinity chromatography has become the standard for antibody platform purification from cell culture medium and has been used widely.[23–28] By incorporating the redox system (cysteine/cystine pair) as a wash step during the capture chromatography, we provide an efficient way of producing intact mAb product from the reduced mAb via on-column disulfide reoxidation. The feasibility of integrating the redox wash in the Protein A platform method was assessed by evaluating whether the reoxidation reactivity was sufficient to achieve high product purity and by process performance, to achieve acceptable yield and impurity clearance. The robustness of the on-column reoxidation was assessed using multiple molecules, different load LMW levels, and various downstream process intermediates at bench and pilot scales. Biochemical and biophysical characterizations were performed to demonstrate comparability between the rescued mAb drug substance and the reference material.

Results

Proof of concept run of mAb disulfide re-formation on Protein A column

Previously we studied disulfide bond formation both in solution and on Protein A resin (MabSelect SuRe LX, the same Protein A resin was used in this study), and found that the disulfide formation rate on Protein A resin was higher than in solution (Tang et al., mAbs, in press). In that study, different factors (including cysteine concentration, cystine concentration, and pH) were taken into account to optimize the redox condition, and we found the optimal redox condition, composed of 1 mM cysteine and 0.3 mM cystine, pH 8–10, achieved high disulfide formation rate and high intact mAb recovery percentage. This optimal redox composition was applied to a Protein A column step with an expectation of reforming mAb disulfide bonds by implementing a wash buffer containing redox components. The reoxidation reaction time was determined by the contact time between the reduced mAb and the redox buffer, which was defined as the residence time plus static hold time (if applicable) of the redox wash buffer. In the first experiment, we used highly reduced mAb-1 sample (<10% intact) as the load material and applied a 1-hr wash using three wash conditions. As shown in Figure 1(a), the redox wash (1 mM cysteine +0.3 mM cystine, pH 8) achieved >90% intact mAb purity of the eluate, compared to ~75% purity and <10% purity when 1 mM cysteine buffer and phosphate-buffered saline (PBS) buffer were used as wash, respectively. This result demonstrated that the previously developed redox condition could be applied as a wash buffer during Protein A step to achieve high intact mAb purity (Tang et al., mAbs, in press).

We subsequently evaluated the dynamic binding capacity (DBC) of reduced mAb on Protein A resin in order to establish an appropriate column loading. MAb-2 material containing three different LMW levels (90%, 50%, and 1%) was used for the DBC study. At 10% breakthrough and 4-min residence time, we achieved DBCs of 58.6, 58.6, and 58.5 g/L_{resin}, respectively. The data seem to suggest that the DBC of the antibody is not affected by its reduction. This result is not surprising given that the main affinity interaction between antibody and Protein A resin is through the Fc region,[24,26] whose tertiary structure is not expected to be affected by the breakage of interchain disulfide bonds (Tang et al., mAbs, in press).[29–31]

Adaptation to platform process for mAb disulfide reformation on Protein A column

The disulfide bond formation study was carried out on Protein A chromatographic resin using the platform conditions shown in Figure 1(b), which includes successive washes after loading followed by predefined low pH elution. The bridging buffer (e.g., acetate buffer, pH 5.5) was used for a pH transition from the high pH wash 2 buffer to the low pH elution. The redox components were added into the wash 2 buffer. Figure 1(c–e) shows the schematic three-step representation of on-column disulfide bond formation on Protein A chromatography: 1) Load the reduced mAb onto Protein A column at a defined loading capacity; 2) Apply the optimal redox wash to promote on-column disulfide bond formation; and 3) Elute the reformed mAb from the column for subsequent downstream operations.

To adopt this wash step to the platform process, three mAbs (mAb-2, mAb-3, and mAb-4) in different IgG subclasses (as indicated in Table 1) were tested. The mAb drug substance containing different levels of LMW were diluted to ~5 g/L using PBS buffer and loaded onto Protein A column at 35 g/L_{resin} loading. By following the steps defined in Figure 1 (b), we carried out Protein A runs using either a PBS control wash or the pH 8 redox wash. The total mAb/redox contact time (dynamic plus static hold) for wash 2 was kept constant at 1 h. Figure 2 shows the mAb purity of Protein A load materials of the three aforementioned mAbs and their corresponding Protein A eluates. With the redox wash, the mAb purities were improved from as low as <5% in the load to >90% in the elution, while the mAb purities remained unchanged for the run with PBS control wash. This demonstrated that the redox wash was able to restore intact purities of all three mAbs tested.

