

Affinity Precipitation of a Monoclonal Antibody From an Industrial Harvest Feedstock Using an ELP-Z Stimuli Responsive Biopolymer

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ABSTRACT: In this work, a proof of concept elastin-like polypeptide-Z domain fusion (ELP-Z) based affinity precipitation process is developed for monoclonal antibody (mAb) purification from industrial harvest feeds. Greater than 99% mAb recoveries are obtained during the initial binding step of the process for both pure mAb and the mAb harvest feeds. Great than 90% overall mAb yields are also obtained for the subsequent elution step of the process with no measurable mAb aggregation. The process is shown to result in more than 2 logs of host cell protein (HCP) and more than 4 logs of DNA clearance from the harvest feed. While the overall mAb yield and HCP clearance for the affinity precipitation process was comparable to Protein A chromatography the DNA clearance was clearly superior. Performance is maintained for mAb final elution concentrations up to 20 g/L, demonstrating the ability of the process to both concentrate and purify the mAb. Effective ELP-Z regeneration is also demonstrated using 0.1 M NaOH with no adverse effect on subsequent capture efficiency. Finally, the reusability of the ELP-Z construct and robustness of the process is demonstrated for up to three purification-regeneration cycles with minimal product and impurity carryover and high yields and purity. This work demonstrates that the ELP-Z based precipitation approach can be successfully employed as an affinity capture step for industrial mAbs.

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Introduction

Monoclonal antibodies (mAbs) constitute a major portion of the biopharmaceutical industry (Reichert, 2012, 2013). Protein A chromatography is extensively employed as the capture step for industrial mAb purification due to its high affinity and specificity towards a range of mAb types (Fassina, 2001; Hober et al., 2007; Huse et al., 2002). While Protein A chromatography results in high product yields and impurity clearance in a single step, relatively high resin costs and slow volumetric throughputs have adversely impacted the economics and scalability of the process. Accordingly, there has been a significant interest in the development more economical and scalable non-chromatographic mAb recovery processes (Knevelman et al., 2010; Low et al., 2007; McDonald et al., 2009; Thömmes and Etzel, 2007; Venkiteswaran et al., 2008).

Affinity precipitation has several attractive features for the initial capture step such as ease of scale-up and processing simplicity along with high selectivity (Hilbrig and Freitag, 2003). Elastin-like polypeptides (ELPs) are stimuli responsive smart biopolymers that can reversibly aggregate above a critical inverse transition temperature (T_i) (Urry, 1997). ELPs consist of elastin-based pentapeptide repeating motifs (VPGXG) (typically between 20 and 300 pentapeptide repeats), where X is the guest amino acid residue. The effect of ELP chain length and guest amino residue on the phase transition properties of ELP has been studied in depth (Meyer and Chilkoti, 2004). In addition, the ELP phase transition has also been shown to be affected by solution conditions such as pH, salt type and concentration (Cho et al., 2008). The ability to tune the phase transition properties of ELPs have generated interest in its potential for downstream bioprocessing applications (Banki et al., 2005; Ge et al., 2005; Kim et al., 2005; Meyer and Chilkoti, 1999; Stiborova et al., 2003; Sun et al., 2005).

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The Z domain is an engineered B domain from staphylococcal Protein A (Nilsson et al., 1987). It consists of an antiparallel three-helix bundle containing 58 amino acid residues that selectively binds to the Fc portion of IgGs with high affinity ($K_D = 10\text{--}50$ nM) (Braisted and Wells, 1996). The helices are in close contact with one another, forming a hydrophobic core that provides a great deal of stability to this domain (Linhult et al., 2004). In addition to the high selectivity, the Z domain also exhibits high tolerance to proteolysis, extremes of pH and high concentration of denaturants such as urea and guanidine hydrochloride, which makes it attractive for mAb purification (Linhult et al., 2004).

While, the purification of target proteins fused to ELPs has been previously demonstrated (Meyer and Chilkoti, 1999), this approach typically requires proteolytic cleavage of the target protein from the ELP prior to final recovery. Banki et al. (2005) and Ge et al. (2005) demonstrated the purification of target proteins by fusing them to ELPs via a self-splicing intein domain which eliminated the need for a proteolytic cleavage step. However, this approach can result in relatively low titers making it less amenable for large scale mAb production and purification.

Kim et al. (2005) have employed an ELP-Protein A fusion for the purification of mAbs via affinity precipitation. In this approach, the mAb is first selectively captured by binding to the ELP-Protein A fusion, followed by precipitation of the complex. The precipitant is then re-solubilized in an appropriate elution buffer to dissociate the ELP-Protein A-mAb complex, followed by a final precipitation of the dissociated ELP-Protein A fusion. A significant advantage of this affinity precipitation approach is that the ELP-Protein A fusion is produced independently of the mAb thus not affecting mAb production and titers.

