Development of an economical and green chromatographic system for lanthanide purification

and

Progress toward protein-polymer hybrids from estrogen receptors for the detection and removal of organic pollutants from water

by Troy Moore

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate Division of the University of California, Berkeley

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Abstract

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The expensive separation procedures necessary to produce pure metals have resulted in a small number of entities producing nearly all of global demand. Market control by only a few entities has, in turn, led to price instability. Increasing global consumption of lanthanide-based materials has highlighted the need for parity in lanthanide production. As more entities attempt to enter the market, they face the significant challenges of overcoming the financial and environmental costs of lanthanide separation. To address these obstacles, a new chromatographic system is created from a cheap, commercially available, and widely produced polystyrene resin. This resin was further synthetically modified by various methods, and chromatographic performance tested. One such synthetically modified resin was used as a new solid phase in a chromatographic system, which exhibits excellent separation of an industrially relevant lanthanide mixture. Furthermore, cost, operating conditions, and the waste stream of this system are extremely competitive with currently employed industrial methods.

The concentration of estrogenic compounds in fresh water sources and observed correlation with deleterious effects in wildlife has been met with increasing concern. As these estrogen concentrations grow, so does the need to detect estrogen concentrations in the field. A reusable protein-polymer conjugate composed of the estrogen receptor and synthetic polymers is proposed to meet this need. For use as the protein component, three different estrogen receptors have been expressed in *E. coli*. Two of these receptors have been successfully modified specifically at the C-terminus, and one at the N-terminus, with ketones to facilitate the attachment of the polymer component. A new monomer has also been synthesized. Further work in constructing and testing the material is underway.
For my mother and my sisters, Sacha and Taryn.
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Chapter 1

An overview of the chemistry and history of the rare earth elements

1.1 Abstract

The rare earth elements present a complex separations problem due to their highly similar chemical nature and the circumstances of their natural occurrence. Despite these complications, the value of rare earth metals in hi-tech materials is sufficiently high to encourage a vast global industry. However, global production has recently been narrowed due to the relatively high cost of producing purified rare earth materials in certain countries. This trend is specifically related to the cost of separation of the rare earth elements. The following serves to examine the economic and environmental challenges associated with state-of-the-art separation methods, as well as the underlying implications for improving upon the current methodology.
1.2 Introduction

Every first year chemistry student has to learn the periodic table. Some of us only learn the parts that will be addressed in our first semester of general chemistry. More ambitious students, or perhaps those with crueler instructors, learn every last chemical symbol. In either case, after the first semester of balancing chemical equations and converting units, many seem to forget a multitude of the names and symbols so frantically scribbled down onto flashcards just a few months prior. Among those easily forgotten, perhaps due to their virtual absence in introductory curriculum or perhaps just due to the spelling challenge they present, are the rare earth elements.

The rare earth elements are comprised of the lanthanides—lanthanum through lutetium—as well as yttrium and scandium. While the rare earths may not hold the same prevalence in the popular psyche and industrial sector as iron, for instance, they are ubiquitous in hi-tech components. Computer displays, refrigeration, permanent magnets, data recording devices, lasers, and microwave equipment are just a small cross-section of an expanding list of applications for these metals.¹ Perhaps a more concrete example of the ubiquity of these metals is demonstrated by the Toyota Prius. To construct the motor of this popular hybrid car, between 2 and 4 pounds of the rare earth neodymium is required, while the battery requires an additional 10 to 15 pounds of lanthanum.²,³ Given such a significant requirement for a single, popular consumer good, it becomes easy to accept that the demand and use for these metals is quite high.

This high demand has become one of the defining characteristics of rare earth elements. To satisfy this need, various processes have to work efficiently and at low cost. As is the case with many other mined materials, there must be a mine from which to extract the rare earth elements, a rough processing step, and purification, which will be further discussed later. In the case of rare earth elements, however, purification provides an especially difficult challenge given the nature of the chemistry and natural occurrence of the rare earth species.⁴ Consequently, we, along with many others, sought to meet this challenge of purification by improving current purification systems used within the industry via a chromatographic approach.

First, however, it is useful to introduce the basis of the challenges in rare earth separation. Generally, in order to justify a specific course of research in separation methodology, there are really only three things that must be addressed: the nature of the species being separated, the benefit of separating these species, and the feasibility of the separation. If reasonable explanations exist for these points, then the rest of the details are secondary. Accordingly, the following serves to satisfy these conditions with explanations. First, the nature of the species to be separated, the rare earth elements, will be addressed by examination of their natural occurrence and chemistry. Subsequently, the myriad uses of the separated materials, and high demand for these materials, will be discussed. Finally, a selection of separation techniques will be covered, with special attention given to scale, cost, and efficacy.
1.3 Natural occurrence and abundance

While one might surmise that the moniker of “rare,” and the frequent absence in general chemistry courses, would mean that these elements are, in fact, scarce, they really aren’t. These elements make up a total of 4.14% of the total composition of the Earth’s crust, mainly owing to the huge abundance of yttrium, scandium, and the lighter lanthanides, lanthanum through neodymium (Figure 1-1).5 To demonstrate rare earth abundance in more relatable terms, simple comparison is useful. Total rare earth crustal abundance is 205.68 ppm, slightly higher than that of carbon (200 ppm). Cerium, the most abundant in the set, at 60 ppm, approaches the 70 ppm concentration of zinc. Finally, thulium and lutetium, the rarest in the set, comprising a combined concentration of approximately 1 ppm, are half as common as tin, at a concentration of 2 ppm.5

As indicated, rare earths are rather prevalent, occurring in deposits around the world and in various geological configurations. Predominantly, rare earth mixtures are found in hard rock sands and placer deposits, the latter due to the relatively high density of the elements. As of the release of the United States Geological Survey Circular 930-N, 123 unique rare earth deposits in 20 countries spanning 5 continents were known.6 Among these numerous deposits and minerals found within, three principal ores have emerged: monazite, bastnaesite, and xenotime. These ores are important due to their reasonably high occurrence, relative to other rare earth-containing minerals, and the distribution of rare earth elements within each. Monazite and bastnaesite are rich in lighter rare earths, while xenotime provides a good source of heavier rare earths and yttrium (Figure 1-2).7,8

As is evident in Figure 1-2, the commercially significant ores of monazite, bastnaesite, and xenotime, contain an uneven mixture of their rare earth components. This is inherently problematic in any mining operation. A mixed ore, such as these, requires further and typically more complicated purification than minable metals that do not occur as complex, multicomponent mixtures. For instance, copper often occurs and is mined as chalcocite (CuS) or chalcopyrite (CuFeS2), from which the significant component of copper can be recovered through straightforward metallurgical processes.9 Unfortunately, owing to the multicomponent nature of rare earth ores, as well as the similar chemical properties of the rare earth elements, purification is much more technically intensive.

1.4 Chemical nature of the rare earth elements

The f orbitals are a defining characteristic of the lanthanides, and a significant factor in the high degree of chemical similarity across the group. When it is considered that the lanthanides make up most of the rare earth elements, this feature is especially relevant to the topic. As the period is traversed beyond cerium, the 4f electrons contract to a lower energy level than 5d and 6s electrons, due to the strong interactions of the 4f electrons with the increasingly positively charged nucleus.10 This results, generally, in the lack of participation of the 4f electrons in any sort of bonding or complexation, as they are held tightly by the nucleus in a sufficiently low energy state. Accordingly, while various other
Figure 1-1. (a) Total crustal abundance of rare earth elements relative to all other elements. (b) Distribution of lanthanides in Earth’s crust. (c) Absolute concentration of each lanthanide found in Earth’s crust.
Figure 1-2. Rare earth element distributions in principal mined ores. Monazite and bastnaesite are principal sources for light and medium rare earths, while xenotime provides a viable source for heavy rare earths.
oxidation states are possible, a preferred +3 oxidation state is adopted by the lanthanides, following the general electron configuration of [Xe]4f

This preferred oxidation state, seen frequently in natural formations of rare earths, coupled with the chemical similarity of the 3+ lanthanide ions (Ln³⁺), imposes a greater challenge to rare earth separation by complexation.

Somewhat mitigating the separation challenges brought about by this charge state preference and resulting similarity is the reduction in atomic radius across the period; more familiarly, the lanthanide contraction. While the 4f electrons in the lanthanides penetrate strongly to the nucleus, they also shield the (usually) higher energy 5d and 6s electrons poorly from nuclear charge. This results in a gradual decrease in atomic radii across the period. Moreover, this trend holds for the Ln³⁺ ions (Figure 1-3). While size differences between neighboring ions are not particularly disparate, the 22% radial decrease across the period, from La³⁺ to Lu³⁺, is sufficient to impact complexation behavior. Because of this, as well as small differences in electronegativity, complexing methods and agents can be used to differentially bind Ln³⁺ ions.

1.5 Resource economics

As previously mentioned, rare earth deposits are known in locations scattered across the globe. Prior to roughly 1997, the United States shared global production of rare earths, with other major contributors including China, France, Japan, and Russia, among others. Around this time, domestic demand began to decrease slightly, prompting a decrease in domestic production. This coincided with the turning point in globally shared production: the rise in Chinese-controlled production of rare earths. The impetus for the domestic, and eventually global, trend of increasing reliance on the growing Chinese rare earth market is starkly apparent by examination of total rare earth production in the US versus that of China over the short period of 1986 to 2008 (Figure 1-4). By 2009, according to the USGS, China mined 120,000 metric tons of rare earths, slightly less than 97% of globally, commercially available rare earths, while holding reserves of 36,000 metric tons, amounting to nearly three times that of the United States. This drastic transformation in production and import dynamics of the rare earth commodities market has had a profound impact on domestic and global commerce. When China began to overtake the production market in the mid-1990s, the level of Chinese production greatly offset the previous global production balance. Additionally, due to many factors, including relaxed environmental standards, Chinese rare earths began to enter the market at a significantly lower cost than those produced in the US or other countries. By 2008, worldwide consumption of rare earths was 129,000 metric tons, being almost entirely supplied by Chinese operations. With the global market empty of competitors, and China in control of the vast majority of production, they gained the ability to unilaterally set prices, which were often quite high. In general, this global dependence on Chinese rare earths has led directly to price volatility, owing to the Chinese-controlled near monopoly on supply. To put this into perspective, from 1998 to 2004 the cost of neodymium oxide rose from about $22.00 to $28.50 per kilogram.
Figure 1-3. Atomic radii of rare earth cations (M³⁺) demonstrate lanthanide contraction and similar size between cations across the lanthanide group. Circular, graphical representations are drawn to scale.

<table>
<thead>
<tr>
<th>Element</th>
<th>Radii (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>La</td>
<td>1.061</td>
</tr>
<tr>
<td>Ce</td>
<td>1.034</td>
</tr>
<tr>
<td>Pr</td>
<td>1.013</td>
</tr>
<tr>
<td>Nd</td>
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</tr>
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<tr>
<td>Tb</td>
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<td>Dy</td>
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<tr>
<td>Yb</td>
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<td>Lu</td>
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<td>Sc</td>
<td>0.680</td>
</tr>
<tr>
<td>Y</td>
<td>0.880</td>
</tr>
</tbody>
</table>
Figure 1-4. Annual rare earth production comparison between the United States and China from 1986 to 2008.
In 2008, this same quantity was sold at $60.00 per kilogram. Subsequently, in 2011, neodymium metal reached a high of about $270 per kilogram. Currently, the metal is valued at close to the 2008 price. This new standard of highly unstable prices and artificially restricted supply has rekindled interest in and economic feasibility of reopening closed mines and processing plants in other countries, including the United States.

1.6 Separation

As has been established, rare earth materials are in high demand and there is sufficient mining production to satisfy the need. However, ores are mined exclusively as mixtures, which are not useful in mature applications. Therefore, rare earth separation methods are a crucial component in the production workflow. Unfortunately, owing to the reasonably high degree of chemical similarity between the lanthanides, separation is also one of the greatest challenges in the process of creating rare earth compounds of value.

Historically, minute differences in solubility were employed to separate the lanthanides from one another. In 1911, James published the first successful isolation of thulium from rare earth ores by means of fractional crystallization. This work included the extremely tedious recrystallization of lanthanides from the acid extracts of large quantities of rare earth ores, separation of lanthanide-containing crystals into subgroups based on solubility, and continuous analysis of mother liquors to confirm the total extraction of thulium. Ultimately, after a colossal amount of bench work and over 15,000 recrystallizations of the final product, pure thulium was isolated.

While James’s work was a great achievement in both chemistry and dedication, the method clearly did not lend itself to large-scale application. In the intervening 100 years, methods of rare earth separation have advanced considerably, providing the means to produce hundreds of thousands of metric tons of purified rare earths each year. Newer methods can be split into four loose groups: traditional, gravity-based ion exchange chromatography; modern affinity and ion exchange chromatography assisted by HPLC; fractional extraction; and specialty separation methods. The following will serve to outline each of these categories, and provide representative examples of each, as well as the relevant advantages and disadvantages of the techniques.

1.6.1 Low pressure ion-exchange chromatography

The preferential and highly similar Ln\(^{3+}\) states of water solubilized lanthanides are but one hurdle to overcome in their separation. Fortunately, it was correctly presumed that the ionic radii of the species were sufficiently different from one another to employ coordination-based separation techniques. Early work in this field was done by Ketelle and Boyd in 1947, who developed one of the early ion-exchange chromatography techniques that could separate complex mixtures of lanthanide ions. At the time, interest in separating and purifying lanthanides was largely motivated by an interest in further
studying the pure metals, as virtually no, relative to the current state of the art, consumer materials employed rare earths. Given the ultimate goal of purity, methods of this era were somewhat less concerned with yield and efficiency than is the current standard.

The chromatographic apparatus that Ketelle and Boyd constructed is shown in a simplified diagram to highlight the core components (Figure 1-5). Briefly, a simple glass column was packed with either the commercially available Dowex-50 or Amberlite-1 resin, equilibrated with water, lanthanide solutions adsorbed onto the resin, and the loaded species were eluted with a solution of citric acid and ammonium citrate. These sulfonated polystyrene resins were advantageous in that reasonably concentrated lanthanide mixtures could be strongly adsorbed onto them due to the high degree of oxophilicity of the Ln$^{3+}$ ions and the high exchange capacity of the resins. After adsorption, separation was effected by ion-exchange with ammonium ions, and further enhanced by the solution-phase equilibrium between the Ln$^{3+}$ ion and Ln$^{3+}$-citrate complex. The combination of differential adsorption affinities of each Ln$^{3+}$ ion for the resin, exchange equilibria with ammonium, and solution-phase equilibria between each Ln$^{3+}$ ion and Ln$^{3+}$-citrate complex provided, at the time, excellent resolution of lanthanides by this method.