Optimization of redox wash process parameters for Protein A step

Several factors can affect the purity of the antibody coming off the Protein A eluate, including Protein A loading amount, LMW level of mAb load, and mAb/redox buffer contact time. In order to optimize the process parameters around redox wash implementation, a full factorial design-of-experiment (DoE) approach was used to assess these factors. DoE is a statistical approach that is widely applied in mAb downstream process development. Generally, different aspects such as the number and type of the factors, existing information, and reliability of the results are taken into account in DoE designs. Thus, one set of the experiments can change several factors to evaluate their influences efficiently.[32–34] As illustrated in Table 2, antibody purities of 69% – 97% were achieved in the studied ranges of Protein A loading amount 10–50 g/L resin, LMW level of mAb load 10–90%, and mAb/redox contact time 15–60 minutes. Statistical analysis (Figure 3) showed that the resultant mAb purity was primarily affected by load LMW level (p = .004) and mAb/redox buffer contact time (p = .05). Protein A loading amount (p = .4) showed no statistical significance on mAb purity. Among these DoE runs (Table 2), consistent high mAb purity (>95%) was achieved with 10% load LMW (a very likely scenario with a mild reduction), regardless of conditions used for Protein A loading amount and mAb/redox buffer contact time. To further understand whether there is any potential compounding effect on disulfide formation due to redox-induced and naturally occurred causes, a process control (load material without redox components) was held at room temperature for the duration of the Protein A runs, and the mAb purity in the load remained unchanged (data not shown). This result demonstrated that, without the redox wash, mAb disulfide bonds cannot be regenerated efficiently over time, and the observed enhancements of mAb purity of the eluates in DoE study were primarily attributed to on-column disulfide formation. Load LMW level of mAb is determined by the operating conditions of bioreactor and harvest, while Protein A loading is limited by bioreactor mass and available Protein A column size. On the other hand, mAb/redox buffer contact time is more controllable in downstream operations and easy to implement. In a scenario where mAbs with monomeric content of 10% or greater, 60 min contact time is sufficient to increase mAb purity >90% based on DoE predictions (Figure 3). This redox reaction time is in agreement with the prediction in our previous kinetic model (Tang et al., mAbs, in press). In a process operation, a static hold following 3–4 column volumes of redox buffer wash (with total dynamic and static contact time >60 minutes) could be implemented to minimize buffer consumption (a sample of results is included in Table 1).

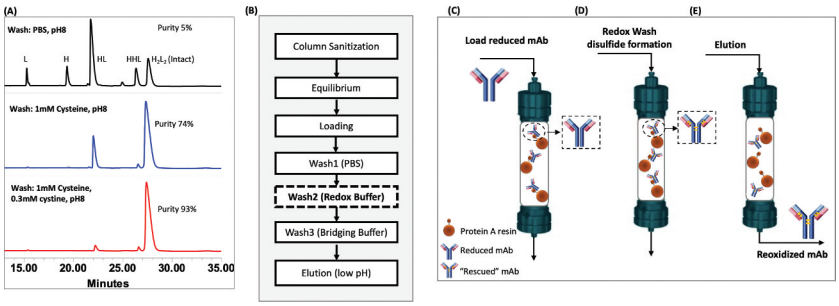

Figure 1. Protein A chromatography step and schematic of on-column disulfide bond formation using redox wash on Protein A chromatography. (a) The electropherograms of Protein A elution pools for mAb-1 that underwent PBS wash, 1 mM cysteine wash, and redox wash, respectively; (b) Integration of redox wash in platform Protein A step; (c) Load partially reduced mAb on the column; (d) Once the reduced mAb molecules are bound to the column, apply redox wash to enhance the interchain disulfide bond formation; (e) Use low pH elution buffer to elute the reoxidized mAb off the column followed by subsequent operations.

Table 1. Quantification of product quality attributes for reformed mAbs in Protein A chromatography using the optimized redox wash condition (1 mM cysteine, 0.3 mM cystine, pH10).

Sample Name	Subclass	Loading material	Load intact mAb (%)	Protein A run condition	Column Size (ID x H, cm)	Product contact time* (hr)	SEC HMW (%)	SEC Monomer (%)	SEC LMW (%)	CE-NR Intact mAb (%)
mAb-1	IgG4	PAE	19.8	Redox, 1-hr flow	1 x 20	1	4.0	96.0	0	92.0
		DS	12.6	Redox, 1-hr flow	1 x 20	1	1.0	98.7	0.3	96.1
		DS	12.6	Redox, 15 min flow + 45 min hold	1 x 20	1	1.0	98.7	0.3	95.9
mAb-2	IgG1	HCCF	76.7	Redox, 1-hr flow	1 x 20	1	3.7	96.3	0	92.8
		DS	72.1	Redox, 1-hr flow	1 x 20	1	1.0	98.9	0.1	97.1
		DS	72.1	Redox, 15 min flow + 45 min hold	1 x 20	1	1.0	98.9	0.1	97.1
mAb-3	IgG4	DS	5.6	Redox, 1-hr flow	1 x 20	1	N/A	N/A	N/A	90.9
mAb-4	IgG1	PAE	4.5	Redox, 2-hr flow	1 x 20	2	2.1	97.8	0.1	94.1
		PAE	4.5	Redox, 4-hr flow	1 x 20	4	1.9	98.0	0.1	96.4
		PAE	4.5	Redox, 4.5-hr flow	1 x 20	4.5	2.0	97.9	0.1	97.3
		PAE	4.5	Redox, 4.5-hr flow	30 x 20	4.5	2.1	97.8	0.1	97.2

* Product contact time was denoted as the time that the protein was contacted with the redox buffer

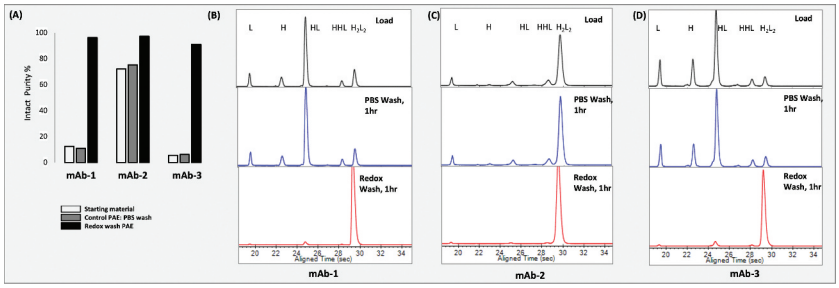

Figure 2. Product purity determined by non-reducing CE-SDS (CE-SDS NR) for partially reduced mAbs and their Protein A elution (PAE) pools by on-column disulfide bond reformation. (a) Intact purities of starting material, PAE of PBS wash, PAE of redox wash; (b) The size variant distributions of mAb-1 starting material, PAE using PBS wash, and PAE using redox wash; (c) The size variant distributions of mAb-2 starting material, PAE using PBS wash, and PAE using redox wash; (d) The size variant distributions of mAb-1 starting material, PAE using PBS wash, and PAE using redox wash.

