ILLUSTRATING CHROMATOGRAPHY WITH COLORFUL PROTEINS

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Advances in biology are prompting new discoveries in the biotechnology, pharmaceutical, medical technology, and chemical industries. Developing commercial-scale processes based on these advances requires that new chemical engineers clearly understand the biochemical principles behind the technology, and develop a firm grasp of chemical engineering principles.[1] To deliver this knowledge to students successfully, engineering educators require additional resources to illustrate relevant biological concepts throughout the curriculum.

In a typical bioprocess, the majority of costs are associated with isolating and purifying the desired biological compound.[2] In many of the later stages of purification, more than 50% use some type of chromatography.[3] Exposing students to biochromatography provides an introduction to bioseparations and the underlying biochemistry concepts. As separation processes are based on the physical and chemical properties of the product and chief impurities, a wide range of concepts can be included, such as overall cell composition, protein biochemistry, recombinant protein production techniques, and bioprocess optimization. Some bioseparation techniques (adsorption, ion exchange, and chromatography), however, are difficult to teach in a lecture-based format because they are rate-based, time-dependent processes.[4]

The use of visually appealing materials has been shown to motivate and captivate students in biology and chemical

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engineering settings.[5-12] To overcome the educational challenges presented by the technical material, an anion exchange chromatography experiment using a pair of colorful proteins was developed. This paper presents a detailed description of the experiment and summarizes the effect of operating parameters on the quality of protein separation. This experiment could be applied in three settings: core chemical engineering courses focused on separation processes, unit operation laboratory courses, and elective courses focused on biochemical engineering or bioseparations.

ION EXCHANGE CHROMATOGRAPHY

Chromatography was developed early in the 20th century by M.S. Tswett, who used the technique to separate plant pigments.[13-15] Two recent articles have outlined the life of Tswett and the development of chromatography, and are available in References 16 and 17. The following quote describes the invention of the term "chromatography" by Tswett:

> *"Like light rays in the spectrum, the different components of a pigment mixture, obeying a law, are resolved on the calcium carbonate column and then can be qualitatively and quantitatively determined. I call such a preparation a chromatogram and the corresponding method the chromatography method."*

The word "chromatography" was an appropriate choice, as it is composed of two Greek roots—"chroma" (color) and "graphein" (to write)—leading to a literal translation of "color writing." Although Tswett theoretically envisioned the concept of elution chromatography, where each compound migrates through the column and exits the column in the liquid phase, this was not actually used until the 1930s by others. Tswett preferred to end his chromatographic separations with the colored rings still on the column, and obtained pure components by pushing the resin out of the column with a wooden rod and slicing off individual bands with a scalpel.

Ion exchange chromatography exploits differences in electrostatic interactions between the various proteins and the resin.[18] In anion exchange chromatography, the resin has a positive charge, and proteins with a negative charge on their surface will exhibit an attraction for the resin. To recover bound proteins, the electrostatic interaction between resin and proteins is disrupted, typically by increasing the salt concentration or changing the pH of the mobile phase. Proteins can be separated based on the strength of their interaction with the resin, as more weakly bound proteins can be easily removed by increasing the salt concentration, while tightly bound proteins require extreme salt concentrations or pH to be removed. Using gradient elution, individual proteins can be recovered in a relatively concentrated pool. This differs from common migration chromatography techniques, such as gas and reversed-phase liquid chromatography, where a short pulse of sample is applied to the column and is diluted as it travels through the column.

Ion exchange chromatography is generally performed in a six-step process using three aqueous solutions: a buffer with a low salt concentration at an appropriate pH, a buffer with a high salt concentration at the same pH, and the protein sample at the same pH and with a low salt concentration (Figure 1). Broad guidelines for the duration of each step are reported in parentheses in terms of column volumes, defined as the product of the cross-sectional area and length of the column. During period "A," low-salt buffer at an appropriate pH is delivered to the column to equilibrate the resin (3-5 column volumes). During period "B," the sample is applied to the column (sample volume). During period "C," additional low-salt buffer is delivered to the column to wash away any unbound protein (1-2 column volumes). During period "D," the concentration of salt in the buffer is slowly incremented to selectively elute the proteins (3-5 column volumes). During period "E," additional high-salt buffer is delivered to remove tightly bound protein (1-2 column volumes). During period "F," the column is re-equilibrated with low-salt buffer (1-2 column volumes). A pH gradient may be used in place of a salt gradient in ion exchange chromatography. Shaped gradients or a series of steps may be substituted for a linear gradient in period "D."