After this system was demonstrated as a viable method for the separation of lanthanide mixtures, Spedding et al. continued to study the parameters of the system in 1950. In this case, efforts were made to determine optimal flow rate, temperature, and pH, in addition to determining a suitable maximum loading capacity. Using resin beds of 2.2 cm x 120 cm, it was found that a very low linear flow rate of 0.5 cm/min with an eluent pH of 3.80 provided optimal separation of gram-scale loads of rare earth oxides. While this finding was encouraging, in that it suggested this type of adsorption chromatography might be used effectively on a preparatory scale, the resolution and throughput of the system was ultimately not sufficient to meet the current demand of purified rare earth metals. Nonetheless, these works provided important, foundational knowledge for more modern techniques, as well as much of the research done in this work.

1.6.2 Modern ion-exchange chromatography

The pace of progress in lanthanide chromatography rapidly increased after initial ion-exchange chromatography work. Efficacious methods for lanthanide chromatography expanded to include thin layer chromatography, numerous and improved column chromatographic methods, and even gas chromatography. Of all of these methods, there was a marked increase in the employment and improvement of column chromatography techniques. This improvement was largely owed to the commercial availability of a variety of specialized resins, the use of high performance liquid chromatography (HPLC), and the discovery of better complexing agents. The advent of HPLC was of particular importance, as it allowed much greater resolution and much faster separations, owing to the amount of pressure that could be applied.

Due to both the greater resolving ability of HPLC and more effective complexing agents,
Simplified schematic of chromatographic apparatus used in Ketelle and Boyd’s adsorption chromatography method. Solubilized rare earths are loaded at the top of the column where they are adsorbed onto the sulfonated polystyrene resin. An appropriate eluent is introduced to effect ion exchange of the adsorbed rare earth ions. Temperature is controlled by maintaining heat exchange with water or steam in the column jacket (purple).

**Figure 1-5.** Simplified schematic of chromatographic apparatus used in Ketelle and Boyd’s adsorption chromatography method. Solubilized rare earths are loaded at the top of the column where they are adsorbed onto the sulfonated polystyrene resin. An appropriate eluent is introduced to effect ion exchange of the adsorbed rare earth ions. Temperature is controlled by maintaining heat exchange with water or steam in the column jacket (purple).
Continuous ion-exchange chromatography became a powerful tool, giving excellent resolution of complex lanthanide solutions. In 1986, Barkley et al. demonstrated how refined this technique had become, crisply resolving the entire lanthanide series, as well as yttrium, uranium, and thorium. In contrast to early experiments, which used gravity-based flow and ion-exchange resin, a hydrophobic HPLC C-18 column, using ammonium N-octylsulfonate as the exchange species, and α-hydroxyisobutyric acid (α-HIBA) as the complexing agent in solution was used. It is worth briefly noting that α-HIBA has for some time been commonly accepted as a gold standard of lanthanide complexing agents, and is extremely important to work described later. Moreover, the entire series was separated in the course of roughly 16 minutes, much faster than the low resolution, low pressure columns previously developed. However, the major drawback of this method, as is the case with all HPLC methods, is the throughput of the technique. In this experiment, only 25 ng of each species was separated. Additionally, while the high pressure used drastically improves resolution, it cannot be reasonably be scaled to accommodate a larger column with a resin bed of sufficient capacity to give comparable separation to that obtained in this work.

Despite the capacity limitations imposed by HPLC-based separation methods, they persist in the field due to the excellent separation they provide, analytical-scale utility, and convenience as a testing method for new chromatographic systems. Recent work with this technique has served to fine tune separation parameters to give the most efficient and highest resolution separations. For example, Datta and coworkers have provided useful speciation and complex stability data for Ln\(^{3+}\)-α-HIBA complexes formed during HPLC separation. Having these data readily available is a key component in designing new rare earth separation systems that utilize α-HIBA as a complexing agent. The effectiveness and availability of α-HIBA, existence of these data, and the continued use of HPLC systems are all helpful in improving the overall state of the art.

### 1.6.3 Extraction

Fractional crystallization is slow, traditional low pressure chromatography provides poor resolution, and HPLC-based methods are extremely limited in separation capacity. While all of these methods have their uses, they inadequately address the problem of scale. Faced with the challenge of separating hundreds of thousands of metric tons of rare earths annually, a large capacity, high-throughput technique is essential. Consequently, extractive methods have emerged as the leading industrial processes to produce rare earth metals in high purity and in vast quantities in a reasonable amount of time.

The impetus for modern extractive separation technologies was largely provided by activity in the field of fractional precipitation. By the late 1950s, slight differences in solubility between lanthanides were being even more effectively exploited by testing more discriminating precipitation media. Carrón and coworkers, in 1958, provided a comprehensive report on fractional precipitation methods for lanthanide separation using phosphoric acid. While the work did not produce exceptionally pure rare earths, it did identify the trend of increasing lanthanide insolubility in phosphoric acid as the atomic
number of the lanthanide increased. Furthermore, this report provided a solid numerical basis for the separation ratios of adjacent lanthanide pairs in phosphoric acid, which was demonstrated as a much more efficient medium for fractional precipitation than that used in mentioned earlier work by James. Finally, and most importantly, the utility of phosphoric acid in separating lanthanides on the basis of solubility informed the selection and testing of organic compounds to be used in current industrial extraction methods, such as tributyl phosphate and di-2-ethylhexylphosphoric acid.30

By the 1960s, continuous countercurrent extraction systems emerged as a solution to the capacity, purity, and efficiency problems that hampered other processes. In 1979, Brown and Sherrington reported on a multitude of extraction setups and methods that had been used effectively on an industrial scale over recent years.31 Since this time, the basic principle and materials have not changed too drastically. By using a continuous, automated system it is possible to obtain rare earths at very high purities. Additionally, these systems are usually closed, or nearly closed during operation, which allows solvents to be constantly recycled, providing some economic and environmental benefit over a chromatographic system.

To illustrate these advantages and separation theory, a simplified schematic of a 100% reflux continuous countercurrent extractor is shown below, as well as simulated separation data (Figure 1-6). While there are a variety of designs, the one shown is a good representation of the key components in the process, and is an apparatus theoretically capable of separating a multicomponent mixture of lanthanides. For simplicity, however, a two component mixture, A plus B, is represented here. In this example, A has greater solubility in the organic phase than B, meaning that A is removed with the organic phase, while B is left over in the aqueous raffinate. After the initial input into a mixer-settler unit of an aqueous solution containing A and B, A is preferentially extracted into the organic phase in the mixer, shown as an x-ray in stage 3. Phase separation then occurs in the adjacent separator, and the mixture is spilled to the next stage. Upon clearing stage 5, the organic phase will be enriched in A, which is then removed from the organic phase and reincorporated into the aqueous phase flowing in the opposite direction (from stage 5 to stage 1). At the end of this direction, B is removed from the aqueous raffinate and added back into recycled organic phase. By executing this process iteratively over days or weeks, a concentration differential will develop with A concentrating toward stage 5 and B concentrating nearest stage 1.

The schematic shown includes only five stages and a two component mixture. However, in practice, an apparatus made up of fifty stages meant to separate a dozen components is not uncommon. Increasing the number of stages in this approach is analogous to increasing the length of a silica flash column to give more theoretical plates. Each additional stage provides another discrete equilibrium, ultimately providing improved resolution of the species initially added. Thusly, complex mixtures of lanthanides, comprised of components of different concentrations with different organic phase solubilities can be separated to high purity by this method.
**Figure 1-6.** Simplified schematic of a 100% reflux continuous countercurrent extraction system separating a two component mixture. The solubility of A and B in the organic phase dictates that A will collect in the organic phase while B will mostly remain in the aqueous raffinate. The diagram (a) shows the manner in which an input mixture of A and B can be separated by five sequential, continuous extraction stages. At each end of the diagram, all of the concentrated solute is placed back into the system. Simulated separation data after a small number of completed steps (b, left) are shown, as well as greater separation after completion of a greater number of completed extraction steps (b, right).
While the total capacity of and purity achieved by countercurrent extraction is exceptional, there are some notable drawbacks in this methodology. First, the time required for purification is an issue. A setup similar to that outlined has been used to adequately separate a three component mixture of lanthanum, praseodymium, and neodymium, a challenging separation to be sure, in 285 hours, or nearly 12 days.\textsuperscript{31} This somewhat large time requirement, coupled with the desired outputs of rare earths, requires that extractors be run on a constant basis whenever ores are being mined. Second, organic phase components and energy requirements can become costly. Both tributyl phosphate and di-2-ethylhexylphosphoric acid are valued at hundreds of dollars per kilogram. Additionally, the energy needed to effect mixing and movement of fluid from one stage to the next is considerable. Finally, the waste streams created by this process are extensive and not environmentally friendly. The sheer volume of organic phase required to separate metric tons of lanthanide is non-ideal, and this is compounded by the corrosive and toxic nature of its components. Thus, while countercurrent extraction provides an economically viable method for rare earth separation, there is significant room for improvement.

1.6.4 Specialty methods

Most rare earth separation techniques fall nicely into the categories already discussed; however, there are examples of those which do not neatly fall into any single box. These types of methods often fuse other approaches, providing a unique blend of resolution, capacity, and efficiency. Currently, industrial processes are mostly devoid of these specialty methods, owing to economic or engineering constraints, but they are worth noting here to provide solid context for the continuously developing state of the art. Consequently, two recent examples representing separation techniques not already encompassed will be discussed here.

The implementation of Molecular Recognition Technology (MRT) chelating agents has been proposed by Izatt and coworkers as a possible powerful method for recovering rare earths from materials at the end of their lifespans.\textsuperscript{32} Additionally, however, this method could be just as easily used for moderate-capacity commercial separation of rare earths. MRT compounds are specifically designed to complex single metals non-ionically at low concentrations in multi-component mixtures. This material lends itself to a method similar to adsorption chromatography, allowing for the total retention of a single metal of interest from a mixture while other components pass through the column. Subsequently, the adsorbed metal can be eluted with a separate eluent. In contrast to traditional ion-exchange chromatography, use of MRT would allow for nearly complete recovery and purification of a single rare earth in a single step (Figure 1-7). While MRT suggests a potentially powerful method for separation and recovery of rare earths, it is somewhat limited by capacity, and severely limited by synthetic challenges, as each compound must be tailor-made to accommodate exactly one species of interest. The potential cost of meeting this synthetic challenge is great, which directly affects the scalability of the method.
Figure 1-7. Comparison of MRT adsorption method (a) to traditional ion-exchange chromatography (b) separation methods for rare earth elements. MRT column (a) specifically adsorbs species A, which is eluted after species B and C are not adsorbed. Ion-exchange column (b) adsorbs and separates A, B, and C simultaneously based on exchange and complexation equilibria of each species with the mobile phase. Usually, some overlap is observed, impacting purity and recovery.
At the interface of continuous countercurrent extraction and chromatographic methods, countercurrent chromatography has developed. As the name suggests, this method combines the iterative resolution enhancement of countercurrent extraction with the highly tunable nature of chromatography. Recently, Krättli et al. reported a highly successful separation of lanthanum, cerium, and praseodymium using a multicolumn countercurrent solvent gradient purification apparatus. Simplistically, the system passes the mixture over the first column, which partially resolves the three species, shunting pure fractions to collection. Mixed fractions are then passed onto a pre-equilibrated second column with a solution that promotes adsorption. After the entirety of the sample clears the first column, the process is repeated, with the second column acting as the resolving column, diverting pure fractions to collection containers, and passing the mixture back to the first column. The process continues to cycle in this manner until the shunted, purified fractions contain most to all of the original sample components. In this example, recoveries of 75% with purities in excess of 95% were achieved in three iterations. Unfortunately, this method is also hampered from industrial scalability due to limited capacity, as this system is essentially an HPLC-based method.

1.7 Conclusion

Rare earth elements have been established as a separation challenge. The chemical nature of the lanthanide series is prohibitive to facile resolution by standard methods, and the natural occurrence of mixed rare earth ores compounds this problem. However, given the high demand for rare earth materials, separation is an economically worthwhile endeavor. Recent price fluctuations and the related cornering of the rare earth commodities market by Chinese interests have served to further highlight this point. Accordingly, cheaper, greener separation methods must be developed as a means to globally diversify production, reestablishing a competitive market price for rare earths. Historical and modern examples of rare earth separation techniques have been outlined to provide a basis for the challenges facing this objective. This has served to illustrate that there remain both room and necessity for improvement in these methods.
1.8 References

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Chapter 2

Developing a new solid phase resin for lanthanide separation

2.1 Abstract

The challenges of lanthanide separation are many, while new and economical strategies to overcome these challenges are few. An inexpensive sulfonamide polystyrene resin, based on existing “safety catch” type resins, is proposed and developed as a platform for the production of new resins capable of separating lanthanides via a chromatographic approach. Several new separation moieties are synthesized and evaluated based upon ease of synthesis, cost, and ability to separate lanthanides as indicated by small-scale chromatography. Ultimately, the sulfonamide resin created without any further modification is found to effect the best separation, and is carried forward for further chromatographic examination.
2.2 Introduction

The 21st century has seen a marked increase in the appetite for rare earth metals. Global markets clamor for increasing quantities to meet the production demand for cars, generators, cellular phones, television displays, and medical equipment, among other items.¹⁻³ Chinese production has provided over 95% of rare earths on the market in the last ten years to meet this requirement, due to the comparatively low production costs in China.⁴ As a result of this immense market share, price instability of rare earth metals has become a worry and problem for consumers around the world. Consequently, there has been renewed interest in improving the costly processing and separation steps required to bring rare earths to the market at a competitive price, thereby diversifying the market and stabilizing prices.

When the individual costs of rare earth production are examined, separation methods stand out as an area ripe for economic and environmental improvement. Current industrial separation procedures by countercurrent extraction, as outlined in chapter 1, are challenging to replicate accurately on a research lab scale. Additionally, the associated energy costs and current configurations of the devices are problems better addressed by engineers. For these reasons, an extractive approach to lanthanide separation was not a particularly attractive option.

With the dismissal of countercurrent extraction, the most promising target for improving the state of the art is chromatography. Chromatographic techniques for the separation of lanthanides exhibit such major problems of scale that they are often considered to be restricted to analytical settings.⁵ Specifically, many resins do not have sufficient exchange or adsorption capacities to resolve large loads of lanthanides adequately, or associated techniques simply rely on an amount of pressure that is not realistically scalable to industrial application. The drawbacks in this case, however, can be readily addressed. To circumvent the problem of high required pressure, we can simply remove extra applied pressure all together, focusing on a method driven by gravity. Capacity constraints are also easily overcome by utilizing commercially available ion-exchange resins with millimole per gram exchange capacities. Unfortunately, reducing pressure and increasing input concentration have a generally negative effect on resolution. Thus, the resolving ability of the chromatographic system must be improved to counteract these effects.