Robustness demonstration of on-column disulfide formation

Although LMW formation due to disulfide reduction is typically caused by excessive reducing components released by cell lysis in cell culture harvest, LMW can be seen in subsequent downstream in-process pools at times, including the final drug substance. Also, as a critical quality attribute, LMW is usually analyzed in a quality control laboratory after drug substance is generated.[7] It is desirable to have a comprehensive LMW control strategy, including in-process monitoring of LMW and a rescue strategy, that is applicable to each in-process pools. To this end, various downstream in-process pools that contain reduced mAbs were evaluated using the redox wash regime.

Table 2. Design-of-experiment (DoE) results of optimization of on-column redox wash process parameters.

Run #	Starting LMW% in Load	mAb/Redox contact time (min)	Column Loading (g/L_{resin})	Eluate Purity %
1	90	60	50	82.5
2	50	37.5	37.5	90.6
3	90	60	25	88.4
4	10	60	50	97.6
5	10	15	50	95.9
6	90	15	50	59.4
7	50	37.5	37.5	89.2
8	10	60	25	96.7
9	10	15	25	96.6
10	90	15	25	69.4

Table 1 summarizes the antibody purity for four mAbs (mAb-1, mAb-2, mAb-3, and mAb-4) that were rescued through on-column disulfide reformation at bench scale and pilot scale. In addition to harvest, the load materials were also made available from Protein A pool and drug substance. With a wide range of LMW levels (4.5% to 51%) in the load, the >1-hr on-column reoxidation could yield intact mAb purity of >90% for all materials studied, demonstrating that this redox wash regime could be implemented as a rescue strategy to reprocess materials from various stages of downstream operation. While the "rescue strategy" in reprocessing the later in-process intermediates (e.g., reduced drug substance) is technically feasible, from a practical application perspective it has to take into consideration other aspects, such as manufacturing productivity, process economics, and potential regulatory concerns. Alternatively, it is desirable to avoid the reprocessing process by either eliminating the disulfide reduction in the first place or rescuing the reduced mAb in HCCF, which is further discussed in the next section.

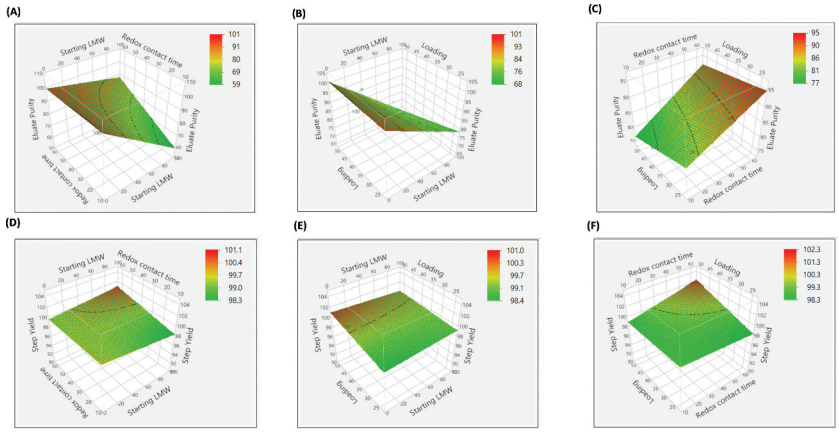

Figure 3. Evaluation of on-column disulfide bond formation using a Design-of-Experiment (DoE) including parameters: Loading amount, low molecular weight (LMW) in starting material, and redox contact time. (a) Elution purity versus Starting LMW and Redox contact time; (b) Elution purity versus Starting LMW and Loading; (c) Elution purity versus Redox contact time and Loading; (d) Step yield versus Starting LMW and Redox contact time; (e) Step yield versus Starting LMW and Loading; (f) Step yield versus Redox contact time and Loading.

Evolution of Protein A platform by integrating redox wash

Previous work demonstrated that an alkaline condition was favorable for disulfide bond formation (Tang et al., mAbs, in press). Meanwhile, a high-pH carbonate wash buffer (pH ≥9) has been demonstrated to effectively remove host cell protein (HCP) and DNA to reduce process burden in the subsequent polishing chromatographic steps.[35] Taken together, it is convenient to integrate the redox components into the high-pH buffer to achieve both high mAb purity and low process-related impurities in Protein A elution pool. Nevertheless, it is essential to assess the impact of the redox buffer on the overall Protein A performance, including product quality and process impurities (HCP and DNA).[36,37] Residual cysteine/cystine is not a concern since any residual redox components may be washed off by the wash buffer succeeding the redox wash.

Figure 4(a) illustrates a comprehensive study plan, in which the HCCFs of three mAbs (mAb-1, mAb-2, and mAb-3) were used for the study. Each HCCF was divided into two pools, which underwent two treatments and storage conditions: 1) air sparging + 4°C; and 2) nitrogen sparging + room temperature (19 ~ 25°C). The first condition, retaining the intact form of the mAb ("good HCCF"), was used to assess whether the redox buffer wash has any negative impact on the product quality (such as disulfide bond reduction and asparagine deamidation) and process impurity removal. The second condition, by generating partially reduced mAb ("bad HCCF"), was intended to demonstrate if redox buffer wash can effectively convert the LMW to intact mAb without sacrificing impurity removal capability.

