Connected flow-through chromatography processes as continuous downstream processing of proteins

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Abstract. Continuous manufacturing is expected to increase the productivity of protein drug production. However, it is not easy to build the continuous process especially for downstream processing as many unit operations (chromatography and membrane filtration) are involved. An operation method known as flow-through chromatography (FTC) is considered to be an efficient method for separating two components as the flow is continuous. In FTC, a target protein is eluted from the chromatography column without adsorption whereas contaminants are strongly bound. Since at least two different modes of chromatography are needed in order to remove contaminants, two FTC columns have to be connected in order to build the continuous process. This is not an easy task since the mobile phase properties (pH, salt, buffer ions) are different for the two columns. In this paper, we investigated how connected FTC columns can remove impurities efficiently from the cell culture broth containing monoclonal antibody. It was found that the sequence (activated carbon - anion exchange chromatography - cation exchange chromatography) is most efficient when the mobile phase pH and conductivity were properly chosen.

1 Introduction

Protein drugs such as monoclonal antibodies (mAbs) are known to be very effective drugs for cancer treatment and autoimmune diseases. However, production of mAbs is very complicated. mAbs are mainly produced in mammalian cells such as CHO cells. After the cell culture (upstream process), mAbs must be purified to a very pure form by using several chromatography and membrane systems. This process called downstream process (DSP) is said to be very costly because of several unit operations involved.

One of the problems of mAbs is its high price. Continuous manufacturing is expected to reduce the cost of goods of mAb production. **Fig. 1** shows a typical platform DSP.

After the cell culture, cells are removed either by centrifugation or filtration in order to produce filtered cell culture supernatant (FCCS). FCCS is then applied to the first chromatography step called "capture step". Protein A chromatography (PAC) is commonly employed as the capture step. Because of very high affinity of protein A to mAbs, PAC can perform both concentration and purification by a simple adsorption/desorption (Bind/Elute) operation.

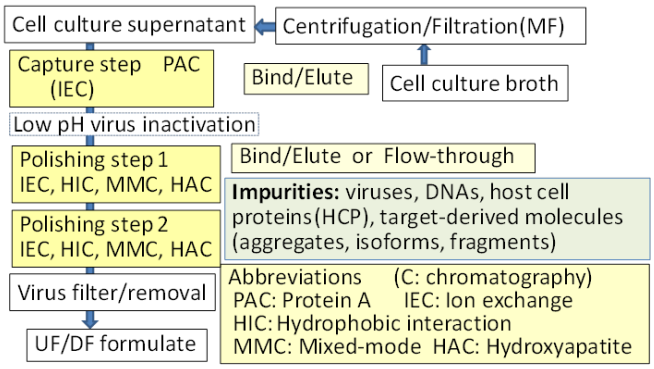

Fig. 1. Typical platform separation (downstream) process for monoclonal antibodies (mAbs).

However, the final product must be purified to a very low impurity level. For that reason, additional chromatography step called "polishing step" is needed. Various types of impurities are included in FCCS such as viruses, host cell DNAs, host cell proteins (HCPs) and product-derived molecules (aggregates, fragments and charge variants). More than one chromatography step is needed as the impurities differ so much in terms of physical and biochemical properties. Ion-exchange chromatography (IEC) [anion exchange (AIEC) and cation exchange (CIEC)], hydrophobic-interaction chromatography (HIC) and mixed-mode chromatography

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(MMC) are commonly used for polishing steps. The standard polishing chromatography sequence is AIEC-CIEC or CIEC-AIEC.

2 Materials and methods

Cell culture broths containing a monoclonal antibody mAb-A (IgG1 pI 7.7) or mAb-B (IgG1 pI 8.2) were used in this study. mAb concentrations in the sample feed were measured byusing analytical protein A chromatography. DNA concentrations were measured by using qPCR. HCP (host cell protein) concentrations were measured by ELISA method. HMW (high molecular weight) species, mAb monomer and LMW (low molecular weight) species were measured by size exclusion chromatography (SEC) using a TSK G-3000 SW_{XL} column. The column volume was 1 mL for anion-exchange chromatography (AIEC), cation exchange chromatography (CIEC) and activated carbon (AC). After the cell culture, the cells were removed by centrifugation and membrane filtration. The clarified liquid was then applied to protein A affinity chromatography. The eluted fraction by using a low pH buffer solution was incubated at low pH for virus inactivation. The feed sample for FTC was taken from the low pH virus inactivation tank, and adjusted to specified pH (5 or 7) and conductivity values (3 mS/cm). The details of the experiments are shown in **[2]**.