Modification of chromatographic resins to separate lanthanides, or many other species, is not a new concept. Inoue and coworkers provided such an example of a custom built nitrilotriacetate (NTA) resin in an HPLC-based lanthanide separation strategy.⁶ While this strategy relied on HPLC and accommodated a correspondingly small input load, it provided excellent separation compared to the commercially available resins of the time using the same method, indicating that synthetic modification of resins is a viable strategy for improving chromatographic resolution. Unfortunately, the synthetic complexity and cost of the resin makes it difficult to envision it used in a large-scale operation.

Similarly, gravity-based and low pressure chromatographic methods have been studied at
some length. Experiments described in chapter 1 utilized very conservative flow rates and pressures. Moreover, due to the simplicity, low energy cost, and rapidity of the method, recent work such as that done by Payne and coworkers has studied the limits of gravity-based lanthanide chromatography. While some degree of separation was achieved, resolution was generally quite poor, especially when juxtaposed with HPLC data obtained for the same experiment. In summary, gravity-based separations typically suffer from poor resolution, but reap the benefits mentioned, and are amenable to many different cheap, commercially available resins.

With the scope of advantages and disadvantages of chromatographic methods in mind, we synthesized a basic framework for the creation of new chromatographic solid phases from a commercially available resin. Our two primary aims were to create a resin that was inexpensive and synthetically versatile (Figure 2-1). By focusing on creating an inexpensive resin, we maintain the ability to scale the material’s capacity all the way up to hundreds of metric tons of separated lanthanides. Synthetic versatility imparts flexibility in our chemical approach, allowing the testing of varied functionalities that impact chromatographic resolution while preserving much of the fundamental chemistry required for attachment of these groups to the resin. The remainder of this chapter will outline the manner in which different resins were synthesized and chromatographically screened to obtain an effective new separation moiety for lanthanides.

### 2.3 Synthesis of inexpensive sulfonamide polystyrene resin

Cost being a central element to the approach, several suitable, commercially available resins and modification methods were examined. Ultimately, Amberlite IR-120 (Na⁺ form) resin was selected due to its low cost, robust nature, and extremely high exchange capacity provided by sulfonate groups. The sulfonate groups also provided an accessible synthetic foothold for the creation a “safety-catch” type sulfonamide resin, giving a known method for both covalent attachment to and cleavage of various functionalities from the resin, facilitating analysis of these functionalities (Figure 2-2a, b). It should be noted that extremely similar resins to that proposed are commercially available, but lower levels of functionalization and much higher costs make these resins unsuitable for large-scale use (Figure 2-2c).

The basic route proposed in Figure 2-2a was initially selected as a good candidate to make sulfonamide resin 3 due to the generally high conversion to the sulfonyl chloride resin 2, as well as the high reactivity of the group. This was essential to ensure that a maximum of sulfonate groups were converted to sulfonamides, preserving a high and uniform exchange capacity. Accordingly, a variety of preparations using thionyl chloride, chlorosulfonic acid, or cyanuric chloride as chlorinating reagents, and ammonium hydroxide, neat ammonia, or ethylenediamine as aminating reagents were tested to determine the best possible conversion to the sulfonamide. Initially, IR spectroscopy was implemented as a qualitative gauge for the efficacy of each set of reaction conditions, as the sulfur-oxygen double bond stretch of the sulfonamide was uniquely identifiable. When this stretch was observed, precise yields were determined by elemental analysis,
Figure 2-1. Strategy for creating new low cost, high capacity lanthanide separation resins. A flexible resin modification strategy allows for the facile attachment of a diverse set of molecules to enhance lanthanide binding and separation.
Figure 2-2. Schematic of proposed resin and comparison to commercially available “safety-catch” resin. (a) A possible route to the synthesis of a sulfonamide resin from Amberlite IR-120 resin via the sulfonyl chloride. (b) Proposed cleavage of groups from sulfonamide resin based on Kenner’s “safety-catch” resin chemistry. (c) Comparison of cost and capacities of proposed resin and comparable, commercially available alternative.
comparing nitrogen to sulfur content. Ultimately, it was determined that reaction of the Amberlite IR-120 resin with chlorosulfonic acid, followed by ammonium hydroxide yielded excellent conversion of the sulfonate to the sulfonamide (96%) in five hours.

While these reaction conditions were not overtly complex, preparation was somewhat problematic when using these reagents. Specifically, the use of chlorosulfonic acid in the first step resulted in a significant quantity of sulfuric acid byproduct trapped within the water-swellable resin. Direct addition of this resin to ammonium hydroxide resulted in a large local temperature increase attributable to the neutralization of the sulfuric acid within the resin. In most cases, the temperature increase was severe enough to exceed the glass transition temperature of polystyrene, causing the resin to lose its defining porosity, size, form, and amenability to filtration. To avoid this, sulfonyl chloride resin was washed thoroughly with isopropanol cooled over dry ice, which removed a significant portion of sulfuric acid while circumventing hydrolysis of the sulfonyl chloride. Subsequently, resin was slowly added in small batches (~50 mg) to 0 °C ammonium hydroxide to further control temperature. The combination of the washing and addition protocols resulted in minimal resin degradation as a function of temperature increase and excellent sulfonamide conversion due to the avoidance of sulfonyl chloride hydrolysis.

2.4 Peptoid-based functionalities for lanthanide binding

Following the successful synthesis of a sulfonamide-modified solid phase, we turned our attention to the types of functional groups that could be used to separate the lanthanide species. Initially, we targeted moieties containing multiple carboxylates, mimicking binding motifs like ethylenediaminetetraacetate (EDTA) or NTA, which have exhibited previous success in this and related applications. A library-based approach for creating a diverse set of tripeptoids (poly-N-substituted glycine) was an attractive means to achieve this. By using relatively cheap amines and amino acids, it was possible to quickly synthesize a diverse set of resins containing variable numbers of carboxylates (Figure 2-3). Furthermore, peptoids are resistant to many harsh conditions, as well as bacterial degradation, as a function of their structural difference from peptides, making them more amenable to long-term use in a separation plant.

To determine if sulfonamide resin was a suitable platform for peptoid chemistry, comparison experiments between sulfonamide resin and Rink amide resin, a standard in peptoid synthesis, were carried out. Short test peptoids were synthesized on both Rink amide and sulfonamide resin, cleaved by standard methods, and analyzed by MALDI-TOF. Representative examples of both a Rink amide-based peptoid and sulfonamide-based peptoid are shown (Figure 2-4). As anticipated, there was little difficulty in synthesizing on and cleaving the desired peptoids from Rink amide resin, however the sulfonamide-based peptoids were synthesized in comparatively low yields with an apparent multitude of side products, truncations, and additions. In fact, the MALDI spectrum of the peptoid shown in Figure 2-4b is one of the tidier spectrums obtained from peptoids synthesized on sulfonamide resin.
Figure 2-3. A library-based tripeptoid approach affords a large number of rapidly synthesized resin-bound tripeptoids with three variable positions (a). A single member of the library (b) can be as functionally diverse as the component amines (c) used in the library. Even with just five different amines, as shown here, 125 unique trimers can be synthesized.
Figure 2-4. Representative examples of synthesized and cleaved peptoids from (a) Rink amide and (b) sulfonamide resins. The top peptoid (a) cleaved from Rink amide exhibits a fairly clean MALDI trace, showing only one major truncation product corresponding to the failed incorporation of the final benzylamine unit (including the starting acid). The benzylamine pentamer peptoid (b) synthesized on the sulfonamide resin appears in the MALDI trace among many unidentified products, but is not the major product.
Based on the generally poor results obtained in trying to cleave a pure peptoid species from the sulfonamide solid phase, it was determined that this type of chemistry was not ideal for our purposes. Without the ability to uniformly convert each resin functional group to the desired peptoid and confirm by mass spectrometry, batch consistency could not be guaranteed, which can be catastrophic for separation media. The Rink amide resin did provide a good synthetic platform for these types of peptoids. However, the high cost of Rink amide resin of $62.30 per gram and the delicate nature of its acid-labile linker are problematic, making it equally unsuitable.

2.5 Single step syntheses to give lanthanide binding groups

Following the inconsistent resins obtained by peptoid synthesis, a simplified approach to modify sulfonamide resin with multiple carboxylates was attempted. With peptoid yields being as apparently poor as they were, we turned to a single step method, rather than the multi-step, iterative process of peptoid synthesis. EDTA dianhydride and diethylenetriaminepentaacetic (DTPA) dianhydride were the first reagents used to accomplish this. As shown in Figure 2-5, addition of EDTA dianhydride or DTPA dianhydride yields three or four carboxylates, respectively, in a single step. This is clearly a much more efficient way to introduce carboxylates, as the construction of a peptoid trimer with three carboxylates from bromoacetic acid and alanine, for instance, would require a total of six sequential synthetic steps, followed by deprotection. However, the possible diversity of the species obtained in a single step is greatly limited when compared to peptoid libraries previously discussed.

After various synthetic optimizations, the conversion of sulfonamide groups to the tetraacetate species (Figure 2-5, 5) reached a maximum of 20%, as determined by elemental analysis. Slightly better, however, was the triacetate species (Figure 2-5, 4), at 40% conversion of the total starting sulfonamides. Due to the expected strong lanthanide complexation abilities of the carboxylate groups, these results were not immediately discouraging. Accordingly, warranting further examination, these resins were selected for preliminary chromatographic testing.

In addition to synthesizing resin groups containing multiple carboxylates to mimic known metal complexation agents, single carboxylate species were also targeted for more efficient syntheses. Various examples of sulfonate groups being exploited to separate lanthanides exist in the literature, and are described in chapter 1. Consequently, we expected that a single carboxylate group might be similarly adequate to affect some level of separation. Again recognizing the unsuccessful nature of peptoid chemistry on the sulfonamide resin, a single step synthetic approach utilizing the much more reactive sulfonyl chloride group and widely available amino acids was employed to create various single carboxylate functionalized sulfonamide resins. The route shown in Figure 2-6 demonstrates this method with a protected amino acid, but this route is amenable to any similarly protected molecule, providing much more potential diversity than the EDTA dianhydride and DTPA dianhydride chemistry already covered. While several of these types of resins were constructed using different amino acids, serine resin 6, with 30% of
Figure 2-5. General synthetic scheme for simultaneously attaching three or four carboxylates to sulfonamide resin using EDTA dianhydride or DTPA dianhydride, respectively.
Figure 2-6. General synthetic scheme for creating various single carboxylate resins for lanthanide separation in one step using protected amino acids.
the starting sulfonyl chloride groups converted to the functionality shown, was ultimately the most chromatographically successful, so it will henceforth serve as a representative case for these resin types.

2.6 Small-scale chromatographic testing of new resins

With an assortment of resins in hand, a small-scale chromatography system was devised to test the ability of each resin to separate lanthanides. Typically, this type of testing would be done on an instrument to control the applied gradient and flow rate, however, our goal was to apply little to no pressure and only have rough gradient control to better represent the eventual scaled up process. Accordingly, after preliminary experiments, the five resins that follow were screened in 300 mg columns to examine their separation abilities: 4, 5, 6, 1, and 3 (Figure 2-7).

In conjunction with the testing of different resins, a variety of mobile phases were explored. In general, step gradients of aqueous solutions of complexation agents, which were used to satisfy the roles of both the exchange species and solution complexation agent, were used. Ammonium sulfate, nitric acid, citrate, dipicolinic acid, EDTA, tartrate, α-HIBA, and lactic acid were screened as potentially acceptable mobile phases on the basis of similarity to effective complexation agents used in previous literature (Figure 2-8). While most of these species provided little to no resolution of mixed lanthanide solutions, α-HIBA and lactic acid were found to give an acceptable degree of separation. Additionally and notably, dipicolinic acid was found to be an extremely potent exchange and complexation agent capable of stripping tightly bound metals from all resins tested, making it potentially useful as a resin regeneration agent should the need arise.

Finally, in the design of these preliminary screens, a less challenging mixture of lanthanides was used. With a few exceptions, neighboring lanthanides expectedly tend to behave much more similarly to each other than non-neighboring lanthanides. For this reason, an equimolar standard of neodymium, terbium, and ytterbium was used to provide minimum difficulty in quickly assessing the separation abilities of the resin/mobile phase system being tested.

To illustrate the separation efficacy of each system, similar experiments are shown in Figure 2-7 for each of the five resins mentioned above. Both the EDTA and DTPA resins (4 and 5) exhibited strong retention of all lanthanide species, requiring dipicolinic acid to liberate the vast majority of metal ions loaded onto the resin. Furthermore, no appreciable separation of species was effected by either of these resins (Figure 2-7, a, b). Serine resin 6 gave reasonable separation of lanthanides, as well as excellent recovery of all species without the use of a regeneration agent (Figure 2-7, c). Similarly, sulfonate resin 1 effected partial separation of the species, resulting in broad, overlapping peaks (Figure 2-7, d). Interestingly, separate experiments performed in conjunction with the Alvarez-Cohen group indicated that sulfonate resin 1 was also exceptionally useful for specifically removing lanthanide ions from multicomponent aqueous solutions. Lastly, and serendipitously, sulfonamide resin 3 gave excellent lanthanide separation.
Figure 2-7. Small-scale separation experiments for (a) EDTA resin, (b) DTPA resin, (c) serine resin, (d) sulfonate resin (Amberlite), and (e) sulfonamide resin.
Figure 2-8. Mobile phase additives tested in diagnostic column experiments.
and recovery, with minimal overlap between peaks (Figure 2-7, e). This final result was very much unanticipated, as it was presumed that stronger or a larger number of complexation groups on the resin would result in better separation. Furthermore, this simple sulfonamide resin had not been studied as a lanthanide separation medium, while many more complex structures based on this structure, or containing this as a substructure had been studied. Therefore, the simple sulfonamide resin made an excellent candidate for additional exploration of similar functionalities, scale up, and further separation experiments.

### 2.7 Conclusion

Amberlite IR-120 has provided a convenient, inexpensive, and commercially available means to synthesize a sulfonamide resin. This sulfonamide resin, in turn, gave a synthetically flexible means of producing various resins for use in the chromatographic separation of lanthanides. Several types of functional moieties constructed on this resin have been conceptualized and produced, including peptides, peptoids, multi-carboxylate chelating agent mimics, and those based on simple amino acids. Of all resins produced and tested, including polystyrene sulfonate resin, which is commonly used as a lanthanide separation medium, an unmodified sulfonamide resin effects the most impressive separation of lanthanides. This discovery has led to the further interest in exploring this resin and functionality type in more directed lanthanide separation experiments.