Recent work has replaced Protein A (42 kDa) with the Z domain (6.6 kDa) allowing for higher elastin-like polypeptide-Z domain fusion (ELP-Z) (39.4 kDa) production yield (Madan et al., 2013). In addition, an HTS protocol has been developed to rapidly identify optimal operating conditions for an ELP-Z based affinity precipitation process (Sheth et al., 2013). Pure mAbs were employed in that study and the results indicated that the process could result in high mAb yields and low levels of aggregation under appropriate conditions.

The current investigation examines the potential of the ELP-Z based affinity precipitation technique for processing industrial mAb feed stream and examines impurity clearance, product quality, ELP-Z regeneration, and reusability. Initially, mAb binding and co-precipitation efficiency of the ELP-Z is initially determined. Elution of the mAb from the ELP-Z-mAb complex is then examined using a range of conditions and the mAb yield, purity (HCP and DNA clearance), and aggregation at the end of the process are determined. In addition, process performance at various mAb elution concentrations is examined. ELP-Z regeneration to remove residual mAb and impurities is then evaluated using a range of regeneration conditions. Finally the mAb binding efficacy of ELP-Z after NaOH treatment and its reusability over multiple cycles is demonstrated. This study provides a

comprehensive evaluation of the use of ELP-Z for mAb purification from an industrial mAb harvest.

Materials and Methods

Materials

A Jupiter[®] 5 μm C4 300A column (4.6 mm \times 50 mm) was purchased from Phenomenex (Torrance, CA). A TSK gel G3000SWxl SEC column (7 mm \times 300 mm) with accompanying guard column was purchased from Tosoh (Tokyo, Japan). A POROS[®] A 20 μm Column (4.6 mm \times 50 mm) and all the SDS PAGE gel supplies were purchased from Life Technologies (Carlsbad, CA). *Escherichia coli* strain BL21 (DE3) cells containing the ELP-Z (78 pentapeptide (VPGVG) repeats) plasmid were constructed as described elsewhere (Kim et al., 2005). Bacto tryptone and yeast extract were purchased from BD Biosciences (Franklin lakes, NJ). Glycerol, ampicillin, and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Pittsburgh, PA). CHO HCP ELISA kit, 3G was purchased from Cygnus Technologies (Southport, NC). Sodium hydroxide, potassium phosphate (mono and dibasic), citric acid, sodium phosphate (dibasic), arginine hydrochloride, acetic acid, phosphoric acid were purchased from JT Baker (Phillipsburg, NJ). Potassium chloride, sodium chloride, acetonitrile (ACN), urea, guanidine hydrochloride, sodium citrate dihydrate, and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). Sodium sulfate was purchased from EMD Chemicals (Billerica, MA).

ELP-Z Expression and Purification

The protocol for expression and purification of ELP-Z (39.4 kDa) has been described in detail previously (Sheth et al., 2013). Briefly, *E. coli* strain BL21(DE3) containing the ELP-Z plasmid was grown in Luria Broth (LB) with 100 $\mu\text{g}/\text{mL}$ ampicillin at 37°C and 250 rpm for 16 h. The grown cultures were then sub cultured into Terrific Broth (100 $\mu\text{g}/\text{mL}$ ampicillin) and grown for 48 h at 37°C and 250 rpm. The cells were harvested by centrifugation and the cell pellets were re-suspended in phosphate-buffered saline (PBS) containing 1X protease inhibitor cocktail. The cells were disrupted by sonication and the cell debris was removed by centrifugation. The inverse phase transition property was then employed for the purification of the ELP-Z fusion (Sheth et al., 2013). This protocol typically resulted in ELP-Z production yield of 0.5 g/L media.

Experimental Procedures

Binding and Precipitation

For the mAb binding and precipitation studies, 2 mL of either pure mAb or mAb harvest was mixed with 2 mL of ELP-Z at different concentrations (in PBS) to achieve the desired ELP-Z:mAb molar ratios (between 8:1 and 1:1) in 15 mL

centrifuge tubes. The solutions were equilibrated for 10 min to allow ELP-Z-mAb binding. To initiate the precipitation of the ELP-Z-mAb complex, 1 mL of 1.25 M sodium sulfate (Na_2SO_4) (in PBS) was added to the ELP-Z-mAb mixtures to achieve 0.25 M final Na_2SO_4 concentration. The tubes then were incubated at room temperature for 15 min followed by centrifugation for 30 min at 4,000g to recover the ELP-Z-mAb precipitates. The supernatants after centrifugation were collected and analyzed using analytical Protein A chromatography and reversed phase liquid chromatography (RPLC) (discussed below) for the presence of leftover mAb and ELP-Z, respectively.