Anion exchange chromatography resin and chromatography columns are available from a variety of sources. In this paper, DEAE Sepharose Fast Flow resin (GE Healthcare, catalogue# 17-0709-10, $50 for 25 mL) and 24 mL low-pressure Kontes columns (Fisher, catalogue# K420401-1030, $20.17 per column) were used. Chromatography resin was prepared and packed into a column using the directions supplied with the resin. A variety of fluid delivery systems can be used, including pipette and gravity-fed flow, peristaltic pumps, and complete chromatography systems such as the Akta Basic from Amersham Biosciences (results in Figures 3 and 4, page 245). Additional information on the theory of ion exchange chromatography and equipment needs can be found in bioseparation or biochemical engineering textbooks.[18-20]

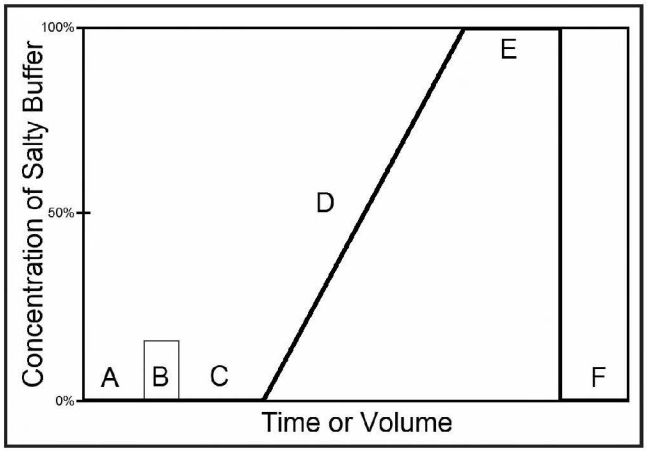

Figure 1. *Outline of general gradient-based chromatography method.*

COLORFUL PROTEINS

Colorful proteins with different physical properties were selected for the experiment. In order to illustrate the challenging nature of biological separations, two proteins with similar ionic properties were chosen. Table 1 describes the physical properties of the two proteins.

DsRed2 is a large, tetrameric fluorescent protein that absorbs light at 558 nm and emits light at 583 nm, giving the protein its characteristic reddish color.[22] EGFP is a smaller, monomeric fluorescent protein that absorbs light at 488 nm and emits light at 508 nm, giving the protein its characteristic green color.[23] Both proteins are very bright, with extinction coefficients over 40,000 $M^{-1}cm^{-1}$.[23,24]

At Rowan University, these proteins have been produced by students in Junior and Senior Clinic through recombinant protein expression in bacteria. DsRed2 is also available from commercial sources (*e.g.*, Clontech, catalogue# 632436, $300 for 100 µg). Many variants of EGFP, which should display similar purification behavior, are commercially available (*e.g.*, Clontech, catalogue# 632369 for GFPuv, $293 for 100 µg). Recombinant protein expression in bacteria is inexpensive, as expression of colorful protein DNA (with *E. coli* BL21(DE3) cells transformed with pET21d plasmid containing the subcloned colorful protein DNA) using standard recombinant DNA techniques[7,8] has resulted in a protein cost of roughly $2 per mg. The results in Figures 3 and 4 were obtained using approximately 500 µg of each protein.

TABLE 1
Physical Properties of the Colorful Proteins

Protein	Color (λ_{max})	Molecular Weight[21]	Isoelectric Point[21]
DsRed2	Pink (558 nm)	103 kDa	6.3
EGFP	Green (488 nm)	27 kDa	5.6

TABLE 2
pK Values for Side Chains of Amino Acids[27]

Amino Acid	pK	Number in Protein
Carboxy terminal	2.34	$n_1 = 1$
Aspartic acid (Asp, D)	3.86	n_2
Glutamic acid (Glu, E)	4.25	n_3
Cysteine (Cys, C)	8.33	n_4
Tyrosine (Tyr, Y)	10	n_5
Amino terminal	9.69	$n_6 = 1$
Histidine (His, H)	6	n_7
Lysine (Lys, K)	10.5	n_8
Arginine (Arg, R)	12.4	n_9