### 2.8 Materials and methods: general methods

All chemicals were obtained from commercial sources and used without any further purification unless otherwise stated. Water used in all experiments was deionized water (dH2O).

### 2.9 Materials and methods: instrumentation and sample preparation

**Mass spectrometry.** Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectra were obtained using a Voyager-DE from PerSeptive S2Biosystems (Applied Biosystems). Samples were co-crystallized using a solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 50% water with 0.1% trifluoroacetic acid.

Liquid Chromatography Inductively Coupled Plasma Mass Spectrometry (LC-ICP-MS) spectra were obtained by Negassi Hadgu using an Agilent 7700 Series ICP-MS (Agilent Technologies). Lanthanide samples were prepared by diluting them to a concentration between 0 and 1000 ppb and volume of 5 mL in an aqueous solution containing 1% HNO3 and 0.5% HCl.

**IR spectroscopy.** IR spectra were obtained using an Alpha FTIR system (Bruker).
Figure 2-9. Synthesis of sulfonamide polystyrene resin 3 from polystyrene sulfonate resin 1 (Amberlite IR-120).
Elemental analysis. Samples were prepared for elemental analysis by drying resins under reduced pressure for 30 minutes and grinding into a uniform powder with a mortar and pestle. Elemental analysis data were obtained by Elena Kreimer using a Perkin Elmer 2400 Series II combustion analyzer capable of determining mass percentages of C, H, N, and S.

2.10 Materials and methods: synthesis of sulfonamide polystyrene

Preparation of Amberlite IR-120 resin (1). To a 250 mL round bottom flask equipped with a magnetic stir bar was added 50 mL of methanol, followed by 15 g of Amberlite IR-120 (Na⁺ form, 1). The mixture was then stirred while boiling in a 70 °C oil bath for 3 h. Subsequently, the mixture was removed from the bath and cooled to ambient temperature. The resin was transferred to filter paper, and the methanol was filtered off by gravity. Two 20 mL portions of THF were used to wash the resin. Finally, the resin was transferred to a 100 mL round bottom flask, which was then attached to a vacuum manifold. The resin was dried in vacuo for two hours.

Synthesis of sulfonyl chloride resin (2). To a 100 mL round bottom flask equipped with a magnetic stir bar was added 25 mL of chlorosulfonic acid, followed by 5 g of freshly dried Amberlite IR-120 resin, (Na⁺ form, 1). While stirring at ambient temperature, 4 mL of DMF was added as gas visibly evolved. A water cooled reflux condenser was attached to the flask, and the mixture was submerged in a 70 °C oil bath for 4 h while stirring. The mixture was then removed from heat and cooled to ambient temperature. Approximately half of the chlorosulfonic acid/DMF supernatant was decanted, while the remaining resin slurry was filtered through filter paper by gravity. The resin was then rinsed with two 15 mL portions of isopropanol that had been cooled over dry ice. The resin was transferred to fresh filter paper, briefly blotted dry, and immediately used in the next reaction.

Synthesis of sulfonamide resin (3). To a 100 mL round bottom flask equipped with a magnetic stir bar was added 30 mL of aqueous ammonia. The aqueous ammonia was stirred at 0 °C for 15 min prior to the slow addition of the sulfonyl chloride resin. Gas visibly evolved during this procedure. The mixture was then stirred at 0 °C for 1 h, at which point it was warmed to ambient temperature and stirred for an additional 2 h. The mixture was then stirred at 0 °C for 1 h, at which point it was warmed to ambient temperature and stirred for an additional 2 h. The mixture was transferred to filter paper, filtered by gravity, and washed with two 20 mL portions of dH₂O and one 15 mL portion of DCM. The wet resin was dried briefly in air and transferred to a scintillation vial containing a magnetic stir bar. A quantity of 2 M HCl was added to submerge all of the resin, and the mixture stirred at ambient temperature for 5 min. The resin was then filtered again on filter paper by gravity and washed with one 10 mL portion of water. After drying in air, the sulfonamide resin was ready for use. Resin 3 was characterized using both IR spectroscopy and elemental analysis. Creation of the sulfonamide group was established by a medium-strength absorption peak centered at 1316 cm⁻¹, corresponding to the sulfur-oxygen double
bond stretch in a sulfonamide (Figure 2-13). Elemental analysis: 43.82% C, 5.82% H, 7.11% N, 16.92% S. Based on the ratio of N to S from elemental analysis, conversion to sulfonamide from sulfonate groups was 96%.

2.11 Materials and methods: general procedure for synthesizing and cleaving resin-bound peptoids

**Synthesis and cleavage of peptoids from Rink amide resin.** The general procedure for synthesizing and cleaving peptoids on Rink amide resin was carried out according to a previously published procedure.19

**Synthesis of peptoids on sulfonamide resin 3.** To a 2 mL fritted plastic cartridge was added 20 mg of resin 3. To the resin was added 170 μL of a 0.6 M solution of bromoacetic acid in DMF, 20 μL of DMF, and 20 μL of diisopropylcarbodiimide (DIC). The cartridge was then sealed and agitated on an orbital shaker for 30 minutes. Liquids in the cartridge were drained into waste and the resin washed with two 2 mL portions of DMF. Subsequently, the same quantities of bromoacetic acid solution, DMF, and DIC were added to the cartridge. The cartridge was again capped and agitated as before, followed by an identical draining and washing step to give the acylated product. To amidate the resin, 200 μL of a 2 M solution of amine was added to the resin and the cartridge agitated on an orbital shaker for 2 h. The solution was then drained and resin washed in two 2 mL portions of DMF to give the amidated product. These steps were repeated as necessary for each sequential amidation.

**Cleavage of peptoids from sulfonamide resin 3.** To a 2 mL fritted plastic cartridge was added 20 mg peptoid resin followed by 0.85 mL NMP, 0.1 mL diisopropylethylamine (DIPEA), and 0.1 mL iodoacetonitrile. The cartridge was covered in aluminum foil and agitated on an orbital shaker for 22 h. Liquids were then drained from the cartridge, 1 mL of NMP was added to the resin, and the cartridge agitated by orbital shaker for 10 minutes. This NMP rinse was repeated two additional times. NMP was then drained from the cartridge and the resin washed with three 0.5 mL portions of NMP, two 0.5 mL portions of DCM, and three 0.5 mL portions of dioxane. To the cartridge was then added 0.66 mL dioxane and 0.33 mL of an aqueous solution of 3 M NaOH. The resin cartridge was then agitated by orbital shaker for 22 h. The liquids in the cartridge were drained into a 15 mL conical vial. The resin was then washed with two 1 mL portions of 2 M NaOH, two 1 mL portions of HCl, and two 0.75 mL portions of DCM. These washes were collected in the same 15 mL conical vial as before. The aqueous layer in the vial was then acidified to pH < 1 with 8 M HCl, and the vial vigorously shaken. The organic layer from the vial was removed to a 25 mL Erlenmeyer flask, to which was added sodium sulfate and an additional 1 mL of DCM. The dried organic phase was evaporated under nitrogen to near dryness and added to a 1 mL portion of diethyl ether. White precipitate was collected from the ether by filtration and isolated as the cleaved peptoid.

2.12 Materials and methods: functionalization of sulfonamide
Figure 2-10. Synthesis of EDTA-based resin 4 from sulfonamide polystyrene 3.
Figure 2-11. Synthesis of DTPA-based resin 5 from sulfonamide polystyrene 3.
Figure 2-12. Synthetic scheme for creating various single carboxylate resins for lanthanide separation in one step. Serine-based resin is shown here, but any protected amino acid is possible.
polystyrene

Synthesis of EDTA-based resin (4). To a 20 mL scintillation vial equipped with a stir bar was added 5 mL NMP, followed by 50 mg of sulfonamide resin 3, and 153 mg of ethylenediaminetetraacetic dianhydride. The mixture was then submerged in an oil bath and stirred at 90 °C for 17 h. The mixture was transferred to filter paper, filtered by gravity, and washed with two 20 mL portions of DMF. The resulting resin was swelled in a quantity of DCM sufficient to submerge it for 1 h. DCM was removed by gravity filtration and the resin briefly blotted dry on filter paper. Subsequently, the resin was further dried in vacuo for 20 minutes. Elemental analysis: 49.81% C, 6.19% H, 8.98% N, 11.32% S.

Synthesis of DTPA-based resin (5). To a 20 mL scintillation vial equipped with a stir bar was added 5 mL NMP, followed by 50 mg of sulfonamide resin 3, and 213 mg of diethylenetriaminepentaacetic dianhydride. The mixture was then submerged in an oil bath and stirred at 90 °C for 17 h. The mixture was transferred to filter paper, filtered by gravity, and washed with two 20 mL portions of DMF. The resulting resin was swelled in a quantity of DCM sufficient to submerge it for 1 h. DCM was removed by gravity filtration and the resin briefly blotted dry on filter paper. Subsequently, the resin was further dried in vacuo for 20 minutes. Elemental analysis: 49.37% C, 5.93% H, 8.27% N, 11.80% S.

Synthesis of serine-based resin (6). To a 20 mL scintillation vial equipped with a stir bar was added 18 mL DMF, followed by 2 mL TEA (10%), and 1.015 g of (S)-tert-butyl 2-amino-3-hydroxypropanoate hydrochloride. After stirring for five minutes, 500 mg of sulfonyl chloride resin 2 was added and the mixture submerged in an ice bath. The mixture was stirred at 0 °C for 2 h, removed to ambient temperature, and allowed to stir for an additional 16 h. The mixture was transferred to filter paper, filtered by gravity, and washed with two 20 mL portions of dH₂O. The resulting resin was briefly blotted dry on filter paper and allowed to dry in air. Resin was transferred to a 2 mL fritted cartridge, to which 0.5 mL TFA was added. The cartridge was capped and stirred on an orbital shaker for 15 minutes. TFA was drained from the cartridge and three 5 mL portions of dH₂O were used to wash the resin. Deprotected resin 6 was briefly blotted dry on filter paper and allowed to dry in air. Elemental analysis: 44.51% C, 6.35% H, 1.89% N, 12.37% S.

2.13 Materials and methods: small-scale lanthanide chromatography

Construction and use of small-scale columns. Small columns for preliminary separations analysis were constructed by removing the plunger and flanges from a 1 mL syringe. A small wad of glass wool was then used to plug the syringe barrel near the tip of the syringe. 300 mg of resin was added to the syringe barrel and deionized water added to fill the entire syringe. The water was allowed to flow through the barrel by gravity, fully immersing the resin, and the syringe was capped. The capped syringe was inserted into
a 15 mL conical vial, counterweighted with another vial, and centrifuged for 1 minute to pack the resin into place.

Columns were run by first equilibrating the resin with either water or aqueous 50 mM sodium citrate, pH 4.5. Subsequently, aqueous lanthanide samples in 10 mM sodium acetate, pH 4.5, were added to the top of the resin bed via micropipette, allowing the solution to settle into the resin before addition of the mobile phase. Further mobile phase was added manually by micropipet, meaning that all gradients used were step gradients. For the experiments listed explicitly in Figure 2-7, the gradient composition was composed of 1 mL 100 mM α-HIBA, pH 4.3; 1 mL 200 mM α-HIBA, pH 4.3; and 3 mL 400 mM α-HIBA, pH 4.3. Fractions were collected sequentially in graduated vials beginning with the first drop from the column after addition of the lanthanide solution and ending at a volume of 5 mL, in most cases.

The lanthanide standard used in this experiment was 10 mM in each of Nd$^{3+}$, Tb$^{3+}$, and Yb$^{3+}$. Hydrated chlorides or nitrates of these metals were obtained and added to a 10 mM solution of aqueous sodium acetate, pH 4.5. As volumetric glassware was not used for the making of this solution, the standard solution was volumetrically diluted and analyzed alongside the collected fractions by LC-ICP-MS to determine the amount of each lanthanide in solution and ultimate recovery from the columns.
2.14 References

Figure 2-13. IR spectrum of sulfonamide polystyrene resin. 3.
Chapter 3

Construction of a new lanthanide chromatography system based on an inexpensive sulfonamide resin

3.1 Abstract

A selection of resins is synthesized, characterized, and chromatographically tested. Based on these results, a sulfonamide resin is selected for scale up and implementation in preparative-scale chromatography. Parameters for the system are determined and optimized for a separation of a monazite-based mixture of lanthanides with a focus on isolating didymium. Using this system and an α-HIBA-containing mobile phase, didymium is isolated at a purity of 95.5%. Use of lactic acid as a mobile phase additive yields didymium at a purity of 92.8%. Rough analysis of the cost, efficiency, and environmental impact of this system is done, and it is determined to be amenable to industrial-scale application.
3.2 Introduction

While lanthanide separation chromatography has been a topic of study for researchers since the discovery of lanthanides, it has only garnered interest from corporate and governmental entities since the advent of high tech lanthanide-based materials. Combined investigation has generated numerous approaches to the optimization of chromatographic separation, such that it can be utilized at minimum cost and maximum output. So far, cost and output are lacking in an industrial setting, with chromatography comprising only a small section of purification that is mostly accomplished by continuous countercurrent extractors.\(^1\)

Many resin types, including anion exchange, cation exchange, reverse phase (C18), and custom built resins, have been put forth to improve the state of the art and overcome the hurdles of expense and scale in rare earth processing.\(^2\)\(^-\)\(^5\) To date, few of these resins have seen significant industrial use in rare earth processing. This has been due largely to the fact that the lanthanide separation performance of these resins is highly dependent on the use of HPLC, a fundamentally difficult technique to scale. For this reason, the performance of the sulfonamide resin synthesized and tested in chapter 2 is of particular interest, as it provides partial separation of the lanthanide set tested without the use of a pump or overly complicated gradient control.