Figure 4(b–e) shows product quality and process impurities using two Protein A wash arms for the three mAbs (mAb-1, mAb-3, and mAb-4) in both intact and reduced forms. The purity and HMW% of the Protein A pools from these three mAbs using different washes are presented in Figure 4(b,c), respectively. First, we observed that "good HCCFs" for all three mAbs maintained high mAb purity using both wash arms, suggesting that air sparging (oxidative condition) and chilled storage temperature of harvested bulk were able to prevent the disulfide bond reduction prior to Protein A step. Additionally, maintaining high mAb purity for wash arm 2 indicated that the redox wash did not pose a risk to the molecular integrity. In contrast, "bad HCCFs" using the control wash condition (arm 1: high pH without redox wash) showed low mAb purity (<50%) for all three mAbs. However, high purity product was achieved by using the redox wash (arm 2), demonstrating the effectiveness of redox wash to enhance the disulfide bond reformation on the Protein A column. In addition, comparable HMW levels among different washes demonstrated that the incorporation of the redox wash had no negative impact on protein aggregation.

The effects of the redox system on clearance of process-related impurities (HCPs and DNA) were also evaluated (Figure 4(d,e)). Although no distinguishable difference of HCP levels was seen for "good HCCFs" between the two wash arms, an overall lower HCP level was observed for Protein A elution (PAE) pools from "bad HCCFs." For both "good HCCFs" and "bad HCCFs" the residual DNA levels were lower using wash arms 2 across the three mAbs, suggesting that the redox wash may play some role in DNA removal.

Product quality attributes of rescued mAb

One concern emerging from this rescue strategy is whether the rescued intact mAb was comparable in terms of biochemical and biophysical properties to a typical reference mAb that was not subject to reduction/reoxidation. Multiple analytical techniques were used to characterize the rescued mAb product and compare to reference material. Table 3 summarizes quality attributes, analytical methods, and assessment results of the comparability study between reference material and the rescued mAb-4 drug substance generated at pilot scale using on-column reoxidation rescue strategy.

Size variants and charge variants

The size variants and charges variants of the rescued mAb-4 drug substance were analyzed and compared to the reference material. The size variants were determined by non-reduced capillary electrophoresis SDS (CE-SDS, NR) (Figure 5(a)) and

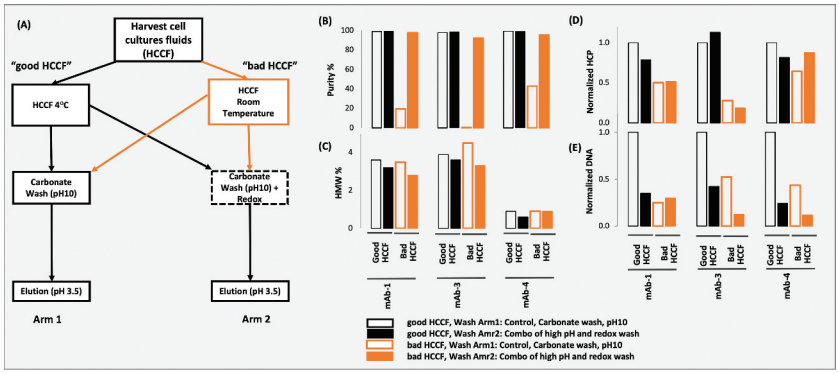

Figure 4. Comprehensive evaluation of using redox wash system in the platform Protein A chromatography. The study was performed using three mAb harvest cell cultures according to the design including two arms. The protein A pools from each run was tested for product quality attributes. (a) Comprehensive study: Arm 1, control; Arm 2, combined wash step; (b) Intact mAb impurity; (c) Aggregates; (d) Host cell proteins (HCP); (e) residual DNA.

size exclusion chromatography (SEC) (Figure 5(b)). Although the HMW% of the rescued mAb-4 determined by SEC was slightly higher than the reference material, the amount was still within the pre-defined range of ≤4%. The 3.6% LMW content of the rescued mAb-4 was slightly higher than the reference material (0.8%), but satisfied the specification of intact content ≥92%. Given that the load material consisted of <5% purity to start, it was a significant improvement for the rescued mAb-4 with >95% intact content. To assess the similarity of charge variants between the rescued mAb-4 and the reference material, imaged capillary isoelectric focusing (icIEF) analysis was performed. The distribution of charge variants in the rescued mAb-4 based on the side-by-side icIEF analysis was comparable to the reference material (Table 3 and Figure 5(c)). The cation-exchange chromatography- high-performance liquid chromatography (SEC-HPLC) analysis also revealed a high similarity of charge profile between the rescued material and the reference material (Supporting information).

Primary structure

Antibody primary structure was determined by intact mass and peptide mapping analysis. Intact mass analysis is the measurement of the molecular weight of whole protein. The molecular weight of the predominant species was determined by liquid chromatography (LC) and mass spectrometry (MS). Deconvoluted mass spectra of samples are shown in Figure 6. The predominant species correspond to the molecular weight of mAb-4 amino acid sequence plus the indicated N-linked

Table 3. Summarized Product Quality Attributes of the Reduced mAb, Rescued mAb, and Intact Reference Material (mAb-4).