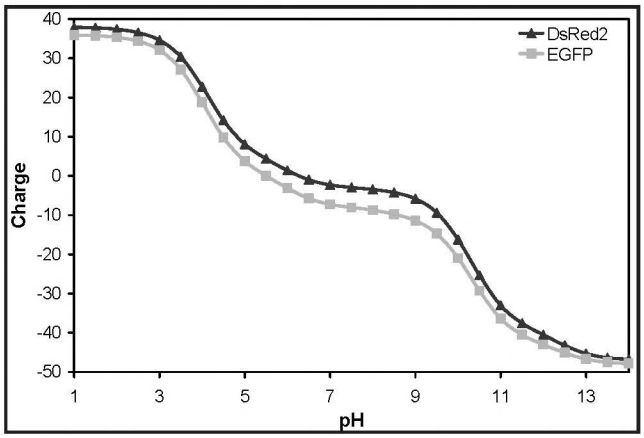

Figure 2. *Protein titration curves for EGFP and DsRed2.*

CHROMATOGRAPHY METHOD DEVELOPMENT

Separating proteins during the gradient portion of an ion exchange separation requires two elements. For the proteins to bind to the charged resin, they must have an oppositely charged patch on their surface. For the proteins to elute at different positions in the gradient, they must have different binding affinities for the resin. The net charge over the entire protein can be used as an initial estimate of the surface ionic character of the protein.

The isoelectric point is defined as the pH at which the protein has no net charge. Above the isoelectric point, the protein will adopt a net negative charge. The isoelectric point and molecular weight of the protein monomers were calculated from amino acid sequences using the Web-based program ProtParam.[21]

In addition to the isoelectric point, it is also important to consider the bulk protein charge over a range of pH values when designing a separation based on ion exchange. A protein titration curve can be constructed using a Web-based program or by building a spreadsheet to perform the calculation.[25, 26] Briefly, the bulk protein charge at a given pH can be calculated from the pK values for the ionizable amino acid side chains using the information in Table 2 and Eq. (1).

$$\text{protein charge} = -(n_1 + n_2 + n_3 + n_4 + n_5) + \sum_{i=1}^{i=9} \frac{n_i * 10^{-pH}}{10^{-pH} + 10^{-pK_i}} \quad (1)$$

To match the Web-based program, pK values from Lehninger are reported.[27] Values from other biochemistry textbooks may be substituted. Computing the protein charge over a range of pH values leads to a protein titration curve (Figure 2). Examination of this curve can help identify a useful pH range for separation, where the proteins will bind to the resin with different affinities. This requires that the signs of individual protein charges are the same, but the magnitudes are different. For the EGFP and DsRed2 case, a pH value between 6.5 and 8.5 is appropriate for anion exchange.

QUANTIFYING CHROMATOGRAPHIC SEPARATION

The quality of a chromatographic separation can be quantified by a resolution calculation. This is illustrated in Eq. (2).[18,28]

$$\text{resolution} = \frac{V_{max,b} - V_{max,a}}{0.5(w_{b,a} + w_{b,b})} \qquad (2)$$

$V_{max,i}$ represents the volume at which peak i displayed maximum signal, and $w_{b,i}$ represents the baseline width of peak i, based on the intersection of peak tangents with the baseline. When the resolution is one, the peaks have an overlap of about 2%. As the resolution decreases, the peaks overlap further, until, at a resolution of zero, the peaks elute at exactly the same position. Examples of resolution calculations can be found in the Sample Calculations section of this article and in textbooks on separation processes.[28]

EXPERIMENTAL INVESTIGATION

Table 3 summarizes the materials used in this experiment. For columns with smaller diameters, less material is required. The majority of materials can be reused. As long as the maximum pressure is not exceeded, the column should last indefinitely. The resin can be cleaned according to the manufacturer's recommendations, and proteins can be recovered and reused for many experiments. An additional option is to produce the proteins in-house through recombinant protein production methods, which essentially eliminates the protein cost.

Anion exchange chromatography experiments were developed to show that a mixture of DsRed2 and EGFP can be selectively eluted at different salt concentrations, providing a powerful demonstration of the principles of protein binding and elution. This style of experiment is suitable for unit operation laboratories and upper-level elective courses with laboratory components. To illustrate the importance of process parameters on ion exchange chromatography performance, two proteins with similar ionic properties were chosen. This resulted in a challenging protein separation that was sensitive to process conditions.