In addition to the resin used, the mobile phase is a vital component in making chromatographic techniques viable for industrial-scale use. Primarily, the eluent used must be reasonably inexpensive, assist in resolving lanthanide mixtures, and be environmentally friendly, as large quantities of eluent will be necessary for separation procedures. Moderately concentrated aqueous solutions of α-HIBA (0.1-1.0 M) are commonplace in lanthanide chromatography due to the excellent distribution coefficients exhibited between differential Ln\(^{3+}\)-α-HIBA complexes and reasonably low environmental impact.\(^6\) However, at the concentrations and volumes necessary, the cost of α-HIBA at $1.33 per gram is potentially problematic (Figure 3-1). Kuroda and coworkers have also demonstrated excellent resolution using much lower concentrations of aqueous nitrilotriacetic acid (0.005 – 0.050 M; $0.10/g) and 1-octanesulfonate (0.010 M; $10.20/g), lowering cost somewhat, but producing a non-ideal waste stream.\(^7\)

While α-HIBA and nitrilotriacetic acid are reasonable choices, the structurally similar lactic acid has many advantages. Lactic acid is produced, on an industrial scale, predominantly as a byproduct from the bacterial fermentation of sugars, with applications ranging from cheese production to polymer synthesis.\(^8\)\(^-\)\(^10\) Accordingly, a global market and supply route already exist. Massive quantities of lactic acid are produced each year, over 300,000 metric tons in 2010, providing a dependable supply and low market price of $0.00145/g.\(^11\) Lactic acid is a compound of minimal environmental concern, making the waste stream produced nearly ideal.\(^12\)

Being very structurally similar to α-HIBA, lactic acid has been previously studied, albeit not very extensively, for use as a mobile phase in lanthanide chromatography. Vera-Avila
Figure 3-1. Structures and prices for common mobile phase additives.
and Camacho demonstrated, in one of the few examples published, that lactic acid can be effectively used as an eluent in an HPLC-based system, though it does not provide the same degree of resolution between the lanthanides. Ideally, lactic acid could be incorporated into an industrial-scale chromatography strategy.

Weighing the challenges of creating a bench-scale prototype for an industrial-scale purification method, we divided our efforts among several specific goals. First, upon encountering the success of the sulfonamide resin characterized in chapter 2, we sought to further synthesize and examine similar sulfonamide, alkyl sulfonamide, and amino sulfonamide functionalities. Second, after determining the best solid phase, we had to develop a reproducible chromatographic system to properly analyze gradient composition, eluent composition, flow rate, column pressure, loading capacity, resin longevity, and lanthanide mixture composition. Finally, with the details of the chromatographic system worked out, we needed to confirm that the cost and environmental impact of the process were amenable to large-scale separations.

### 3.3 Synthesis and evaluation of alkylated sulfonamide resins

Based on the excellent retention of Ln$^{3+}$ ions by carboxylate and sulfonate-containing resins synthesized and tested in chapter 2, it was quickly realized that less strongly complexing resins would be required. Fortified with the separation success of the sulfonamide polystyrene resin, an assortment of similar resins was created for additional examination (Figure 3-2). The primary aims in selecting these new functionalities were the use of short, simple synthetic methods for appending of the functional groups to the sulfonamide resin, and reducing or not significantly contributing to the lanthanide complexing ability of the resin. With these aims in mind, resins 2, 3, 4, and 5 were synthesized in a single step from sulfonyl chloride resin 1. Resin 6 was previously synthesized by the method described in chapter 2.

It was already known that conversion of sulfonyl chloride resin 1 to sulfonamide resin 6 was excellent, but elemental analysis did not yield the same high conversion for all of resins 2-5. As shown in Figure 3-2, conversion of the sulfonyl chloride to the intended sulfonamide occurred in a broad range of 18%-76%, depending on the amine used. Elaborate optimizations were attempted to increase conversion rates, but the values shown here were the best achieved, so the nature of the resins evaluated are reflected in this figure.

Unlike the incomplete conversion of the sulfonyl chloride resin to the serine-based resin in chapter 2, incomplete conversion of sulfonyl chloride resin 1 to sulfonamide resins 2-5 was, in this instance, a point of concern. In the case of the serine-based resin, unconverted sulfonyl chlorides were likely hydrolyzed back to sulfonates after prolonged exposure to water during the synthetic cleanup of the resin. This would provide a resin with functional groups of a mixed nature, containing a combination of sulfonates and sulfonamide-linked β-hydroxy carboxylates. Both of these groups would be expected to complex Ln$^{3+}$ ions strongly and somewhat similarly, mitigating excessive peak-
Figure 3-2. Modification of sulfonyl chloride polystyrene resin with non-complexing amines.
broadening or the creation of bimodal peaks during chromatography. This prediction correlates well with what was observed in small-scale chromatographic experiments shown in chapter 2. However, in the case of resins 2-5, incomplete conversion would lead to resins of mixed functionalities comprised of the intended, weakly complexing groups in Figure 3-2, and the strongly complexing sulfonate groups. The disparity in the strength of Ln$^{3+}$ ion complexation between the two groups would be expected to produce broader, or possibly bimodal, peaks, decreasing resolution.

In order to verify that resolution was indeed affected by incomplete conversion of sulfonil chloride resin 1 to intended resins 2-5, small-scale chromatography experiments were performed to evaluate the separation capabilities of each resin. As before, a mixture of relatively simple-to-separate lanthanides, containing equimolar amounts of neodymium, terbium, and ytterbium, was analyzed on a 1 mL column containing approximately 300 mg of each resin. The results of these experiments are outlined in Figure 3-3, juxtaposed with previous experimental results for sulfonamide resin 6. Phenylsulfonamide resin 2, benzylsulfonamide resin 3, and methoxyethylsulfonamide resin 4 all demonstrated partial separation of the analytical lanthanide mixture. In each case, they did not perform as well as sulfonamide resin 6, and exhibited comparatively significant peak broadening and overlap, supporting the predicted differential complexation nature of the heterofunctionalized resins. Interestingly, ethylsulfonamide resin 5, despite having the highest conversion from the sulfonyl chloride, exhibited the broadest peaks and poorest resolution of the lanthanide mixture. This is likely due to the fact that it forms the weakest complex with Ln$^{3+}$ ions of the set, exacerbating the disparity in complex strength between the ethylsulfonamide and sulfonate moieties on this resin (Figure 3-3, d, e). We expect this effect on resolution was not observed as drastically for resins 2, 3, and 4 because each was capable of forming stronger complexes via cation-π or cation-dipole interactions between Ln$^{3+}$ ions and aromatic groups and Ln$^{3+}$ ions or ether groups, respectively. This would serve to mitigate the differences in complexation strength between sulfonamide-linked groups and sulfonates with the lanthanide ions, similar to the behavior observed for the serine-based resin in chapter 2.

### 3.4 Scale up, determination of chromatographic parameters, and application

The continued superior chromatographic performance of sulfonamide resin 6 coupled with the various synthetic and chromatographic drawbacks of the other resins reinforced that resin 6 was the best candidate to scale up to a larger column size in a more controllable setup. To achieve this, a computer-controlled liquid chromatography (LC) unit with the ability to accommodate many sizes of custom columns was employed. While the LC unit did contain a pump and was controlled via computer, it is important to note that this instrument was not an HPLC instrument. As mentioned, maintaining a low pressure during separation was critical to demonstrate that the system could be scaled to industrial specifications. Furthermore, columns were roughly packed by hand to simulate conditions in a much larger column, as well as provide a maximum separation challenge
Figure 3-3. Small-scale separation experiments for (a) phenylsulfonamide resin, (b) benzylsulfonamide resin, (c) methoxyethylsulfonamide resin, (d) ethylsulfonamide resin, and (e) sulfonamide resin. Sulfonamide resin used and chromatogram (e) are the same as shown in chapter 2 and included here for comparison.
to demonstrate the effectiveness of the system. A typical scaled up column of sulfonamide resin \(6\), as shown in Figure 3-4, is roughly a 15-fold increase in scale from previously described small-scale chromatographic experiments.

In addition to the scale up of sulfonamide resin \(6\), Amberlite IR-120 resin, bearing sulfonate groups, was scaled up and used as a means of comparison. As shown in chapter 2, the Amberlite resin can separate lanthanide mixtures, and is indeed often used for this purpose in the field, but does not perform as well as resin \(6\). To confirm the relative inferiority of Amberlite for lanthanide chromatography, it is included in parallel to resin \(6\) in many of the following experiments.

### 3.4.1 Baseline performance of larger scale system

In order to determine a baseline of performance for the scaled up system, parameters from small-scale columns were approximated and the same mixture of neodymium, terbium, and ytterbium was used. Loading volume of the sample lanthanide solution was increased ten-fold, a linear gradient of \(\alpha\)-HIBA of comparable concentration to small columns was used, and a low flow rate was maintained so as to create no back pressure. The results of these preliminary experiments were generally good (Figure 3-5). Resolution of lanthanides by the column containing resin \(6\) was comparable to small-scale experiments, with peaks maintaining a uniform, somewhat narrow shape (Figure 3-5, a). Comparatively, the same degree of separation was not achieved by the Amberlite \((7)\) column, which gave slightly worse resolution of the lanthanides, and generally broader peaks (Figure 3-5, b). Particularly striking was the tailing observed in all of the peaks, suggesting greater retention of the species on resin \(7\) than was the case in the resin \(6\) column. Overall, both chromatograms demonstrated that this system was acceptable, but in need of optimization.

### 3.4.2 Examining a broader set of light lanthanides

With the basic aspects of this system worked out, our attention turned to a larger set of lanthanides. As covered in chapter 1, most lanthanide ore compositions tend to favor either lighter or heavier lanthanides, often containing more than the three components analyzed above. For this reason, a mixture of lanthanum, praseodymium, neodymium, samarium, and europium (10 mM in each), was separated over both sulfonamide resin \(6\) and Amberlite \(7\) (Figure 3-6). Because the lanthanides used in this set were more similar to each other, resolution was expected to decrease for both resins.

Results indicated that resolution was indeed affected. The resolution of the mixture was not particularly good on sulfonamide resin \(6\), but peak shapes remained much the same as before (Figure 3-6, a). Comparatively, the resolving ability of Amberlite \(7\) was extremely poor. Furthermore, peaks were particularly broad and prone to severe tailing (Figure 3-6, b). This result again suggested that the Amberlite resin, while extremely effective in HPLC, was not performing sufficiently to separate lanthanides by this approach.
Figure 3-4. Image of one of the columns used in scaled up chromatography experiments performed with the use of LC. This column contains a bed of 4.7 g of sulfonamide resin sandwiched between two ground glass frits. All packing was done manually.
Figure 3-5. 5 g column runs of Nd, Tb, Yb mixtures (10 mM in each) for sulfonamide resin and Amberlite. (a) Sulfonamide resin shows resolution and peak shapes comparable to small scale experiments. (b) Amberlite shows similar resolution, but noticeably broader peaks.
Figure 3-6. 5 g column runs of La, Pr, Nd, Sm, Eu mixtures (10 mM in each) for sulfonamide resin and Amberlite. (a) Sulfonamide resin shows poor resolution, but reasonable peak shapes. (b) Amberlite displays very poor resolution with significant tailing.
Finally, these experiments further confirmed that the order of lanthanide elution from the sulfonamide column was actually the same as that commonly observed for columns using Amberlite: heavier lanthanides elute first, and lighter lanthanides elute later.

3.4.3 Modeling monazite and isolating didymium

Monazite, one of the principal commercially important ores in the industry, is composed primarily of the lighter lanthanides (See Chapter 1, Figure 1-2). The majority of this ore is made up of an uneven combination of lanthanum, cerium, neodymium, and praseodymium. These elements comprise the primary commercial importance of the monazite ore. One particularly important application derived from these metals is the production of rare earth-iron-boron permanent magnets. While the rare earth component of these magnets can be made from pure neodymium or praseodymium, it is much cheaper, while still effective, to make them from didymium (neodymium and praseodymium) alloy, which can be derived from monazite ore.\textsuperscript{14}

Having determined the ability of resins \textsuperscript{6} and \textsuperscript{7} to separate a challenging set of equimolar light lanthanides, a more directed goal of isolating didymium from a monazite mixture was targeted. To do this, typical monazite composition was determined by acid digestion of monazite sand, followed by ICP-MS quantitation of each lanthanide. An analytical solution containing quantities of lanthanum, praseodymium, neodymium, samarium, europium, and terbium corresponding to their relative abundances in monazite sand was then created. Notably, cerium, promethium, and gadolinium were excluded from this mixture. Industrially, cerium is typically removed by oxidation to the Ce\textsuperscript{IV} species and subsequent precipitation.\textsuperscript{1} Promethium does not occur naturally, and is therefore not present in the ore. Finally, gadolinium occurs in particularly low abundance in monazite sand, so it was excluded on this basis.

Application of this monazite-based lanthanide mixture to our chromatographic system using sulfonamide resin \textsuperscript{6} and a gradient composed of $\alpha$-HIBA yielded appreciable separation of didymium from other components (Figure 3-7, a). After this single application to the column, 77.3% of the didymium input was isolated at a purity of 92.5%, with samarium and lanthanum comprising the bulk of the 7.5% impurity. This isolated didymium was applied to the same column again to yield the majority of isolated didymium at a purity of 96.4%, which is sufficient for many applications (Figure 3-7, b). The success of this process demonstrates that a given mixture can be sequentially and substantially enriched in a lanthanide or lanthanides of interest with a relatively small number of iterations.

3.4.4 Resin capacity determination

Thus far, all of the lanthanide separations carried out using columns loaded with sulfonamide resin \textsuperscript{6} that have been shown have been loaded with solutions of low total lanthanide concentration. This was useful in that less concentrated solutions help to avoid
**Figure 3-7.** 5 g column runs of La, Pr, Nd, Sm, Eu, Tb mixtures (monazite ratio) for sulfonamide resin with α-HIBA gradient. (a) First run of the mixture provides acceptable purity of isolable didymium. (b) A second run of the combined fractions indicated in (a) is necessary to obtain sufficiently pure didymium.
peak tailing and significant overlap of peaks as a result of the column being overloaded, allowing for the determination of the general behavior of the system. However, if this combination of low pressure LC and sulfonamide resin is to be used industrially, the exchange capacity of the resin must be sufficient to adequately separate large quantities of concentrated lanthanide solutions. Consequently, the diagnostic monazite-based lanthanide solution used in previous experiments was serially multiplied in concentration, and the resulting chromatograms were analyzed (Figure 3-8).

For the purposes of this set of experiments, the starting lanthanide mixture of composition shown in Figure 3-8f was dubbed “1x,” denoting that other solutions were proportionally more concentrated in all species by a factor of n. As before, the chromatogram of the separation of the 1x monazite solution, Figure 3-8a, showed good resolution of didymium peaks from other species, with moderate tailing of all peaks. As the concentration was increased from 10x-50x, Figure 3-8b-d, a somewhat greater degree of peak overlap was observed, most evident in the 50x chromatogram (Figure 3-8, d). Upon doubling the 300 μL loading volume of the 50x solution to obtain a 100x total loading of material, both increased overlap and significant peak broadening were observed, possibly indicating that the combination of the large volume and high concentration of the sample exceeded the resolving capacity of the resin and column dimensions (Figure 3-8, e).