Category	Analytical Methods	Quality Attributes		Reduced mAb	Rescued mAb	Reference Material
Primary Structure	Intact mass analysis under non-reducing conditions	Molecular weight (Da)	G0FG0F	-	147079	147082
			G0FG1F	-	147242	147245
			G0FG2F/G1FG1F	-	147404	147408
			G1F/G2F	-	147567	147570
	Peptide mapping by LC-MS	Methionine oxidation	Met253	-	3.6%	2.7%
			Met359	-	1.0%	0.8%
			Met434	-	1.9%	1.4%
	Peptide mapping by LC-MS	Deamidation	HC Asn316	-	2.2%	2.1%
			HC Asn326	-	4.9%	3.7%
			HC Asn385 and Asn390	-	7.7%	7.6%
	Peptide mapping under non-reducing condition	Disulfide linkage mapping		-	Highly similar, No disulfide scrambling	
	Elman's reagents	Free sulfhydryl group	Avg thiol/IgG	2.5	0.2	0.3
High-order Structure	Far- and near-UV CD	Protein secondary and tertiary structure		-	Highly similar	
	DSC	Thermodynamic stability	T_{m1}, T_{m2}, T_{m3}, T_{m4}	-	69.7, 77.5, 80.9, 87.0	69.7, 77.5, 80.9, 87.0
Size heterogeneity	SEC	High molecular weight	HMW	1.5%	2.0%	0.9%
			Monomer	98.2%	97.8%	98.4%
			LMW	0.3%	0.3%	0.7%
	Non-reducing CE-SDS	Low molecular weight		4.5%	3.6%	0.8%
Charge heterogeneity	CEX-HPLC, iclEF	Acidic and basic variants	Acidic	72.0%	46.3%	47.6%
			Main	26.5%	50.7%	49.2%
			Basic	1.5%	3.0%	3.2%
Fab-related biological activity	ELISA-based binding	Binding activity		-	107%	100%

glycans. The theoretical masses and observed masses of the predominant species obtained from LC-MS quadrupole time-of-flight intact mass analyses for mAb-4 samples are presented in Table 3, which shows that the measured molecular weight of the reformed mAb-4 and reference material are comparable.

Peptide mapping provides in-depth information about post-translational modifications of the primary sequence including methionine oxidation and asparagine deamidation (Table 3). Disulfide linkage was analyzed by disulfide mapping using trypsin digestion (Figure 7) and free thiol quantification (Table 3). No significant difference was observed between the rescued mAb-4 drug substance and reference material base on total ion chromatogram mass spectra. All predicted disulfide-bonded peptides were recovered in correspondence to their expected mass, which indicated the disulfide bonds were reformed correctly in mAb-4. This is further evident from the comparable free thiol levels of the reformed mAb-4 and the reference material (Table 3).

High-order structure

High-order structure and thermal stability were determined using circular dichroism (CD) and differential scanning calorimetry (DSC).[38] The overlaid spectra for the rescued mAb-4 and reference material for both far- and near-UV CD support high comparability in secondary and tertiary structures (Figure 5(d,e)). Protein thermal stability and associated structural transitions were determined by DSC, which measures changes in T_m values.[39–42] The overlaid thermograms exhibit four dominating peaks around 70°C (T_{m1}), 77.5°C (T_{m2}), 80.9 (T_{m3}), and 87°C (T_{m4}) (Table 3 and Figure 5(f)). The shapes of thermograms for the rescued mAb-4 and reference material indicate high comparability in thermal stability.

Enzyme-linked immunosorbent assay binding activity

The biological activity of the rescued mAb-4, determined by enzyme-linked immunosorbent assay, showed comparability to the reference material (Table 3).

Summary

Table 3 summarizes the properties of the rescued mAb-4 and its reference material determined by a number of analytical techniques. MAb-4 rescued through disulfide reformation is comparable to its reference material (i.e., mAb-4 not subjected to the rescue practice) based on the biochemical and biophysical testing performed.

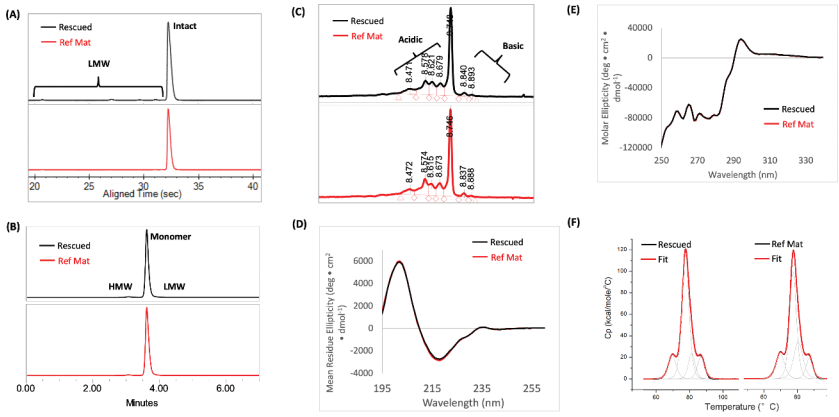

Figure 5. Comparison of the rescued mAb-4 drug substance (DS) and mAb-4 reference material. (a) Intact mAb purity determined by non-reduced CE-SDS (CE-SDS NR); (b) high molecular weight species (HMW), monomer and low molecular species (LWM) determined by size exclusion chromatography (SEC); (c) Charge variant profile determined by Imaged capillary isoelectric focusing (icIEF); (d) Secondary structure determined by far-UV circular dichroism (CD); (e) Tertiary structure determined by near-UV CD; (f) Thermal stability determined by differential scanning calorimetry (DSC).