3 Results and discussion

3.1 Polishing chromatography steps

Let us take a look at standard IEC polishing steps. An IEC column is first equilibrated with a low salt buffer solution (mobile phase) at a fixed pH in order to bind proteins and impurities. After the sample loading, the column is washed to remove unbound or weakly bound impurities with the same buffer solution. Then, the elution buffer of high salt concentration at the same pH is fed to the column to desorb (elute) the product (mAb). Tightly bound impurities are then stripped from the column with a very high salt concentration buffer and sodium hydroxide solution. The column is then re-equilibrated for the second run. This operation is schematically shown in **Fig. 2.**

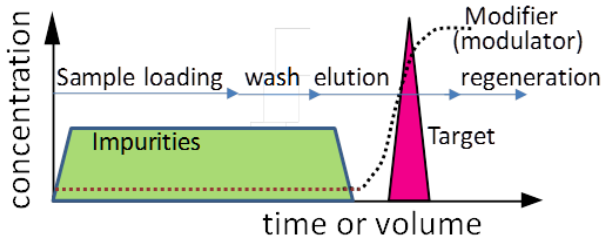

Fig. 2. Stepwise elution (bind/elute) chromatography.

Proper choice of the buffer pH and salt concentrations for both adsorption (bind) and elution (elute) is very important. In addition, the sample load and the flow-velocity are important parameters to increase the process efficiency (productivity). However, in most cases they were determined by trial and error approaches.

The efficiency of typical three chromatography DSPs is low. In order to increase the efficiency of DSP various continuous chromatography operation methods have been proposed. For capture chromatography, multi-column switching techniques called “periodic counter-current (PCC)” chromatography operation is known. Polishing chromatography steps can be also operated by using PCC. However, since the purpose of polishing steps is to remove a small amount of impurities from the sample feed it is possible to use negative adsorption operation mode called flow-through chromatography (FTC). While the sample feed is continuously fed to the column, the impurities are adsorbed and the flow-through stream from the outlet contains purified mAb in FTC **(Fig. 3)**. Although FTC is not a true continuous operation (the column has to be replaced after the breakthrough of the impurities), it is regarded as a pseudo-continuous operation.

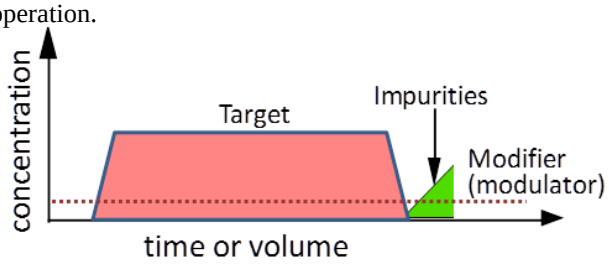

Fig. 3. Flow-through chromatography (FTC) operations.

3.2 Flow-through chromatography operations

In order to design the optimized FTC process it is needed to know the mobile phase condition where the target mAb is not adsorbed whereas the impurities are tightly bound. For IEC, the retention is basically understood based on the concept shown in **Fig. 4.**

Both AIEC and CIEC are needed to remove the impurities having different isoelectric points (pIs) by using FTC operations. Additional FTC of different adsorption mode (affinity interaction) such as activated carbons is efficient for removing the impurities that cannot be easily captured by AIEC and CIEC.

3.3 Polishing FTC of monoclonal antibody (mAb)

We have carried out FTC processes with two or three columns for removing impurities from the pooled fraction by protein A chromatography.

Table 1. Summary of purified product specifications **[2].**

Protein[1]	Process step[2]	Concentration (mg/mL)[3]	DNA[4] pg/mg	HCP[5] ng/mg	HMW1 (%)[6]	HMW2 (%)[7]	Monomer (%)[8]	LMW1 (%)[9]	LMW2 (%)[10]
mAb -A	1	2.55	< 11.8	< 0.4	0.02	0.49	98.4	1.08	0.01
	2	2.67	< 11.2	31.0	0.00	0.31	98.6	1.04	0.01
	3	2.43	< 12.3	< 0.4	0.00	0.07	98.8	1.13	0.02
	4	1.78	< 16.8	< 0.6	0.00	0.36	98.6	0.94	0.06
mAb -B	1	3.95	< 0.76	< 0.25	0.00	0.07	98.2	1.68	0.03
	2	3.06	< 0.98	< 0.33	0.00	0.57	98.2	1.16	0.03
	3	3.11	1.10	2.00	0.00	0.06	98.5	1.44	0.04
	4	3.60	0.70	< 0.28	0.00	0.29	98.2	1.44	0.04

[1]mAb-A IgG1 pI 7.7, mAb-B IgG1 pI 8.2
[2]See Fig. 5
[3] mAb concentrations in the sample feed were measured by using analytical protein A chromatography.
[4] DNA concentrations were measured by using qPCR.
[5] HCP (host cell protein) concentrations were measured by ELISA method.
[6-10] HMW species, mAb monomer and LMW species were measured by size exclusion chromatography (SEC).

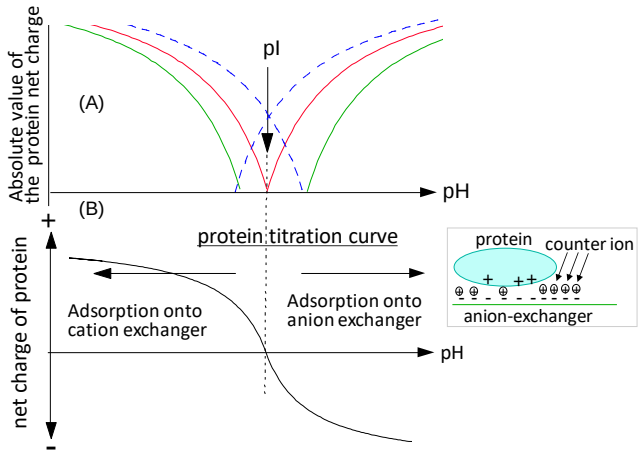

Fig. 4. Binding of proteins in ion-exchange chromatography **[1]**.