Generally, these experiments demonstrated that the exchange capacity of resin 6 was large enough to be amenable to significant increase in the concentration of lanthanides separated in a single run. Based on the exchange capacity of the starting sulfonamide resin and elemental analysis data of resin 6, the 4.7 g column of resin 6 that was used should have a total exchange capacity of 10.74 milliequivalents. The 100x sample only contained a total of 1.96 mmol of soluble lanthanide species. In light of this, it is likely that the volume of the loaded samples is the major factor in the observed performance decrease between the 50x and 100x samples (Figure 3-8, d, e). Therefore, if it is possible to lower the loading volume of a concentrated sample, the effective capacity of the system may increase.

### 3.4.5 Further gradient optimization

After determining a set of basic parameters and successfully implementing this chromatographic system in the applied isolation of didymium from a monazite-based mixture, further optimization of parameters was enacted. Throughout earlier experiments, it was observed that modification of the gradient was a simple, yet powerful tool to drastically change separation performance. Using this knowledge, a variety of gradient types and refinements were attempted and the resulting chromatograms were analyzed. The best performance was yielded by a simple step gradient beginning with pH 4.3 α-HIBA; 200 mM, and stepping to pH 4.3 α-HIBA; 400 mM at the middle of the run.

The implementation of this gradient resulted in a drastically improved separation of the monazite-based lanthanide solution (Figure 3-9). The use of α-HIBA in the mobile phase yielded near-complete separation of didymium from lanthanum, with some overlap
Figure 3-8. 5 g column runs of La, Pr, Nd, Sm, Eu, Tb mixtures (monazite ratio) for sulfonamide resin with α-HIBA gradient to test resin capacity. Loaded sample for these runs begins with (a) diagnostic “1x” mixture and increases to (b) 10x, (c) 25x, (d) 50x, and (e) 100x. 1x-50x loading volumes are 300 µL, while 100x loading volume is 600 µL (doubled volume of 50x solution). Solution composition of a 1x monazite load is tabulated in (f).
**Figure 3-9.** 5 g column runs of La, Pr, Nd, Sm, Eu, Tb mixtures (monazite ratio) for sulfonamide resin with α-HIBA step gradient and lactic acid step gradient. (a) α-HIBA step gradient shows excellent separation of La from didymium. (b) Lactic acid step gradient gives excellent separation as well, but with significant shouldering late in the didymium peaks.
between the samarium and didymium curves (Figure 3-9, a). In a single run, 80.8% of didymium loaded onto the column was isolated at a purity of 95.5%, with a primary impurity of samarium. The quantity of didymium isolated in this example exceeds that obtained in two consecutive runs using the previously referred to linear gradient. Additionally, the purity is less than 1% lower than that obtained with two iterations of the linear gradient. Simply changing to this linear gradient effectively doubles the efficiency of the system, and likewise reduces operating costs.

The performance of lactic acid as a mobile phase component was markedly less impressive than α-HIBA throughout all of the previous experiments performed. However, in response to the extreme increase in resolution using this step gradient, lactic acid was also tested. The results, similar to α-HIBA, were excellent. Again, lanthanum separation from didymium was nearly complete, but samarium did tend to tail into didymium peaks (Figure 3-9, b). Unlike the α-HIBA run, both the neodymium and praseodymium peaks exhibited abrupt shouldering near an elution volume of 50 mL. This was likely due to a non-ideal positioning of the step in the gradient for lactic acid. Because lactic acid exchanges and complexes lanthanide ions more weakly than α-HIBA, a longer time spent at the lower concentration step is necessary to completely elute the didymium species. The too early increase in lactic acid concentration disrupts and accelerates the elution of didymium species while simultaneously causing lanthanum to elute. Consequently, recovery can be improved by simple modification to this gradient. Nonetheless, in a single run, 58.2% of didymium loaded onto the column was isolated at a purity of 92.8%. Considering these results, the extremely low price, and minor environmental impact of lactic acid, this system lends itself well to intended industrial-scale purposes.

3.5 Cost analysis

The final piece of demonstrating the utility of this resin and system lies in determining how economically and environmentally viable it is. To properly determine this, comprehensive analysis is currently underway. In the meantime, it is still useful to roughly assess these aspects. For comparison, we can examine extractive methods which, while they provide a product of excellent purity, are problematic on the grounds of environmental impact, cost, and separation time. For the purposes of comparing extraction to this newly developed chromatographic system, qualitative comparisons will be drawn and lab-scale costs will be scaled linearly to give a rough estimate of an industrial-scale process.

Relative to currently used extractive methods, this system compares quite well with respect to environmental impact. While extraction produces large amounts of aqueous and organic waste streams containing various harmful organic acids, the chromatographic system produces only an aqueous waste stream with a modest amount of the considerably “greener” lactic acid. Furthermore, if it is desired, much of the waste feed can be recycled back into the system after fairly minimal treatment, as it undergoes no chemical changes throughout the process. Finally, the resin used in these experiments was recycled dozens of times with no change in chromatographic performance, which leads us to believe that
this resin can be used for an extensive number of cycles. Consequently, the environmental impact of producing the resin is negligible.

Though precise costs for lanthanide purification by extraction are not readily available for comparison, lab-scale costs of this chromatographic system are instructive. To illustrate the point, we can assume a somewhat large column and examine the amount of material input and capital required to isolate 1 kg of didymium from a monazite-based input mixture. For this purpose, 72 kg of resin would be required, with a bench-scale production cost of $28,000. Considering the robust nature and consequent longevity of the resin, this can be counted as a minor cost on a per run basis. To separate a concentrated solution of monazite proportions, 173 L of lactic acid mobile phase, valued at $72, would be required. The output, 8 minutes later, would be 1 kg of didymium, 1 kg of lanthanum, and 173 L of slightly acidified, recyclable waste. If the current prices of lanthanum and didymium metals are considered, $13/kg and $100/kg, respectively, the profit margin on a single column run is $41.\textsuperscript{15,16} Moreover, the short run time of 8 minutes allows for 180 runs in one day, producing a profit of $7380 per day. At this rate, the cost of the resin is offset after four days of operation.

3.6 Conclusion

The scale-up and optimization of sulfonamide-based lanthanide chromatography have been describe and further developed. This system gives excellent separation of monazite-based inputs using either α-HIBA or lactic acid-based mobile phases. Using α-HIBA, 80.8% of didymium loaded onto the column was isolated at a purity of 95.5%. Using lactic acid, 58.2% of didymium loaded onto the column was isolated at a purity of 92.8%. Based upon these results and laboratory-scale cost, it was further determined that this system is extremely viable in an industrial capacity on the basis of cost, efficiency, and environmental impact.

3.7 Materials and methods: general methods

All chemicals were obtained from commercial sources and used without any further purification unless otherwise stated. Water used in all experiments was deionized water (dH\textsubscript{2}O).

3.8 Materials and methods: instrumentation and sample preparation

**Mass spectrometry.** Liquid Chromatography Inductively Coupled Plasma Mass Spectrometry (LC-ICP-MS) spectra were obtained by Negassi Hadgu using an Agilent 7700 Series ICP-MS (Agilent Technologies). Lanthanide samples were prepared by diluting them to a concentration between 0 and 1000 ppb and volume of 5 mL in an aqueous solution containing 1% HNO\textsubscript{3} and 0.5% HCl. Determination of the composition of monazite sand was carried out by Negassi Hadgu according to previously published
Elemental analysis. Samples were prepared for elemental analysis by drying resins under reduced pressure for 30 minutes and grinding into a uniform powder with a mortar and pestle. Elemental analysis data were obtained by Elena Kreimer using a Perkin Elmer 2400 Series II combustion analyzer capable of determining mass percentages of C, H, N, and S.

Liquid chromatography. Preparative-scale liquid chromatography (LC) was carried out using 5 g RediSep columns with a CombiFlash Companion purification system (Teledyne ISCO).

3.9 Materials and methods: synthesis of sulfonamide polystyrene resins

Synthesis of sulfonyl chloride resin (1). This resin was prepared according to the procedure outlined in chapter 2.

Synthesis of phenylsulfonamide resin (2). To a 20 mL scintillation vial equipped with a magnetic stir bar was added 10.19 mL of a 2 M solution of aniline in isopropanol (20 mmol, 21 eq). The vessel was submerged in an ice bath at 0 °C and stirred for 15 min. Subsequently, 400 mg of sulfonyl chloride resin 1 was added slowly, and the mixture stirred at 0 °C for an additional 1.5 h. The mixture was then removed to ambient temperature and stirred for 16 h. The mixture was transferred to filter paper, filtered by gravity, and washed with two 20 mL portions of dH$_2$O and one 15 mL portion of DCM. The wet resin was dried briefly in air and transferred to a scintillation vial containing a magnetic stir bar. A quantity of 2 M HCl was added to submerge all of the resin, and the mixture stirred at ambient temperature for 5 min. The resin was then filtered again on filter paper by gravity, washed with one 10 mL portion of water, and dried in air. Elemental analysis: 42.72% C, 5.45% H, 0.98% N, 12.67% S. Based on the ratio of N to S from elemental analysis, conversion to sulfonamide from sulfonyl chloride groups was 18%.

Synthesis of benzylsulfonamide resin (3). To a 20 mL scintillation vial equipped with a magnetic stir bar was added 9.80 mL of a 2 M solution of benzylamine in isopropanol (20 mmol, 21 eq). The vessel was submerged in an ice bath at 0 °C and stirred for 15 min. Subsequently, 400 mg of sulfonyl chloride resin 1 was added slowly, and the mixture stirred at 0 °C for an additional 1.5 h. The mixture was then removed to ambient temperature and stirred for 16 h. The mixture was transferred to filter paper, filtered by gravity, and washed with two 20 mL portions of dH$_2$O and one 15 mL portion of DCM. The wet resin was dried briefly in air and transferred to a scintillation vial containing a magnetic stir bar. A quantity of 2 M HCl was added to submerge all of the resin, and the mixture stirred at ambient temperature for 5 min. The resin was then filtered again on filter paper by gravity, washed with one 10 mL portion of water, and dried in air.
Elemental analysis: 42.16% C, 5.68% H, 1.24% N, 12.13% S. Based on the ratio of N to S from elemental analysis, conversion to sulfonamide from sulfonyl chloride groups was 23.4%.

**Synthesis of methoxyethylsulfonamide resin (4).** To a 20 mL scintillation vial equipped with a magnetic stir bar was added 10.01 mL of a 2 M solution of methoxyethylamine in isopropanol (20 mmol, 21 eq). The vessel was submerged in an ice bath at 0 °C and stirred for 15 min. Subsequently, 400 mg of sulfonyl chloride resin 1 was added slowly, and the mixture stirred at 0 °C for an additional 1.5 h. The mixture was then removed to ambient temperature and stirred for 16 h. The mixture was transferred to filter paper, filtered by gravity, and washed with two 20 mL portions of dH\textsubscript{2}O and one 15 mL portion of DCM. The wet resin was dried briefly in air and transferred to a scintillation vial containing a magnetic stir bar. A quantity of 2 M HCl was added to submerge all of the resin, and the mixture stirred at ambient temperature for 5 min. The resin was then filtered again on filter paper by gravity, washed with one 10 mL portion of water, and allowed to dry in air. Elemental analysis: 37.26% C, 6.70% H, 1.58% N, 11.22% S. Based on the ratio of N to S from elemental analysis, conversion to sulfonamide from sulfonyl chloride groups was 32.2%.

**Synthesis of ethylsulfonamide resin (5).** To a 20 mL scintillation vial equipped with a magnetic stir bar was added 10.00 mL of a 2 M solution of ethylamine in isopropanol (20 mmol, 21 eq). The vessel was submerged in an ice bath at 0 °C and stirred for 15 min. Subsequently, 400 mg of sulfonyl chloride resin 1 was added slowly, and the mixture stirred at 0 °C for an additional 1.5 h. The mixture was then removed to ambient temperature and stirred for 16 h. The mixture was transferred to filter paper, filtered by gravity, and washed with two 20 mL portions of dH\textsubscript{2}O and one 15 mL portion of DCM. The wet resin was dried briefly in air and transferred to a scintillation vial containing a magnetic stir bar. A quantity of 2 M HCl was added to submerge all of the resin, and the mixture stirred at ambient temperature for 5 min. The resin was then filtered again on filter paper by gravity, washed with one 10 mL portion of water, and allowed to dry in air. Elemental analysis: 44.01% C, 6.59% H, 3.92% N, 11.80% S. Based on the ratio of N to S from elemental analysis, conversion to sulfonamide from sulfonyl chloride groups was 76.0%.

**Synthesis of sulfonamide resin (6).** This resin was prepared according to the procedure outlined in chapter 2.

### 3.10 Materials and methods: small-scale lanthanide chromatography

**Construction and use of small-scale columns.** Small columns for preliminary separatory analysis were constructed by removing the plunger and flanges from a 1 mL syringe. A small wad of glass wool was then used to plug the syringe barrel near the tip of the syringe. 300 mg of resin was added to the syringe barrel and deionized water added to
Columns were run by first equilibrating the resin with either water or aqueous 50 mM sodium citrate, pH 4.5. Subsequently, aqueous lanthanide samples in 10 mM sodium acetate, pH 4.5, were added to the top of the resin bed via micropipette, allowing the solution to settle into the resin before addition of the mobile phase. Further mobile phase was added manually by micropipet, meaning that all gradients used were step gradients. For the experiments listed explicitly in Figure 3-3, the gradient composition was composed of 1 mL 100 mM α-HIBA, pH 4.3; 1 mL 200 mM α-HIBA, pH 4.3; and 3 mL 400 mM α-HIBA, pH 4.3. Fractions were collected sequentially in graduated vials beginning with the first drop from the column after addition of the lanthanide solution and ending at a volume of 5 mL, in most cases.

3.11 Materials and methods: preparative-scale lanthanide chromatography

Construction and use of preparative-scale columns. Empty 5 g RediSep columns were packed by hand with 4.7 g of resin, and ground glass frits were fitted to either side of the resin bed. Flow rates of 5-15 mL/min were used, which kept back pressure below 10 psi. Two different two-component (A: dH₂O, B: 400 mM α-HIBA, pH 4.3 or 400 mM lactic acid pH 4.3) gradients were used for the experiments covered in this chapter. The first gradient consisted of a 2 minute isocratic step at 25% B, followed by a 14 minute linear gradient from 25% to 100% B, and a final 4 minute isocratic step at 100% B. The second gradient consisted of a 10 minute isocratic step at 50% B, followed by a 10 minute isocratic step at 100% B.