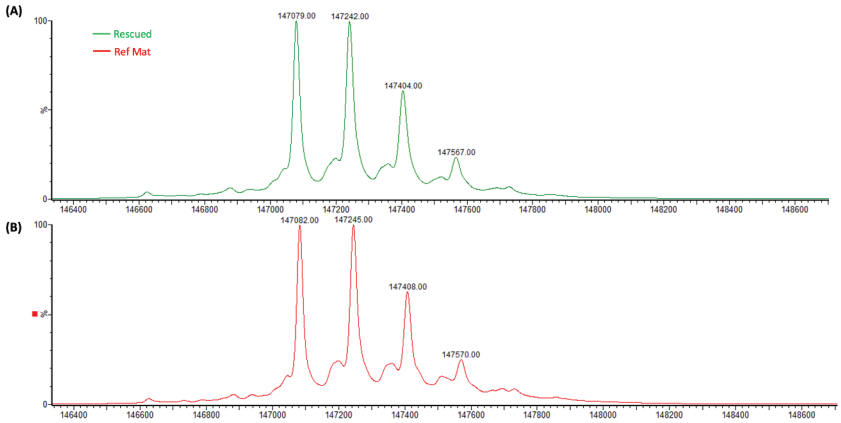

Figure 6. Comparison of intact mass profiles of the rescued mAb-4 and the reference material under non-reduced condition.

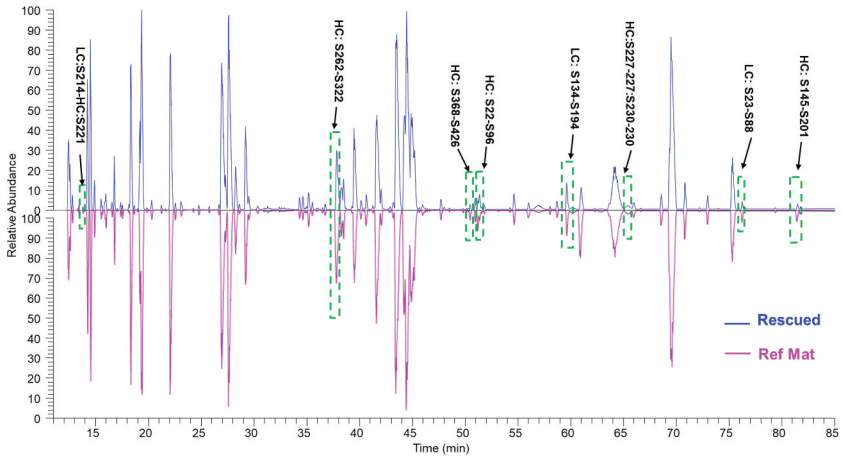

Figure 7. Comparison of Trypsin-digested peptide mapping profiles of the rescued mAb-4 and the reference material. Mirror images of base peak profiles from non-reduced disulfide bond mapping.

Discussion

In our previous study (Tang et al., mAbs, in press), we gained a fundamental understanding on reformation of interchain disulfide bonds and identified a fast way to generate intact mAb from the reduced form using a redox system on Protein A resin. Therefore, in this study, we directly applied the optimal redox condition established in test tubes to fit into a Protein A chromatography platform process. The feasibility, applicability, and manufacturability of using on-column reoxidation in manufacturing processes were demonstrated using multiple reduced mAbs and various downstream in-process samples. Using three mAbs in both reduced and native forms, the robustness of on-column reoxidation was evaluated holistically to achieve high product purity and acceptable process impurity removal capability.

The comparability study was performed to ensure safe implementation of this redox wash strategy in biologics manufacturing. We demonstrated that the rescued mAb-4 is comparable to its reference material based on all the biochemical and biophysical testing (Table 3). Yet, given that the nature of this application is to reoxidize the free sulfhydryl groups using a redox system at a high pH, close attention must be paid to particular product quality attributes, including primary sequence modifications (e.g., methionine oxidation, asparagine deamidation) and disulfide bond integrity, as they may influence protein stability, aggregation, and affinity.[6,43,44]

A reduced peptide map showed comparable oxidation and deamidation levels between the rescued mAb-4 and the reference material (Table 3), suggesting that the on-column disulfide reoxidation using the redox buffer has negligible impact on protein primary sequence and post-translational modification. Conversely, peptide mapping under a non-reduced condition revealed complete reformation of disulfide bonds for the recovered material, as was evident from the comparable base peak profile (Figure 7). All eight pairs of interchain disulfide bonds were fully identified. In addition, both the rescued mAb-4 and reference material showed 0.2–0.3 free thiols per mAb molecule,[11] consistent with observations for a typical drug substance. Relatively lower thiol observed for the rescued mAb-4 than the reduced mAb (2.5 free thiols per mAb) further confirmed the effectiveness of the on-column disulfide reoxidation.

Our rescue strategy was implemented in the Protein A chromatography platform by combining the optimized redox condition (1 mM cysteine, 0.3 mM cystine, pH 10) with a high-pH wash step. We demonstrated that the on-column redox wash has no negative impact on mAb product quality, but with additional benefit of recovering mAb product by reoxidizing the reduced mAb, if present. Furthermore, the DNA levels were significantly lower for samples that underwent redox wash, suggesting that the redox wash may potentially disrupt associations between DNA and mAb or DNA and Protein A resin. As both DNA and certain viruses are negatively charged at pH 9 ~ 10 condition,[45] this high pH + redox components may be helpful for virus removal in Protein A step as well, which warrants future study. Taken together, the benefits of product quality improvement and potential enhancement of impurity removal by using on-column redox wash may lead to further evolution of Protein A platform process. The benefits of adopting redox wash to platform operation include: 1) when there is disulfide bond reduction, "waste" reduced mAbs can be saved; 2) the risks of mAb disulfide bond reduction in downstream process steps are further lowered for the samples that originally do not have disulfide bond reduction; and 3) no extra equipment nor extra operation steps are required.