Elution and Precipitation

For the mAb elution screens, the precipitates obtained from the mAb binding and precipitation step were resolubilized in appropriate volumes of various elution buffers (50 mM citrate and acetate buffers between pH 3.6 and 5) to achieve the desired mAb concentrations in the elution pools. These elution pools were then allowed to equilibrate at room temperature for 10 min to allow for mAb elution from the ELP-Z-mAb complex. ELP-Z precipitation was then initiated by the addition of Na_2SO_4 (in the appropriate elution buffers) to 0.25 M final concentration. The solutions were incubated at room temperatures for 15 min followed by centrifugation at 4,000g for 30 min to recover the ELP-Z precipitates. The supernatants after centrifugation were subsequently analyzed for mAb yields (using Protein A), mAb aggregation (using SEC), host cell proteins (HCP), and DNA content all described below.

ELP-Z Regeneration

ELP-Z was regenerated after each mAb elution to remove residual mAb and impurities present in the ELP-Z. The ELP-Z precipitates were re-solubilized in various regenerant solutions (50 mM citrate, pH 2.5; 50 mM citrate, 1 M guanidine, pH 2.5; 50 mM citrate, 1 M urea, pH 2.5; 100 mM acetic acid, pH 2.9; 100 mM phosphoric acid, pH 1.6; 120 mM phosphoric acid, 167 mM acetic acid, pH 1.7 and 0.1 M NaOH) and incubated at room temperature for 20 min. Na_2SO_4 was then added to a final concentration of 0.25 M to initiate ELP-Z precipitation (note: for ELP-Z regeneration with 0.1 M NaOH, the solution was first titrated to pH 7). The resulting suspensions were incubated at room temperature for 15 min followed by centrifugation at 4,000g for 30 min to recover the ELP-Z precipitates. The final resulting ELP-Z precipitates were then re-solubilized in PBS pH 7.4 and analyzed for the presence of mAb and host cell impurities.

Analytical Methods

Reversed Phase Liquid Chromatography (RPLC). RPLC was used to analyze the mixtures (supernatants and resolubilized precipitates) from the mAb affinity precipitation experiments

and to quantify the ELP-Z and mAb recoveries. RPLC was carried out using a C4 column (4.6 mm \times 50 mm) at a flow rate of 1 mL/min with an A buffer of deionized water and 0.1% TFA and a B buffer of 90% ACN, 10% deionized water and 0.1% TFA (all v/v). The column was first pre-equilibrated with 35% B which caused the mAb to come out in the column flow through (monitored at 280 nm). This was followed by a step change to 100% B for two column volumes which resulted in the ELP-Z elution (monitored at 214 nm).

SEC. Size exclusion chromatography (SEC) was employed to analyze the final supernatants after the mAb elution screens and to determine the mAb aggregate content. A carrier buffer of 50 mM phosphate with 200 mM arginine and 100 mM NaCl, pH 6.5, was used at a flow rate of 1 mL/min. The column effluents were monitored at 280 nm. The samples were maintained at 4°C in a temperature controlled auto injector (Waters 2695) to minimize aggregation of the product prior to analysis.

Analytical Protein A Chromatography. Analytical Protein A chromatography using a POROS[®] A 20 μm column was carried out to determine mAb yield/recoveries during the affinity precipitation processes when the mAb harvest feed was employed. PBS buffer pH 7.4 was used for loading the samples to allow the mAb to bind to the column while the other impurities flowed through. 100 mM Glycine with 100 mM NaCl, pH 2.5 was employed for mAb elution from the column and the column effluents were monitored at 280 nm.

ELISA Host Cell Protein Analysis

A 3rd generation generic ELISA kit from Cygnus Technologies was employed to quantify CHO HCP levels in various samples. A standard assay protocol provided by the supplier was employed for the assay. Briefly, samples were reacted with a horseradish peroxidase (HRP) enzyme labeled anti-CHO antibody (goat polyclonal) in microtiter strips coated with a capture anti-CHO antibody. The resulting solid phase antibody-HCP-enzyme labeled antibody complex was then washed to remove unbound species. Tetramethylbenzidine (TMB) was then added to the microtiter wells and the hydrolysis reaction was terminated after 45 min by the addition of 0.5 M sulfuric acid. The developed color which corresponds to the amount of captured HCP was then analyzed using a microtiter plate reader at 450 nm. The assay quantification limit was 1 ng/mL.

DNA Analysis

The residual DNA content in the mAb elution pools were determined using a quantitative PCR (qPCR) method developed in house at BMS. 2 qPCR measurements were performed for every sample and the DNA assay variations were ~5%. The quantification limit was 0.2 pg/mL.

Results and Discussion

The ELP-Z based affinity precipitation process involves the binding of ELP-Z to the mAb followed by precipitation of the complex. The precipitates are then re-solubilized in the elution buffer where the mAb is eluted from the complex. This is followed by precipitation of the ELP-Z to recover the purified mAb in solution and to obtain the precipitated ELP-Z for subsequent use. ELP-Z phase transitions can be carried out using an appropriate temperature change, addition of salt or a combination. Our previous studies (Sheth et al., 2013) indicated that operating at higher temperature (37°C) with low salt could have adverse effects on product quality resulting in elevated aggregate content. In contrast, that work showed that operation at room temperature (22°C) with higher salt concentrations resulted in significant reductions in product aggregation. Clearly, room temperature operation has many advantages from a bio-manufacturing perspective. Accordingly, the affinity precipitation study with an industrial mixture described in the current work was developed for a room temperature process.