Columns were run by first equilibrating the resin with either dH₂O. Subsequently, aqueous lanthanide samples were added directly to the top of the frit above the resin bed via syringe, allowing the solution a moment to settle into the resin before application of the mobile phase. Fractions were collected sequentially in 13x100 mm culture tubes beginning with the first drop from the column after addition of the lanthanide solution and ending at a volume of 100 mL, in most cases.

Several lanthanide standards were used in chromatography experiments. The first was 10 mM in each of Nd³⁺, Tb³⁺, and Yb³⁺. The second was 10 mM in each of La³⁺, Pr³⁺, Nd³⁺, Sm³⁺, and Eu³⁺. The third was 15.7 mM La³⁺, 2.8 mM Pr³⁺, 12.3 mM Nd³⁺, 1.8 mM Sm³⁺, 0.02 mM Eu³⁺, and 0.09 mM Tb³⁺, reflecting the relative proportions of these lanthanides in a typical sample of monazite sand. In all cases, solutions were made and used as follows: hydrated nitrates of these metals were obtained and added to a solution of aqueous 10 mM sodium acetate, pH 4.5. As volumetric glassware was not used for the making of this solution, the standard solution was volumetrically diluted and analyzed.
alongside the collected fractions by LC-ICP-MS to determine the amount of each lanthanide in solution and ultimate recovery from the columns.
3.12 References

Chapter 4

Progress toward protein-polymer hybrids from estrogen receptors for the detection and removal of organic pollutants from water

4.1 Abstract

Growing concerns about the contamination of drinking water with estrogentic compounds have spurred a need for materials capable of rapid detection of estrogens in water. To satisfy this need, a protein-polymer conjugate composed of a recombinantly expressed estrogen receptor and a synthetic polymer component is proposed. Estrogen receptors ERα-LBD, 3ER-LBD, and xER-LBD, which undergo major conformational change upon binding estrogens, are designed, expressed, and characterized to facilitate use of each as the protein component in the proposed material. Through the use of an intein-chitin binding domain purification strategy and previously designed piperidone cysteine, 3ER-LBD and xER-LBD are converted to an oxime at the C-terminus in yields up to 75%. Furthermore, complete modification of the N-terminus of xER-LBD is effected by PLP-mediated transamination followed by oximation. Lastly, a 4-arm PEG is synthesized and characterized for use as the oximation-amenable polymer component in the proposed material.
In recent years, attention to the persistent concentrations of hydrophobic organic contaminants, such as hormones and chlorinated aromatic compounds, in water supplies has grown due to the deleterious biological activity of these contaminants. For instance, estrogenic effects in marine life, including intersexuality, vitellogenesis in males, and skewed gender ratios, have been observed and linked to increased estrogen concentrations in lakes and rivers. While these animals do endure greater total exposure to contaminated water, there is still further concern that negative effects will extend to exposed human populations, based on available data.

Such concerns over high estrogen exposure in humans center mainly upon potential carcinogenic effects. In 2002, the NIH released a full report on the carcinogenicity of estradiol, noting similar exposure risks pertaining to other common estrogens. Parallel research has also identified multiple mechanisms of estrogen-stimulated carcinogenesis in humans and animals. Increased relative endometrial and breast cancer risk in women who use oral contraceptives or undergo hormone replacement therapy is similarly well-documented and widely accepted.

Observed estrogenicity in lakes and rivers is indicative of estrogenicity in municipal wastewater. This is of concern, as wastewater treatment and reclamation have become necessary processes to meet the ever-growing demand for clean water for general consumption. Typical municipal wastewater has been shown to be contaminated with pollutants such as heavy metals, bacteria, organic solvents, cosmetics, illegal drugs, prescription drugs, antibiotics, pesticides, halogenated aromatics, detergents, and various natural and synthetic hormones (Figure 4-1). The removal of these contaminants is a crucial step in reintegrating wastewater into the supply. While effective large-scale methods exist for the extraction or destruction of heavy metals, bacteria, detergents and some organics—such as biodegradation, chlorination, filtration, addition of cationic detergents, foaming, etc.—methods for specific removal of other compounds, specifically hydrophobic organic compounds, are still developing.

Developing methods for estrogen removal from water include ozonation and repeated reverse osmosis, which have demonstrated high removal (>99%) of estrogens in spiked wastewater. However, they exhibit low specificity, can be expensive to implement, and leave behind low but still biologically harmful concentrations of hormones. Adsorption onto granular activated carbon (GAC) has also been proposed as a low-cost method for removal of estrogens, but results suggest that while it does strongly adsorb estrogenic compounds, it is hampered by too large a K_d to remove estrogens to an extent deemed environmentally acceptable. Thus, the challenge of specific removal of estrogenic compounds from water has not yet been fully met.

While the presence and toxicity of contaminants like chlorinated organic compounds in drinking water has been well established for some time, the levels of various hormones, specifically estrogens, in drinking water sources have only recently been met with...
Figure 4-1. Structures of estrogens commonly found in drinking water sources.
the same increased attention. This attention has been fostered by the quantification of estrogens in collected water samples, which has led back to the examination of the sources and effects of estrogenicity. Unfortunately, current methods for determining the estrogenicity of water samples rely on complicated analyses that often require sophisticated instrumentation and technical knowledge, which incur significant costs, such as extraction of the hormones followed by bioassay, the use of mass spectrometry, or modification of estrogens to facilitate detection via fluorescence. In short, a simple, inexpensive, and portable method for estrogen detection of field samples would be useful for estrogen detection in the field.

Faced with challenges in the detection and removal of estrogens from water, we turn to human estrogen receptor alpha (ERα)-based materials (Figure 4-2). ERα is a native estrogen receptor, capable of tightly binding estrogens of interest, including estrone, estradiol, estriol, and ethinylestradiol, with dissociation constants ranging from 0.1-10 nM. Tangentially, ERα is also known to specifically bind some toxic, chlorinated organics known to be present in municipal waters, such as dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs). While ERα affinity for these molecules is at least 1000-fold less than for estrogens, it does increase the overall utility of ERα in the realm of detection and contaminant removal.

In the past, we have reported the covalent, site-specific incorporation of pollutant-binding proteins into hydrogels. Briefly, the protein of interest is expressed as an intein fusion, allowing for subsequent modification of the C-terminus. Ketones are then installed on the C- and N-termini through expressed protein ligation with a modified cysteine and a pyridoxal phosphate-mediated transamination, respectively. Finally, oximation can be employed to crosslink the protein into an appropriately designed aminooxy-containing polymer system, thereby creating a hydrogel (Figure 4-3). By applying this procedure, we seek to develop an analogous material through incorporation of ERα into a hydrogel, thereby imparting mechanical stability and greater utility to the receptor protein, while preserving its ligand binding functionality.

ERα is well suited for integration into the protein-polymer hybrid system. Past successfully integrated proteins have all been relatively small, high affinity binders of their ligands, and have been typified by facile recombinant expression in E. coli. We are, however, presented with a challenge in that ERα is a mammalian nuclear receptor and a necessarily larger protein than those previously used, as it contains a ligand binding domain, a DNA binding domain, and other domains relevant to its function. Fortunately, work in other labs has confirmed that the native protein can be significantly truncated to the ERα ligand binding domain (LBD) while maintaining the receptor’s binding affinity. This allows an avenue for modification of the native receptor to make it more suitable for the existing system.

While this device could theoretically be used as part of a water treatment process to remove estrogenic contaminants from municipal wastewater, the scale of such an endeavor is a limiting factor. To illustrate the point: an amount of material containing
Figure 4-2. Depiction of a dimer of ERα ligand binding domain subunits with estradiol ligands bound (green).
Figure 4-3. Strategy for construction of a well-defined ERα-LBD-polymer conjugate.
100 mg of ERα ($k_a = 1.3 \times 10^6 \text{M}^{-1}\text{s}^{-1}$, $k_d = 1.2 \times 10^{-3} \text{M}^{-1}\text{s}^{-1}$, $K_d = 0.9 \text{nM}$)\(^2^5\) has an estrogen capacity of at most 932 ug. A 1000 L quantity of water with a typical contaminant concentration in the Rhine River of 66 ng/L\(^1^6\) of estradiol could theoretically form the bound complex (i.e. recover estradiol) at an initial rate of 32 ug/s. However, real recovery rates would surely be slower due to lack of distribution and reduced accessibility of the ERα. Furthermore, once the capacity is reached, the material would have to be recycled, which is possible, but likely through time-consuming denaturation and re-folding.\(^2^6\) Thus, the material in its current conception is not ideally suited for large-scale water purification work.

While the hybrid, as designed, is not currently a good fit for large volume water purification tasks, it is well suited for detection. Published crystallographic studies have suggested that the receptor undergoes a dramatic conformational change upon ligand binding.\(^2^7\) In previously reported polymer-protein hybrids, similar conformational changes extended to the larger polymer network, causing a macroscopic contraction upon ligand binding. This allowed for the hybrid to be used as a macroscopic indicator of ligand concentration, reported by the size change of the material.\(^2^1\) Initially, we plan to utilize this property in the construction of a simple detector for ERα ligands. Thus, with small modifications, ERα can be optimized for construction of a simple, yet powerful protein-polymer hybrid for use as a tool for the detection of estrogens in the field.

### 4.3 Design and expression of estrogen receptor proteins

#### 4.3.1 Construction of the ERα-pTYB1 plasmid

In order to obtain a specifically bifunctionalized protein that can be incorporated into the proposed protein-polymer hybrid, we turned to the IMPACT™-CN expression system.\(^2^8\) This system was chosen to facilitate the construction of an easily purified and functionalized intein-chitin binding domain (Int-CBD) fusion to the protein of interest. To this end, we obtained a pUC57 plasmid containing an ampicillin resistance gene and ligand binding domain residues 302-552 of the ERα gene (ERα-LBD) from Genscript. The protein coding region was flanked by a 5’ NdeI restriction site and 3’ SapI site for later integration into the intein fusion plasmid (pTYB1). The restriction site design is unique, as the NdeI site allows for cleavage and ligation at the start codon, while SapI allows for integration of the gene of interest upstream of the intein without retaining any non-native bases that formerly composed the restriction site (Figure 4-4).

Initial construction of the ERα-LBD-pTYB1 plasmid began with double digestion of the ERα-LBD-pUC57 and pTYB1 plasmids with NdeI and SapI restriction enzymes. Each digestion reaction mixture was analyzed by agarose gel electrophoresis. In the case of the ERα-LBD-pUC57 mixture, several smaller fragments were observed, which corresponded to other digestion products given by cleavage at the NdeI and SapI restriction sites within the sequence of the pUC57 vector, but outside of the ERα-LBD gene (Figure 4-5, Lane 1). Fortunately, superfluous digestion products were of a different length than the fragment of interest, allowing for adequate separation of the ERα-LBD
Figure 4-4. General cloning scheme for construction of ERα-LBD-pTYB1 plasmid.
Figure 4-5. Agarose gel of double digestion products indicating fragments purified for subsequent ligation (red arrows). Lane 1: ERα-pUC57 digestion products corresponding to multiple restriction digest fragments resulting from 2 NdeI and 2 SapI sites. The red arrow denotes the ERα-LBD gene. Lane 2: pTYB1 digestion fragments. The red arrow denotes the linearized vector with other band corresponding to the supercoiled plasmid. Length markers are shown in base pairs on the side of each lane.
fragment from its nearest neighbors. In the case of the pTYB1 mixture, linearization was indicated by a larger apparent length compared to supercoiled DNA, as determined by agarose gel electrophoresis (Figure 4-5, Lane 2). Proper cleavage at both restriction sites on the pTYB1 vector, however, was not explicitly confirmed. After identifying the correct digestion products within the gel, each was excised and subsequently purified.

Preliminary attempts at the ligation of the prepared ERα-LBD insert into the pTYB1 host were performed using 30 ng of insert DNA with 100 ng of host DNA, giving a molar ratio of 3:1 insert:host. DNA was mixed and reacted with a proper amount of T4 DNA ligase at ambient temperature, but subsequent addition of the ligation mixture to competent cells, followed by growth on ampicillin-containing selection plates, yielded no transformants. Accordingly, different factors were screened to improve transformation efficiency, including ligation time, ligation temperature, addition of phosphatase, ligation reaction scales, and competent cell types. Subsequent transformations yielded either no colonies or a modest number of colonies containing recircularized host vector.

Repeated failed transformations using ligation reaction mixtures prompted closer examination of the supplied sequence of the ERα-LBD-pUC57 plasmid. It was quickly discerned that the non-palindromic SapI restriction site was constructed in the opposite orientation of that which was originally ordered. Pursuant to this construction, any cleavage performed by SapI at this site would yield a fragment of nearly intended length, containing a non-complementary set of overhanging bases relative to the sticky end left behind on the pTYB1 vector after SapI cleavage. Additionally, the SapI restriction site would be left connected to the C-terminus of the ERα-LBD insert.

Using the unmodified pTYB1 vector, and designing primers to reverse the SapI restriction site in the ERα-LBD gene, the insert and host fragments were digested and purified as before. Ligation reaction conditions, including insert:host ratio and quantity of ligase used, were screened on a 200 ng scale. Subsequently, NEB-5α sub-cloning grade E. coli were transformed with the ligation mixture. Following ampicillin selection, modest numbers of transformants were observed in all cases but one. Presence of the desired ERα-LBD insert within the purified DNA was screened by colony PCR, using the same primers used to amplify the insert (Figure 4-6). The insert was present in 7 of the 18 colonies screened. These 7 were submitted for sequencing, which indicated that 2 of the samples submitted contained the proper sequence.

4.3.2 Expression and purification of ERα-LBD from ERα-LBD-Int-CBD

Expression of the ERα-LBD-Int-CBD fusion protein was first attempted with the general conditions of growth at 37 °C until optical density at 600 nm (OD₆₀₀) reached 0.5, followed by induction of the cells by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) and overexpression for 4 h at 30°C. Aliquots of cells taken immediately after IPTG addition and 4 h after induction were analyzed by SDS-PAGE to check expression.
Figure 4-6. Analytical agarose gel of colony PCR screening for determination of the presence of the ERα-LBD insert. Lanes marked in red indicate the presence of the correct gene through appearance of an extra band near 750 base pairs, the length of the ERα-LBD gene. Length markers are given on the side of the gel in base pairs.
Gel analysis yielded the expression band expected (85 kDa) from the ERα-LBD-Int-CBD fusion in higher quantities after induction.

After confirming reasonable overexpression of the correct protein, the cell extract was mixed with washed chitin to facilitate binding of ERα-LBD-Int-CBD, loaded onto a column, undesirable proteins not bearing a chitin binding domain were removed through repeated washings, and purified chitin-bound protein was eluted by adding 2-mercaptoethane sulfonate sodium salt (MESNa, 3), an intein cleaving agent (Figure 4-7). Following cleavage, eluted fractions were analyzed by SDS-PAGE. However, fractions were devoid of cleaved ERα-LBD protein.