Table 1 summarizes the results by FTC polishing processes of mAbs with different sequence and conditions shown in **Fig. 5**. Impurities such as HCP, DNA, HMW and LMW were removed efficiently by using FTC processes (1-4) for both mAb-A and mAb-B. For mAb-A Process 3 was most effective whereas it was not efficient for mAb-B. These results show that impurity removal by FTC process depends on the cell culture liquid.

We also tried to carry out a fully connected FTC process (AC-AIEC-CIEC) at pH 6.0 and 4 mS/cm (**Fig. 6**). The monomer recovery was >95% and the monomer concentration was <95%. The impurities were reduced to levels below the specification (HCP <100 ng/mg, DNA <10 pg/mg).

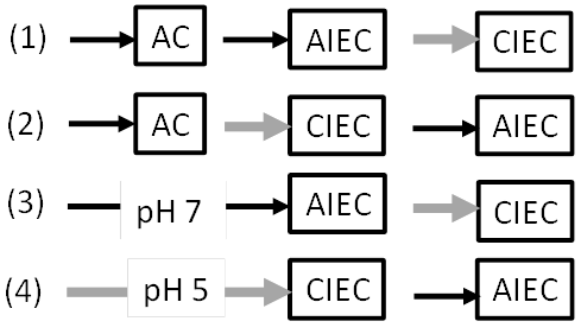

Fig. 5. FTC processes for removing impurities with two or three columns.

Black arrow (pH 7), gray arrow (pH 5).
AC:activated carbon,
AIEC:anion-exchange chromatography,
CIEC: cation exchange chromatography.

The success of FTC process relies on the method for determining proper pH and salt concentration *I* (conductivity) of feed sample. We have established a method for determining the distribution coefficient *K* as a function of *I* from linear gradient elution (LGE) experiments **[1]**. FTC process removing aggregates was successfully designed and carried out by using our method **[3].**

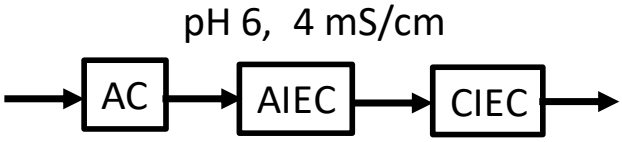

Fig. 6. Fully connected FTC process for removing impurities with three columns. The same sample as in Fig. 5 was used.

FTC is not a complete continuous system as the operation must be stopped before the breakthrough of the impurities as shown in **Fig. 3**. The breakthrough volume depends on the flow velocity due to pore diffusion based band broadening. Models considering the salt concentration dependent distribution coefficient and the zone spreading parameter such as pore diffusion **[1, 4]** are needed in order to design and operate FTC process properly.

Conclusion

We have shown that flow through chromatography (FTC) is an efficient and promising method for removing impurities. However, proper choice of mobile phase conditions such as pH and salt concentration is crucial. In this study three pH levels (5, 6 and 7) and three conductivity levels (3, 5, and 7 mS/cm) were examined. Mechanistic model based approach may make it possible to find the optimum pH and conductivity values. Sequence of different chromatography such as anion and cation exchange chromatography depends on monoclonal antibody and impurities contained in the liquid feed. Simple and reliable methods for designing efficient FTC processes are needed. Since the UV signal does not provide information on the breakthrough of impurities and/or the deterioration of the column, new methods for monitoring of impurities are required. This will enable us to do real-time release testing **[5].**

References

[1] Yoshimoto, N., Yamamoto, S. Simplified methods based on mechanistic models for understanding and designing chromatography processes for proteins and other biological products -Yamamoto Models and Yamamoto Approach- in Preparative chromatography for separation of proteins, Chap.4, pp, 111-157, Wiley, 2017.

[2] Ichihara, T., Ito, T., Kurisu, Y., Galipeau, K., Gillespie, C. Integrated flow-through purification for therapeutic monoclonal antibodies processing. mAbs, 10, 2018, 325-334.

[3] Suda, E. J., Thomas, K. E., Pabst, T. M., Mensah, P., Ramasubramanyan, N., Gustafson, M. E., Hunter, A. K.. Comparison of agarose and dextran-grafted agarose strong ion exchangers for the separation of protein aggregates. J. Chromatography A, 1216, 2009, 5256-5264

[4] Carta, G., Jungbauer, A. Protein chromatography: process development and scale-up. Wiley, 2010

[5] Jiang, M., Severson, K. A., Love, J. C., Madden, H., Swann, P., Zang, L., Braatz, R. D. Opportunities and challenges of real-time release testing in biopharmaceutical manufacturing, Biotechnology and Bioengineering, 114,2017, 2445-2456.