ELP-Z-mAb Binding and Precipitation

The ability of the ELP-Z proteins to bind and co-precipitate the mAb was initially evaluated for pure mAb as well as the mAb harvest mixture. Figure 1 shows the % mAb recovered in this first step of the process as a function of ELP-Z:mAb molar ratios at room temperature using 0.25 M Na₂SO₄ as the precipitant. As can be seen in the Figure, high mAb recoveries were obtained at a 4:1 molar ratio for both the pure mAb (99.3%) and mAb harvest (99.1%) while reduced mAb recoveries were obtained at lower ELP-Z:mAb molar ratios for both samples. Importantly, only minor differences were observed with the pure mAb and the mAb harvest feeds, suggesting that the harvest impurities for this mAb did not inhibit ELP-Z-mAb binding or precipitation to any significant extent.

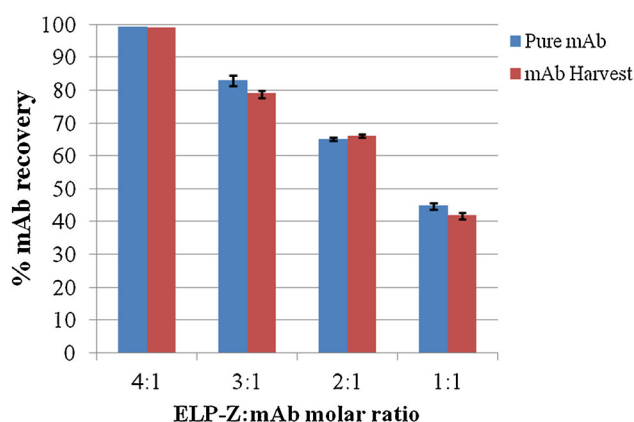


Figure 1. Binding and co-precipitation of the mAb from pure mAb and the mAb harvest feeds using ELP-Z (note: the experiments were performed in triplicates).

While the stoichiometry of binding of the Z domain to mAb should be on the order of 2:1 (Jendeborg et al., 1995), a 4:1 molar ratio was required for high mAb recovery in these experiments. As has been discussed previously, this could be due to steric hindrance effects on ELP precipitation caused by the bound mAb and/or a reduction of the binding affinity of the Z domain to the mAb due to its fusion to the ELP (Sheth et al., 2013).

mAb Elution and Product Quality

In the next step of the process, the precipitates were re-solubilized in the elution buffer, followed by precipitation of the ELP-Z to recover the purified mAb. A range of conditions were first evaluated for mAb elution from the ELP-Z-mAb complex using pure mAb. The ELP-Z:mAb molar ratio of 4:1 identified from Figure 1 was employed for these studies. Since low pH is typically required to elute mAbs from the Z domain, a number of low pH conditions (between pH 3.6 and pH 5) were examined for mAb elution.

Figure 2 shows the mAb yields after elution as a function of elution pH for the citrate and acetate buffer systems which are commonly employed in the industry for Protein A elution. As can be seen in the figure, high mAb elution yields were obtained for pH values of 4.2 and below. In contrast, the elution yields decreased for elution pH values of pH 4.5 and 5. The results for the acetate and the citrate buffer systems were observed to be similar, with the acetate buffer systems showing slightly lower mAb yields at the higher elution pH values (pH 4.5 and 5). These elution pH results are similar to those previously observed in our group using a different model mAb, demonstrating that relatively mild elution pH conditions (e.g., pH 4.2) may be applicable to a broad range of mAb products with these ELP-Z reagents. In addition, the pure mAb feed in the current study contained approximately 0.3% aggregates and the mAb aggregate content after elution was less than 0.3% for all the conditions tested, suggesting

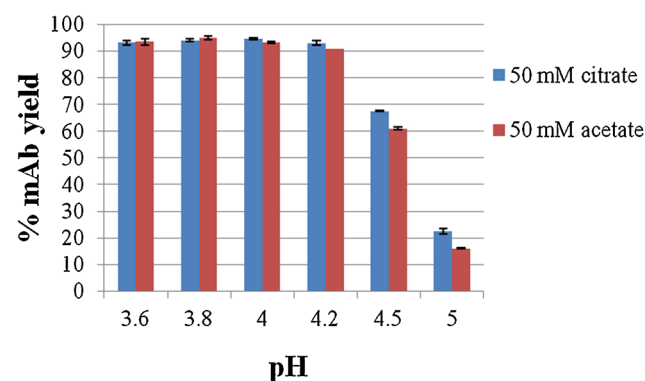


Figure 2. mAb yields after elution from the ELP-Z and the second round of precipitation (note: the experiments were performed in triplicates).

that this mAb was quite stable under the elution conditions employed.