In addition to the chromatography column and related tubing, three solutions are needed for the experiment: a buffer with a low salt concentration (Buffer A), a buffer at the same pH with a high salt concentration (Buffer B), and a separated protein sample (Sample). Chromatography experiments were performed at pH values between 7.5 and 8.5. Buffer A was typically 50 mM Tris (pKa = 8.3) at the pH of interest. Buffer B was typically 50 mM Tris, 300 mM NaCl at the pH of interest. Sample was typically prepared by diluting concentrated stocks of DsRed2 and EGFP into Buffer A. For the experiments, at a pH value of 7.5, 50 mM sodium phosphate was used as the buffer. For experiments at pH values below 7.5 or above 9.0, an alternative buffer should be selected, as buffer pKa should not deviate from solution pH too significantly.

Experiments were performed on an Amersham Biosciences Akta Basic chromatography unit, equipped with a UV detector capable of monitoring three individual wavelengths. Total protein was monitored at 280 nm, EGFP was monitored at 488 nm, and DsRed2 was monitored at 561 nm. Alternatively, the process could be monitored off-line by collecting small fractions and measuring the absorbance on a visible spectrophotometer.

RESULTS AND DISCUSSION

Six methods were evaluated for protein separation effectiveness. For each method, the separation resolution was calculated using Eq. (2). Table 4 compares the resolution for each method, illustrating the effect of buffer pH, salt concentration, and gradient shape on separation quality.

Figure 3 presents a typical chromatogram for method 4. The black curve is the absorbance at 280 nm, which tracks all proteins (A280). The dark gray curve is A561, which tracks

TABLE 3
Materials Required for Experiment

Item	Quantity	Price
Kontes 24 mL column	1	$20
DEAE Sepharose fast flow resin	25 mL	$50
25 mM Tris, pH 8.0	200 mL	$0.06
25 mM Tris, 200 mM NaCl, pH 8.0	100 mL	$0.09
Enhanced green fluorescent protein (EGFP) From vendor Produced in-house	 500 µg 500 µg	 $1,500.00 $1.00
DsRed2 From vendor Produced in-house	 500 µg 500 µg	 $1,500.00 $0.15

TABLE 4
DsRed2 – EGFP Separation Resolution

Method	pH	Salt Gradient	Resolution
1	8.5	Linear from 20 to 300 mM NaCl	0.02
2	8.0	Linear from 20 to 300 mM NaCl	0.32
3	8.0	Steps at 80, 125, 170, 215, 300 mM NaCl	0.58
4	8.0	Steps at 20, 50, 80, 110, 140 mM NaCl	0.48
5	7.5	Linear from 0 to 300 mM NaCl	0.51
6	7.5	Step at 135 and 150 mM, linear to 300mM	0.72
7	7.5	Steps at 30, 60, 90, 105 mM NaCl	0.66

DsRed2, and the light gray curve is A488, which tracks EGFP. Figure 4 presents a time-lapse image of the proteins separating as they move through the column (also available in color as Figure 4 in Reference 12).

Complete separation was never achieved, as the ionic properties of EGFP and DsRed2 are very similar. The quality of separation is strongly affected by buffer pH and moderately affected by the shape and type of gradient.

SAMPLE CALCULATIONS

To illustrate the use of Eq. (1), consider EGFP. This protein contains one carboxy terminal (n_1=1), 18 Asp (n_2=18), 16 Glu (n_3=16), two Cys (n_4=2), 11 Tyr (n5=11), one amino terminal (n_6=1), nine His (n_7=9), 20 Lys (n_8=20), and six Arg (n_9=6) residues. Using Eq. (1):

$$\text{protein charge} = -(1+18+16+2+11) + \frac{1*10^{-\text{pH}}}{10^{-\text{pH}}+10^{-2.34}} + \frac{18*10^{-\text{pH}}}{10^{-\text{pH}}+10^{-3.86}} + \ldots$$

At a pH of 9.5:

$$\text{protein charge} = -48 + 6.9\text{x}10^{-8} + 4.1\text{x}10^{-5} + 9.0\text{x}10^{-5} + 0.13 + 8.4 + 0.61 + 2.8\text{x}10^{-3} + 18 + 6.0$$

$$\text{protein charge} = -14.7$$

To illustrate the use of Eq. (2), consider the separation shown in Figure 3. For EGFP, $V_{max,B}$ = 64.5 mL and $w_{b,b}$ = 16.8 mL. For DsRed2, $V_{max,A}$ = 57.2 mL and $w_{b,a}$ = 13.5 mL. Using Eq. (2):

$$\text{resolution} = \frac{64.5\text{mL} - 57.2\text{mL}}{0.5(16.8\text{mL} + 13.5\text{mL})} = 0.48$$