It is worth noting that the sulfhydryl group of cysteine is highly reactive and cysteine itself can form cystine through natural air oxidation.[27,46,47] Therefore, the stability of the redox buffer needs to be assessed to establish the expiry specification based on its reactivity. For this purpose, the redox buffer stored at room temperature and 2–8°C was used for on-column reoxidation study. It was demonstrated that the redox buffer stored at room temperature could maintain its reactivity for up to 3 weeks (data not shown).

While we have demonstrated disulfide formation on the Protein A column using MabSelect SuRe LX resin, which mainly binds the Fc domain of mAbs, it is possible that a different Protein A resin, which may bind both the Fc domain and V_H domain of mAb, will show different column

performance, including DBC and disulfide formation efficiency.[48,49] Additionally, it is critical to have an alternative resin in manufacturing with a demonstrated comparable performance to avoid any manufacturing disruption caused by a potential raw material supply shortage.[50] The possible mixture mode of binding of a different resin and mAb may cause a different molecular conformation and reoxidation behaviors (e.g., reaction kinetics and preferable pathways) that need further optimization of the redox condition. A comprehensive study is required to evaluate the alternative resin for the process performance and resultant mAb product quality.

In summary, our study has shown that the reduced mAb could be rescued to form intact mAb through on-column disulfide formation using a redox wash in Protein A chromatography. This novel approach is robust and offers a broad spectrum of benefits, including product quality improvement, impurity removal enhancement, and further evolution of Protein A platform. Furthermore, this rescue strategy would achieve significant cost savings by avoiding production of additional batches. Using this rescue strategy, we successfully recovered >800 g mAb-4 drug substance at Pilot Plant. The comparability between the rescued mAb-4 drug substance and the reference material has demonstrated that this rescue strategy could be implemented in the biologics manufacturing to produce acceptable mAb product for potential clinical needs. Implementation of the redox wash in the Protein A chromatography is simple and straightforward, and the post-redox wash buffer washes out excessive redox components; hence, no residual cysteine/cystine remains in the product pool. We are proposing to use this redox wash as our future platform wash buffer for mAb purification in Protein A chromatography step to achieve high mAb purity without going through reprocessing, which can have added cost and potential regulatory implications. Additionally, this on-column disulfide reoxidation method can potentially be used to generate bispecific antibodies based on controlled Fab-arm exchange strategy, which in principle recombines antigen-binding arms (HL pairs) between individually expressed antibodies by reoxidizing two free cysteines on each arm.[51–53] This possibility may be explored in future work.

Materials and methods

Materials

Materials used in this study were generated using Chinese hamster ovary cells in disposable bag bioreactors and underwent different stages of downstream purification steps. The process intermediates of five mAb molecules (mAb-1, 2, 3, 4, and 5) included HCCF, purified PAE pool, and drug substance, with various levels of LMW species resulted from disulfide bond reduction. Prior to use, all materials were stored at < −60°C.

On-column disulfide formation study

The lab-scale purification was performed using an AKTA Avant 150 system (GE Healthcare, Piscataway, NJ) equipped with a 1 cm (Inner diameter, ID) x 20 cm (Height) column packed with MabSelect SuRe LX resin (GE Healthcare, Piscataway, NJ). As a standard Protein A chromatography operation, the column was loaded with the material to be purified, followed by a serial wash steps, including the redox buffer system. The product was eluted with low pH buffer followed by a neutralization to pH 5.5. The study was performed using 4 minute residence time unless specified otherwise. The samples were collected and stored at −80°C prior to analysis.

The pilot-scale purification was performed using a Delta-V system (Siemens, Germany) equipped with a 30 cm (ID) x 20 cm (Height) column packed with MabSelect SuRe LX resin. The rest of operation was conducted in the same manner as the small-scale process.

Dynamic binding capacity determination of reduced mAb

DBCs on MabSelect SuRe LX resin were determined using mAb-2 at three LMW levels (90%, 50%, and 1%), representing worst, moderate, and low severity of disulfide bond reduction, respectively. The 90% LMW drug substance was generated from a pilot run. The 50% LMW was prepared by mixing the 90% LMW and 1% LMW proportionally. All materials were diluted using 20 mM histidine buffer, pH 6 to reach a final protein concentration of ~5 g/L as the loading materials. The study was performed using 4 minutes residence time and DBC was calculated at 10% UV breakthrough.

DoE design for optimization of on-column redox wash

A full factorial design DoE was used to evaluate factors that may affect the on-column reoxidation kinetics. A 1 cm (ID) x 20 cm (Height) column packed with MabSelect SuRe LX resin was used for the experiment. Based on risk assessment and practicality evaluation, we chose the following primary factors and their study ranges: Protein A loading amount (25, 37.5, 50 g/L_{resin}), LMW level of mAb load (10%, 50%, 90%), and mAb/redox buffer contact time (15, 37.5, 60 minutes). All eluates were analyzed for purity by non-reduced CE-SDS method. Statistical analysis was performed using JMP13 software.

Non-reduced capillary electrophoresis SDS (CE-SDS NR) analysis

SDS Microchip-based CE-SDS was performed on a LabChip GXII (Perkin Elmer) under non-reducing condition. Iodoacetamide (IAM) was added into HT Protein Express Sample Buffer (Perkin Elmer) to a final IAM concentration of approximately 5 mM. A total of 5 µL antibody sample at approximately 1 mg/mL was mixed with 100 µL of the IAM containing sample buffer. The samples were incubated at 75°C for 10 min. The denatured proteins were analyzed with the "HT Protein Express 200" program.