A set of conditions (pH 3.6 and 4.0 with both buffers) which provided high mAb elution yields with the pure mAb (Fig. 2) were then employed for elution studies with the mAb harvest solution. The results of these experiments with respect to yield, and clearance of key host cell impurities such as HCP and DNA were determined and compared to those obtained with Protein A chromatography (note: the experimental results are for duplicate measurements of mAb yield and HCP. The Protein A chromatography comparison experiment was carried out once). The mAb harvest feed (3.3 g/L mAb titers) used for these experiments contained 3×10^5 ppm of HCP and 3.6×10^6 ppb of DNA impurities. As can be seen in Table I, high mAb yields (92–94%) were obtained with both buffers at pH 3.6 and 4.0 with comparable results to those obtained with the pure mAb. Importantly, the HCP content in the elution pools for all the conditions tested was between 1,400 and 2,000 ppm which corresponds to a 2.1–2.3 LRV for the HCP impurities. Further, the DNA content in the elution pools was also very low ranging from 35 to 296 ppb which corresponds to a 4–5 LRV for the DNA impurities.

As can also be seen in the table, these results were quite similar to those obtained with Protein A chromatography with respect to mAb yield and HCP clearance. For DNA clearance, the affinity precipitation process was observed to be superior to Protein A chromatography. In addition, there were minimal differences in the impurity clearance levels obtained using the different buffer systems (acetate and citrate) and the elution pH (3.6 and 4). It was also of interest to study the effect of the ELP-Z:mAb ratio on the process since excess ELP-Z could conceivably alter the impurity profile in the elution pool due to non-specific adsorption during the precipitation process. As can be seen in the table, mAb yield, HCP, and DNA contents in the eluted mAb pools were quite similar for the 4:1, 6:1, and 8:1 ELP-Z:mAb molar ratios, suggesting that there was no adverse effect on product quality when using these excess ratios. Overall, the results shown in Table I demonstrate that the ELP-Z based affinity precipitation approach was indeed capable of purifying this mAb from the harvest feedstock at high yield and high levels of impurity clearance. In fact, while the overall mAb yield and HCP clearance for the affinity precipitation process was comparable to Protein A chromatography the DNA clearance was found to be superior.

The experiments shown in Table I were designed for a mAb elution pool concentration of 3 g/L. However, it is clearly desirable to minimize the elution pool volume to facilitate subsequent processing. In fact, mAbs typically get concentrated during elution from a Protein A column resulting in concentrations in excess of 10 g/L. Accordingly, it was of interest to examine the effect of higher final mAb concentrations in the ELP-Z process on product quality and yield. Table II shows the results for final mAb concentrations ranging from 3.5 to 20 g/L using a 4:1 ELP-Z:mAb molar ratio for binding and 50 mM acetate buffer, pH 4, for elution. The desired final mAb concentrations were achieved by re-solubilizing the ELP-Z-mAb precipitates from the binding step in the appropriate volume of the elution buffer. As can be seen in the table, high mAb yields (90.5–93.8%) were obtained for all elution mAb concentrations. Further, excellent HCP and DNA clearances were obtained for all conditions examined. Importantly, the same mAb aggregate content was obtained at all elution concentrations and was essentially the same as that present in the mAb harvest feed (3.9%) and the Protein A elution pool. These results clearly suggest that the performance of this process with respect to mAb yield and quality was conserved across a wide range of elution mAb concentrations, thus allowing for significant flexibility in the operating conditions and a reduction in the processing volumes.

ELP-Z Regeneration and Reusability

The precipitated ELP-Z from the elution step described above was re-solubilized and analyzed for any residual impurities. The results of this analysis indicated that approximately 3,500 ppm HCP and 6900 ppb of DNA were present in the resolubilized ELP-Z solution. Since the ELPs have significant hydrophobicity and salt was used for the precipitation, it is possible that some of the hydrophobic host cell impurities were non-specifically bound to the ELP-Z during the precipitation process. Although these residual impurity levels were low, they might impact the reusability of this construct and thus the economics of the process. In addition, approximately 2–3% mAb (from RPLC analysis) was also present in the final resolubilized ELP-Z at the end of the process.

Carryover of the product or process related impurities to the subsequent purification cycles can potentially adversely affect product quality and impact patient safety. Further, it is

Table I. Effect of elution conditions and ELP-Z:mAb molar ratios on mAb yield and impurity clearance.

Elution condition	ELP-Z:mAb molar ratio	mAb yield (%)	HCP (ppm)	DNA (ppb)
50 mM citrate, pH 3.6	4:1	92.1 ± 0.4	1890 ± 87	260
50 mM citrate, pH 4	4:1	93.0 ± 0.7	1957 ± 135	296
50 mM acetate, pH 3.6	4:1	92.9 ± 0.2	1573 ± 74	55
50 mM acetate, pH 4	4:1	93.4 ± 0.9	1450 ± 96	35
50 mM acetate, pH 4	6:1	93.1 ± 1.1	1486 ± 49	34
50 mM acetate, pH 4	8:1	93.3 ± 0.5	1609 ± 52	70
Protein A chromatography elution pool		95.0	1,787	5,436

Table II. Effects of elution mAb concentration on product yield and quality.