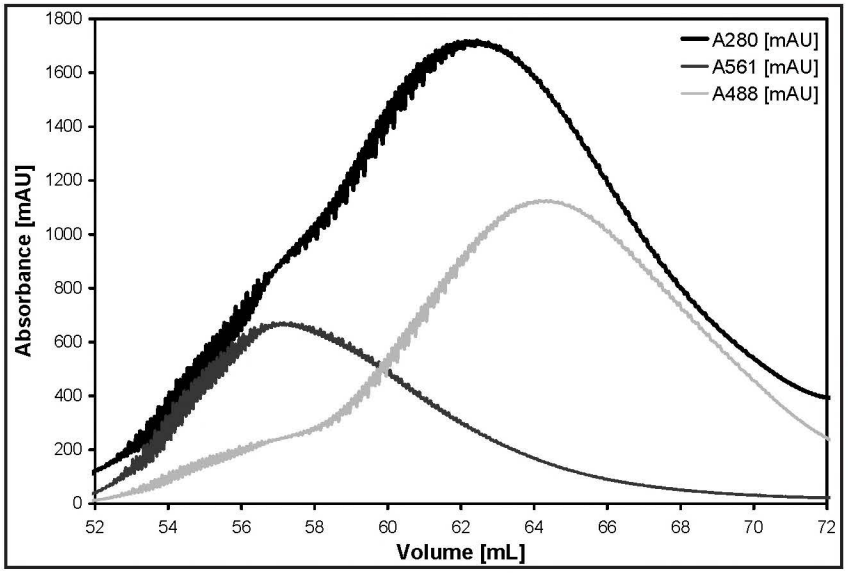

Figure 3. *Chromatogram for step gradient at pH 8.0 (method 4).*

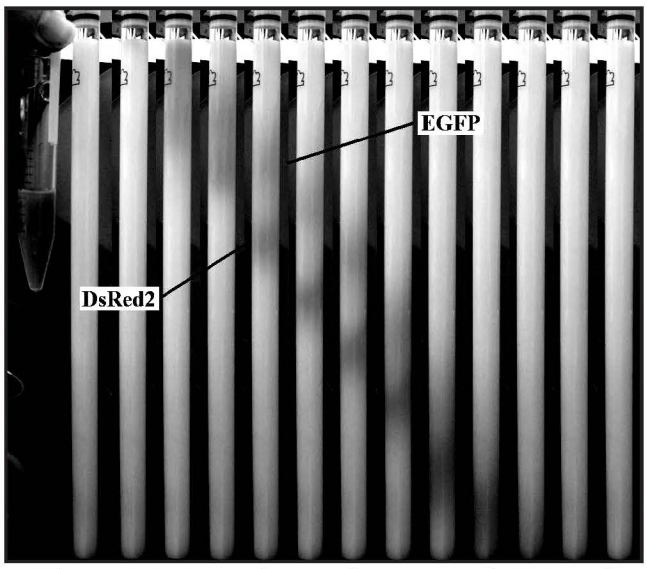

Figure 4. *Anion exchange of a mixture of EGFP and DsRed2 using method 4 (see Table 4). Also available in color as Figure 4 in Reference 12.*

COURSE IMPLEMENTATION

In any setting, this experiment illustrates the effect of protein properties and operating conditions on separation quality. At an introductory level, lecture material focused on protein and chromatography resin properties could be combined with one or two experiments to illustrate a "real" protein separation. This type of coverage may be appropriate for a core separations course. Extended student experimentation, where students evaluate separation quality for multiple methods, would allow students to discover the effect of operating conditions on separation quality. This type of coverage may be appropriate for unit operations laboratories. In a biochemical engineering or bioseparations elective, this experiment can be combined with additional material to highlight the need for multiple separation techniques in order to produce a pure protein product. The material on isoelectric point and titration curve prediction can also be used as a stand-alone item in a variety of settings.

SUMMARY

An experiment in anion exchange chromatography using a pair of colorful proteins has been described. This material allows instructors to introduce important biochemical engineering and physical biochemistry principles into the chemical engineering curriculum. The visual appeal and low cost of supplies will make the experiments an effective teaching tool in core courses focused on separation processes. The variety of possible behavior will make the experiments a robust addition to unit operations laboratories or biochemical engineering electives.

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