Size-exclusion high-performance liquid chromatography

SEC-HPLC was performed using a Waters BEH column (4.6 mm x 150 mm, 200 Å, 1.5 µm) with an isocratic gradient

monitored at 280 nm on a Waters ACQUITY UPLC system (Milford, MA). The samples (target load of 50 µg) were injected onto the system at an isocratic flow rate of 0.4 mL/min using mobile phase of 0.1 M sodium phosphate, 0.15 M sodium chloride, pH 6.8.

Charge variants analysis

Charge variants were assayed by icIEF, which was performed on a Protein Simple iCE3 instrument (Bio-Techne) with an Alcott 720 NV autosampler (San Jose, CA). Samples were mixed with appropriate pI markers, ampholytes, and urea and injected into a fluorocarbon coated capillary cartridge. A high voltage was applied and the charged variants migrated to their respective pI. A UV camera captured the image at 280 nm. The main peak was identified and the peaks that migrated into the acidic range and basic range were summed, quantitated, and reported as relative percent area.

Intact mass analysis of non-reduced samples with liquid chromatography–mass spectrometry

LC–MS was performed on a Waters UPLC system and directly connected to a Waters Xevo G2X mass spectrometer equipped with an electrospray ionization (ESI) source. The non-reduced samples were separated with an Applied Biosystems Column (R2/10 2.1 mm x 30 mm) at 50°C with a flow rate of 0.2 mL/min. Mobile phase A 0.1% (v/v) of formic acid in water and mobile phase B 0.1% (v/v) of formic acid in acetonitrile were used for chromatographic separation. MS data were collected in the positive ion mode. The source conditions were source temperature 150°C, desolvation temperature 500°C, desolvation gas 500 L/hr, sample cone voltage 150 V, capillary voltage 3000 V. Spectra were acquired at 1 spectra/sec with mass range 500–4000 m/z. The analysis of the acquired LC/MS data was performed using Masslynx Software (V4.1).

Peptide mapping analysis with LC-MS

The antibody was dissolved in 50 mM ammonium bicarbonate buffer with 0.05% RapiGest surfactant, pH 7.0, to achieve a final protein concentration of 1 mg/mL. Samples were heated at 60°C for 20 minutes. After returning to room temperature, trypsin was added and the mixture was incubated at 37°C for 150 minutes, with the protein–enzyme ratio of 30:1 for protein digestion. Following protein digestion, iodoacetamide solution was added to the final concentration of 10 mM and the sample was shielded from light for 40 minutes. Trifluoroacetic acid was then added to the digested protein samples to quench the reaction. This acid-treated mixture was centrifuged at 12,000 rpm for 10 minutes to remove all matrix components prior to LC-MS analysis.

The tryptic peptides were analyzed on a Waters Acquity UPLC system (Waters, Milford, MA) equipped with a peptide BEH C18 column (150 × 2.1 mm, 300 Å, Waters, Milford, MA) using a linear gradient from 2% to 40% B over 5–95 min. Mobile Phase A was 0.1% formic acid in water, and Mobile Phase B was 0.1% formic acid in acetonitrile. Column temperature was maintained at 45°C and the flow rate was kept at 0.20 mL/min. Digested peptides were detected at 215 nm wavelength using a Photodiode Array Detector. The UPLC system was directly coupled to Thermo Q-Exactive plus Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific) equipped with an electrospray ionization source for mass spectrometry analysis. MS data were acquired and analyzed using the Xcalibur 3.1 software.

Circular dichroism spectroscopy

CD spectra were measured using a Chirascan-auto CD spectropolarimeter (Applied Photophysics) fitted with a 0.5 mm pathlength quartz cuvette. Near-UV (CD) spectroscopy was used to monitor protein tertiary structure. Near-UV CD spectra were collected from 340 to 250 nm on solutions containing 10 mg/mL protein. Far-UV CD spectroscopy was used to monitor the secondary structure of proteins prepared at a concentration of 0.3 mg/mL at a wavelength range of 195–260 nm. All protein solutions were prepared and added to a 96-well plate temperature controlled at 10°C. Spectra were collected in triplicate, baseline subtracted, averaged, and corrected for concentration. For near-UV CD, molar ellipticity was calculated and plotted as a function of wavelength. For far-UV CD, mean residue ellipticity was calculated.

Differential scanning calorimetry

Thermal stability, conformational stability, and structural unfolding transitions were assessed by measuring heat capacity as a function of temperature with a MicoCal-VP capillary DSC instrument (GE Healthcare). Protein solutions were prepared at an approximate concentration of 1 mg/mL and final concentrations were determined using a SoloVPE/UV-VIS instrument (C Technologies) and used to normalize data. Each DSC thermogram was fit with a non-two-state model with four thermal transitions to obtain melting temperature (Tm) values. Reported transition temperatures are averages of duplicate measurements.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Abbreviations

CD	circular dichroism
CE-SDS	capillary electrophoresis with sodium dodecyl sulfate
DBC	dynamic binding capacity
DoE	design of experiment
DSC	differential scanning calorimetry
HCCF	harvested cell culture fluid
HCP	host cell protein
HMW	high molecular weight
HPLC	high-performance liquid chromatography
IAM	iodoacetamide
icIEF	imaged capillary isoelectric focusing
LC	liquid chromatography
LMW	low molecular weight
mAb	monoclonal antibody
MS	mass spectrometry
PAE	Protein A elution
PBS	phosphate-buffered saline
SEC	size exclusion chromatography

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