Elution mAb concentration (mg/mL)	mAb yield (%)	HCP (ppm)	DNA (ppb)	Aggregation (%)
3.5	92.6 ± 0.7	1391 ± 158	78	3.6 ± 0.2
5.3	90.5 ± 1.6	1112 ± 192	45	3.4 ± 0.1
10	92.3 ± 0.4	1580 ± 167	56	3.6 ± 0.1
20	94.1 ± 0.9	1683 ± 121	70	3.8 ± 0.3
Protein A elution pool 23.5 g/L		1,787	5,436	3.9

expected by the regulatory agencies that adequate regeneration procedures are implemented for every purification step and that product and impurity carryover are minimized, characterized, and controlled. In an effort to address these concerns, a study was carried out to establish an appropriate regeneration protocol for the ELP-Z construct.

Low pH conditions are frequently employed to remove any residual mAb bound to Protein A resins between cycles. In addition, sodium hydroxide is widely employed for both regeneration and sanitization operations which is crucial for bio-burden control. Accordingly, NaOH as well as variety of low pH conditions with or without modifiers such as urea and guanidine were evaluated for their efficacy to clean the ELP-Z construct.

mAb affinity precipitation for these studies was performed using a 4:1 ELP-Z:mAb molar ratio for mAb binding and 50 mM acetate, pH 4 for mAb elution. The ELP-Z precipitates after mAb elution were re-solubilized in the different regeneration solutions and incubated for 20 min, followed by an additional ELP-Z precipitation step. The resulting ELP-Z pellets were then re-solubilized in PBS for analysis. After the regeneration incubation with NaOH, the solution was titrated back to pH 7 and then precipitated. Validation of complete precipitation of the ELP-Z under the various regeneration conditions was independently verified using pure ELP-Z.

Figure 3 shows the non-reducing gel analysis of the ELP-Z before and after regeneration under various conditions. As can be seen in the figure, without regeneration, the re-solubilized ELP-Z solution at the end of the process contained the ELP-Z band along with residual mAb (Lane 3, 2–3% mAb as determined by RPLC). In contrast, all of the regeneration procedures resulted in re-solubilized ELP-Z with no detectable mAb bands (Lanes 4–10) and levels of mAb below the limit of RPLC detection (<0.1%). These results demonstrate that all of these regeneration regimens were effective in stripping the residual mAb product from the ELP-Z.

The ELP-Z regeneration protocols were also evaluated for residual HCP and ELP-Z recovery after the entire affinity precipitation process and the results are presented in Table III. The presented ELP-Z yields were for the entire process which included binding, elution as well as the additional regeneration step. The yields were very similar for all conditions examined, with minimal losses occurring

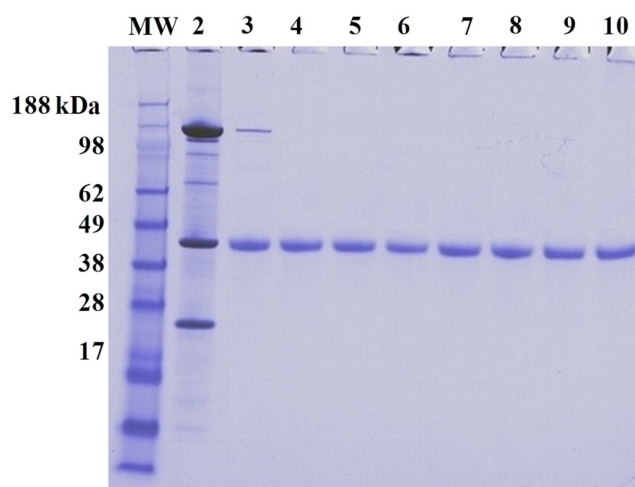


Figure 3. Relative efficacy of various protocols for ELP-Z regeneration; (Lane 1) Protein ladder; (Lane 2) mAb harvest feed; (Lane 3) Resolubilized ELP-Z after the affinity precipitation cycle; (Lane 4) Resolubilized ELP-Z after treatment with 50 mM citrate, pH 2.5; (Lane 5) Resolubilized ELP-Z after treatment with 50 mM citrate, 1 M guanidine, pH 2.5; (Lane 6) Resolubilized ELP-Z after treatment with 50 mM citrate, 1 M urea, pH 2.5; (Lane 7) Resolubilized ELP-Z after treatment with 100 mM acetic acid; (Lane 8) Resolubilized ELP-Z after treatment 100 mM phosphoric acid; (Lane 9) Resolubilized ELP-Z after treatment with a combination of 120 mM phosphoric acid and 167 mM acetic acid, pH 1.7; (Lane 10) Resolubilized ELP-Z after treatment with 0.1 M NaOH.

during the regeneration steps. The minor losses in the ELP-Z can be attributed to irreversible precipitates formed during the affinity precipitation process which will be fully evaluated in a future study. Without regeneration, residual HCP levels were on the order of 4000 ppm. The pH 4 acetate and the 100 mM acetic acid regeneration protocols produced minor clearance of HCPs with levels of 2509 and 930 ppm, respectively. All the citrate buffers were effective in removing the residual HCP, providing more than an order of magnitude clearance over the control. Clearance in the presence of guanidine and urea was slightly better than with citrate alone, however, there was a slightly decreased yield of ELP-Z when these modifiers were employed. The phosphoric acid and the combined phosphoric acid and acetic acid regeneration conditions also exhibited good HCP clearance. Finally, 0.1 M NaOH demonstrated the best HCP clearance with only 60 ppm residual HCP and good ELP-Z yield. In summary, it is clear from the results shown in Figure 3 and Table III, that a number of regeneration strategies can be readily employed for effective stripping of residual mAb and HCP from the ELP-Z between lots. Since NaOH treatment has the additional benefit of sanitization, it was selected for the subsequent studies described below.

Although, the Z domain has previously been shown to be quite stable to NaOH treatment (Linhult et al., 2004) it was of interest to test the efficacy of the ELP-Z fusion after NaOH regeneration. For this study, pure ELP-Z was incubated in NaOH for time intervals ranging from 20 min to 4 h to determine its stability over multiple cycles. The efficacy of the

Table III. Residual HCP in the resolubilized ELP-Z after treatment using various regeneration protocols.

Cleaning condition	ELP-Z recovery (%)	HCP (ppm)
Control	93.0 ± 0.6	4,313 ± 296
50 mM acetate, pH 4	92.1 ± 0.2	2,509 ± 103
100 mM acetic acid, pH 2.9	92.3 ± 0.4	930 ± 82
50 mM citrate, pH 2.5	92.0 ± 0.4	228 ± 35
50 mM citrate, 1 M guanidine, pH 2.5	87.6 ± 0.7	104 ± 23
50 mM citrate, 1 M urea, pH 2.5	87.2 ± 0.5	125 ± 17
100 mM phosphoric acid, pH 1.6	90.2 ± 1.1	117 ± 29
120 mM phosphoric acid, 167 mM acetic acid, pH 1.7	89.3 ± 0.8	102 ± 21
0.1 M NaOH	91.4 ± 0.3	60 ± 14

ELP-Z to bind and precipitate the mAb product was tested after each NaOH treatment and was compared with the control (i.e., ELP-Z without NaOH treatment). As can be seen in Figure 4, complete mAb recovery was obtained for ELP-Z: mAb molar ratios greater than or equal to 4 for all the incubation times tested. At molar ratios below 4 there was a slight decrease in the efficacy for the NaOH treated ELP-Z as compared to the untreated ELP-Z. However, even after 4 h of incubation (equivalent to 12 cycles with 20 min regeneration steps) the ELP-Z efficacy for these lower molar ratios was still well conserved with less than 4% reductions of mAb recovery. These results suggest that the ELP-Z fusion was quite stable to NaOH treatment and can potentially be used over multiple purification cycles with minimal reduction in mAb binding and purification efficacy.

The efficacy of this affinity precipitation process was then evaluated over 3 cycles. In this study, the mAb purification cycle (which includes binding, elution and regeneration) was repeated for one or three times followed by a blank run (no mAb feed) and the elution pool from the blank runs were then evaluated for the presence of mAb and other impurities. In addition, the mAb yield and purity after each purification

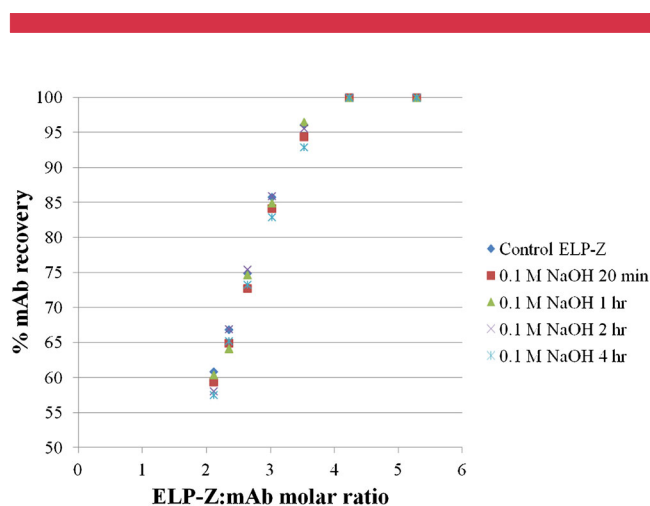


Figure 4. Efficacy of the ELP-Z for mAb binding after extended treatment using 0.1 M NaOH.

cycle was determined. This set of experiments was carried out at an ELP-Z:mAb molar ratio of 6:1 to enable us to fully evaluate mAb carryover without any additional effects due to ELP-Z losses described above.

If the regeneration protocol was robust, one would expect to be unable to detect any mAb or impurity carryover in the elution pools of the blank loads. As can be seen in Table IV, the mAb, HCP, and DNA levels in the blank elution pools (after 1 and 3 cycles) were below the assay detection limits. In addition, high mAb yields and purity were maintained after every purification cycle. While slightly higher DNA content was observed in the elution pool after cycles 2 and 3, this did not result in any detectable DNA in the blank runs. The results shown in Table IV demonstrate that the NaOH treatment was effective in regenerating the ELP-Z and in maintaining good mAb quality over multiple purification cycles.

While the affinity precipitation process resulted in high levels of mAb yields and impurity clearance, the economics of the process are also important to consider before it can be considered as a viable alternative to Protein A chromatography. For a Protein A resin with a 100-cycle lifespan, the costs of mAb purification are roughly \$2/g mAb (Kelley, 2007). Some of the factors that need to be considered for an economic analysis of the proposed affinity precipitation process are the ELP-Z production costs, precipitate recovery costs at the large scale (e.g., via continuous centrifugation) and the reusability of the ELP-Z fusion. The ELP-Z employed for the current study was produced in a non-optimized *E. coli* fermentation and approximately 0.5 g/L media production yields were obtained. Considering bulk TB media costs (without proline) to be roughly \$7.6/L media (Fong and Wood, 2010), the net media costs for ELP-Z production would be \$15.2/g ELP-Z. Assuming that the ELP-Z can be recycled 10 times (based on the 10% loss shown in Table III) the mAb purification costs purely based on media price would be approximately \$1.52/g mAb. There are multiple other factors that would add to the ELP-Z production costs, such as facility costs and ELP-Z purification costs. While facility costs could be substantial, we believe that the ELP-Z purification costs would be minimal due to the ease of ELP-Z recovery using phase transition. Thus, from a purely “material” perspective, at 0.5 g/L ELP-Z production yields, it is likely that there will be minimal if any economic advantage of the ELP-Z process using the current production approach. However, we believe that there is significant room

Table IV. mAb and impurity carryover study during multiple mAb purification cycles.

Reuse condition	mAb yield (%)	HCP (ppm)	DNA (ppb)
Elution after 1 cycle	95.3 ± 0.8	2505 ± 98	160
Blank after 1 cycle	<DL	<DL	<DL
Elution after 2 cycles	92.2 ± 1.3	3036 ± 127	650
Elution after 3 cycles	94.3 ± 0.5	3123 ± 184	800
Blank after 3 cycles	<DL	<DL	<DL

DL, detection limit.

for improving ELP-Z production as well as reusability. Importantly, there are several other factors which come into play in comparing these two processes. Protein A chromatography is often limited by the volumetric throughputs that can be achieved during the loading stage. This requires longer processing time, with associated increases in labor and other costs. On the other hand, affinity precipitation should be readily scalable with less time constraints as compared to Protein A chromatography. In view of these arguments, it is important to continue exploring the potential of this affinity precipitation technology.

Conclusions

The results presented in this paper have demonstrated that ELP-Z based affinity precipitation process can be employed for mAb purification from industrial harvest feeds. High levels of mAb recoveries were obtained during the initial binding step of the process when ELP-Z:mAb molar ratios of 4:1 or higher were employed using both pure mAb and the mAb harvest feed. High mAb yields were also obtained for the subsequent elution step of the process when pH values in the range of 3.6–4.2 were used with no observable mAb aggregation. However, for mAbs which are more prone to aggregation at lower pH (Sheth et al., 2013), a more limited pH elution range may be required. Importantly, the process resulted in more than 2 logs of HCP and more than 4 logs of DNA clearance from the mAb harvest feed in this single affinity precipitation step. In fact, these clearance and yield values were comparable or superior to Protein A chromatography. The performance of the process was maintained for mAb final elution concentrations up to 20 g/L, again providing high yields and purity without measurable increase in soluble aggregate content. This is a very important result, in that the capture step for biologics must result in both concentration and partial purification. Effective ELP-Z regeneration was achieved using several regeneration strategies, with 0.1 M NaOH producing the best results. Further, ELP-Z retained its efficacy to capture the mAb product even after extended treatment (4 h) with NaOH, demonstrating the stability of this construct. Finally, the reusability of the ELP-Z construct and robustness of the process was demonstrated over multiple purification-regeneration cycles with minimal product and impurity carryover and high yields and purity. However, although the efficacy was maintained in repeated studies, the slight loss of ELP-Z during each cycle will have to be addressed in future work.

The results presented in this work clearly demonstrate that this ELP-Z based affinity precipitation approach can efficiently be employed as a capture step for industrial mAbs with performance comparable or better than Protein A chromatography. This study lays the foundation for performing further investigations into the scalability and economics of the process which will be reported in a future publication.

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