Insolubility of the protein was suspected to be the reason for the absence of protein after cleavage and elution. To confirm this, aliquots from the soluble fraction and pellet were obtained immediately after lysis and analyzed by SDS-PAGE. Gel analysis clearly indicated the presence of ERα-LBD-Int-CBD in the insoluble pellet (Figure 4-8, a, left). First attempts to solubilize the ERα-LBD-Int-CBD protein were made by screening the temperature and total time of expression. Optimal IPTG concentrations for induction were screened in tandem. After determining the best time, temperature, and IPTG concentrations independently, these factors were co-screened, yielding optimal expression conditions of 15 °C for 16 h with a total IPTG concentration of 0.5 mM. While these conditions somewhat improved the solubility of the protein, they were not adequate to allow for high yielding purification. Therefore, it was necessary to solubilize the protein by further modifying the expression conditions, lysis, and wash buffers used during purification.

Expression conditions were modified to include the addition of 5% sucrose to the growth media, allowing more cell growth, and the addition of 10 μM estradiol (E2), which has been found to encourage proper folding of the receptor during expression.24 Further expression experiments also demonstrated that addition of E2 to a total concentration of 20 μM at the time of induction facilitated solubilization. E2 was also added to subsequent lysis and wash buffers to maintain solubility, but this alone was not sufficient. Accordingly, other solubilizing co-additives were screened. The screen included SDS, urea, Tween 20, Triton x-100, and 3-(1-pyridinio) propane sulfonate, a non-detergent sulfobetaine (NDSB). Addition of 2 M NDSB or 2 M urea to the lysis buffer and 0.5 M to the wash buffer were found to be the most successful and greatly increased the solubility of the fusion protein during purification (Figure 4-8, b, left). However, the addition of NDSB or urea decreased binding of the fusion to the chitin beads, so the NaCl concentration was decreased from 500 mM to 200 mM to partially offset this increased salt concentration.

Protein expression using the optimized system resulted in significantly increased solubility of the protein after lysis. After binding to and cleavage from the chitin beads, eluted fractions showed a dark band corresponding to the apparent mass of an ERα-LBD monomer (Figure 4-8, b, right). To assess the cleavage efficiency, the used chitin beads were boiled in DTT-containing SDS buffer and analyzed in a gel lane adjacent
**Figure 4-7.** Schematic for purification and C-terminal modification of ERα-LBD by intein method.
Figure 4-8. Polyacrylamide gel of overexpression of ERα-Int-CBD and purified ERα before and after optimization. Prior to optimization, (a) the construct exhibited poor solubility and yields of 1 mg/L. After optimization, (b) solubility was much improved, giving yields of 3 mg/L.
to the eluted fraction. To further discern the identities of the gel bands, each was excised from the gel, digested with trypsin, and subjected to RPLC followed by MS/MS peptide sequencing. Sequencing results confirmed that the bands were correctly identified. Further characterization of the purified, intact ERα-LBD protein was done by mass spectrometry. The obtained spectrum reflected the expected mass of the protein and protein thioester, which has been observed to hydrolyze over time to give the free C-terminal amino acid. Two other significant peaks were also observed, correlating to an addition of two glucose units and the corresponding thioester. This pattern of glycosylation has been previously observed when expressing this protein in *E. coli*.\(^{25}\) Protein yield using this optimized system was 3 mg/L.

### 4.3.3 Construction of the 3ER-pTYB1 plasmid

The successful construction and expression of the ERα protein was encouraging. However, at a yield of 3 mg/L, production efficiency was a moderate concern. In order to create a sufficient amount of material for testing and use, much higher yields would be preferable to avoid large-scale expression and expenditure of resources. Consequently, a strategy for drastically improving the protein yields without sacrificing function was needed.

Fortunately, previous work with the human estrogen receptor, including expression results described above, has indicated that the major impediment to efficient ERα expression is solubility of the protein. Published work by Gangloff and coworkers has indicated that a triple mutant of ERα, made by converting three cysteines to serines, drastically improves solubility and expression yields (Figure 4-9).\(^{27}\) Specifically, these cysteines reside on the hydrophobic face that contacts another monomer in the native dimer form. Thusly, this face typically is not exposed, and the exposure of these cysteines inhibits proper folding when expressing this region recombinantly.

The construction of the 3ER-pTYB1 plasmid was fairly straightforward, as the ERα-pTYB1 plasmid was already in hand. First, C381, C417, and C530 codons were identified as those which were to undergo mutation to serine codons. To make the proper mutations, three sequential rounds of site-directed mutagenesis, adapted from the QuikChange™ technique (Stratagene), were employed.\(^{29}\) Due to the different regions of these mutations, it was not possible to mutate more than one cysteine per iteration. Accordingly, three sets of forward and reverse overlapping primers were designed. Initially, the ERα-pTYB1 plasmid was amplified via QuikChange™ protocol with primers corresponding to C381S, C417S, and C530S mutations, followed by DpnI digestion, and transformation into XL-1 Blue Supercompetent Cells (Stratagene). The C381S mutation yielded 2 colonies, of which one was determined to be the correct, mutated sequence. The C417S and C530S mutations each yielded 5 colonies, of which 5 and 4 colonies were determined to be the correct mutated sequence, respectively.

Having confirmed the efficacy of the primers as designed, a single colony of intended sequence, bearing the C417S mutation, was grown overnight in a 5 mL LB culture at
Figure 4-9. Structure of ERα-LBD with C381, C417, and C530 indicated. Indicated cysteines are mutated to serines to give 3ER-LBD.
37 °C, pelleted, and DNA purified and extracted. This plasmid was subjected to another round of site-directed mutagenesis using primers corresponding to the C530S mutation, transformed, grown, and DNA purified as before. Finally, this plasmid, bearing both C417S and C530S mutations was subjected to a final round of site-directed mutagenesis with the primers corresponding to the C381S mutation. This final mutation yielded many transformants, of which 6 were selected, purified, and sequenced. All 6 sequences indicated that the intended 3ER sequence was achieved.

4.3.4 Expression and purification of 3ER-LBD from 3ER-LBD-Int-CBD

Previous expression of the ERα-LBD protein was used as a basis for expression of the 3ER-LBD protein. Using conditions determined by these screens, 3ER was expressed in prodigious yields, as compared to ERα (Figure 4-10, a). As indicated by SDS-PAGE analysis, purity of the 3ER-LBD protein was very good, showing only a small proportion of Int-CBD impurity, as well as faint bands corresponding to other co-eluting serum proteins. Additionally, the yield of 120 mg/L was excellent, and was more than sufficient for construction of a reasonable amount of the hybrid material from small-scale expression. Finally, confirmation that the correct protein was expressed was provided by mass spectrometry, which indicated a 1 Da deviance from the expected mass within the margin of error of the instrument (Figure 4-10, b).

4.3.5 pH stability of ERα-LBD and 3ER-LBD

Following the successful expression and purification of 3ER-LBD, pH stability of both 3ER-LBD and ERα-LBD was examined in phosphate buffer. This investigation was crucial to ascertain the viability of the proteins during the long transamination and oximation reactions to be carried in preparation of the bifunctionalized products at pH 6-7. In both the 3ER and ERα cases, over 90% of the protein in a solution of 10 mM sodium phosphate buffer, pH 7, had precipitated in 24 h, as determined by absorbance of the supernatant before and after precipitation at 280 nm. Even more precipitation was measured at pH 6.5 and pH 6. This result was not entirely unexpected, as the calculated isoelectric point of ERα is 6.09, and that of 3ER is nearly the same. However, it did underline the importance of carrying these transamination and oximation reactions out in shorter time or at a higher pH. Alternatively, an estrogen receptor stable at lower pH would offer a possible solution.

4.3.6 Construction of the xER-pTYB1 plasmid

To address the problem of pH stability exhibited by 3ER and ERα, one additional estrogen receptor was designed. The primary concerns in the selection of this receptor were a lower isoelectric point, to address the problem of insolubility at below neutral pH, and a reasonably high degree of homology to the human estrogen receptor. Several different estrogen receptors were considered along these lines, and the *Xenopus* estrogen
Figure 4-10. Yield and characterization of 3ER-LBD expression. Sequential, purified fractions analyzed by SDS-PAGE, (a) indicate good purity with yields of 120 mg/L as determined by UV-vis analysis. Subsequent mass spectrometry analysis (b) indicates that the intended protein is the major product of expression.
receptor (xER) was selected (Figure 4-11). While sequence homology of xER to ERα was somewhat less than the chicken estrogen receptor to ERα, it was decided that the lower isoelectric point of xER was more important. Finally, having selected the plasmid, it was further decided to construct the sequence with three serines instead of the three cysteines, corresponding to C381, C417, and C530 in ERα. This was presumed to impart greater stability and solubility to the xER-LBD protein during and after folding.

With the xER sequence determined, the full gene was constructed by overlap extension polymerase chain reaction (OE-PCR), adapting a previously published protocol. A series of overlapping 40-mers, covering the extent of the xER-Int-CBD gene, were ordered and subjected to an extended number of PCR cycles. The resulting reaction mixture was separated by agarose gel, purified, and amplified by PCR using the 5’- and 3’-terminal primers from the 40-mers used in the gene construction. The resulting gene was then extended by PCR with additional primers to include a 3’-terminal PstI restriction site and additional base pairs to facilitate digestion. Subsequently, both pTYB1 and the xER-Int-CBD gene were subjected to a double digest by NdeI and PstI followed by ligation. Ligation was carried out at 16 °C for 16 h with 200 ng of pTYB1 with a 6:1 molar ratio of insert:host. The final ligation mixture was plated on agar and the DNA from 12 resulting colonies purified. This DNA was digested again with NdeI and PstI, followed by separation by agarose gel. In this analysis, a digest product of ~900 base pairs indicated failed incorporation of the xER-Int-CBD gene, while a digest product of ~1600 base pairs indicated successful incorporation. As is seen in Figure 4-12, a single colony of those selected exhibited proper incorporation. Isolated DNA from this colony was used as a template for expression of xER-LBD.

### 4.3.7 Expression and purification of xER-LBD from xER-LBD-Int-CBD

Expression of newly constructed estrogen receptor xER-LBD was carried out using conditions optimized for expression of ERα-LBD and 3ER-LBD and likewise purified. Initial SDS-PAGE analysis of purified fractions showed decent purity of a protein of expected apparent mass at a yield of 5 mg/L (Figure 4-13). Further characterization was done by excising the protein from the gel and subjecting it to tryptic digest followed by MS/MS analysis. Convincing sequence coverage was observed, confirming the expression of the correct protein.

After successful expression of xER-LBD, pH stability was briefly examined by SDS-PAGE analysis. A concentrated sample of protein in pH 8.0 storage buffer was split into two parts. One part was subjected to buffer exchange with 25 mM sodium phosphate buffer, pH 6.5, via 3 kDa MWCO spin filter. The remaining part was used for comparison. After 24 hours, each sample was centrifuged and each supernatant analyzed by SDS-PAGE. No difference in band intensity was observed between the two samples, indicating good solubility in both storage and phosphate buffers at designated pH. Additionally, no visible precipitate was present in either sample after centrifugation. Both
**Figure 4-11.** Table of candidates for an estrogen receptor stable at lower pH than ERα. *Xenopus* estrogen receptor (xER) was selected on the basis of good homology and lower pl.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Length (AA)</th>
<th>Homology (rel. ERα)</th>
<th>pl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>241</td>
<td>1.000</td>
<td>6.09</td>
</tr>
<tr>
<td>Mouse</td>
<td>285</td>
<td>0.971</td>
<td>5.94</td>
</tr>
<tr>
<td>Horse</td>
<td>240</td>
<td>0.959</td>
<td>6.21</td>
</tr>
<tr>
<td>Chicken</td>
<td>285</td>
<td>0.942</td>
<td>5.89</td>
</tr>
<tr>
<td>Frog (Xenopus)</td>
<td>284</td>
<td>0.848</td>
<td>5.62</td>
</tr>
<tr>
<td>Rainbow Trout</td>
<td>299</td>
<td>0.636</td>
<td>6.19</td>
</tr>
</tbody>
</table>
Figure 4-12. Agarose gel of NdeI/PstI digestion products from ligation colonies of xER-Int-CBD and pTYB1. Lane 9, indicated in red, shows incorporation of xER-Int-CBD into pTYB1. Length markers are given on the side of gel in base pairs.
Figure 4-13. SDS-PAGE analysis of purified xER-LBD. Purity was comparable to 3ER, while yields were significantly lower at 5 mg/L.
results indicated that sought-after stability at pH 6.5 had been achieved by this protein.

4.4 Terminal modification of estrogen receptors
4.4.1 C-terminal modification of 3ER-LBD and xER-LBD by expressed protein ligation followed by oximation

Owing to the poor expression yields and related poor solubility of the ERα-LBD construct, the ERα-LBD protein was quickly disqualified from attempts to effect high-yielding terminal modification. While this protein was deemed a poor candidate, both the high expression yields of the 3ER-LBD protein and the excellent solubility of the xER-LBD protein at pH 6.5 made them excellent candidates for C-terminal modification. Accordingly, ketone modification was achieved during purification of each protein by intein cleavage using 3, followed by transthioesterification and rearrangement upon exposure to ketone cysteine 1 (Figure 4-7). This concurrent purification and modification strategy afforded the ketone-modified 3ER-LBD (k3ER) and ketone-modified xER-LBD (kxER).

The extent of ketone modification of each protein was determined by subsequent oximation using an excess of aminooxy poly(ethylene glycol) methyl ether (2 kDa) and SDS-PAGE analysis. After exposure of both k3ER and kxER proteins to a 100 mM solution of 2 kDa PEG-ONH₂ for 18 h at pH 7, the extent of conversion to the ketone and oxime was determined via gel shift assay and densitometry (Figure 4-14). The extent of ketone modification for both kxER and k3ER was calculated to be 75% by densitometry. This extent of ketone conversion is more than sufficient for materials construction when coupled with the high expression yield of k3ER.

4.4.2 N-terminal modification of xER-LBD by transamination followed by oximation

While the expression yields and the extent of ketone modification for 3ER-LBD were both excellent, poor solubility below pH 8.0 was very limiting to our N-terminal modification strategy. In order to make these proteins amenable to N-terminal oximation, the necessary N-terminal ketone or aldehyde first needed to be installed through PLP-mediated transamination. Previous work has determined that the efficiency of this transamination is particularly poor above pH 6.5. Consequently, after preliminary experiments, it was determined that the solubility of 3ER-LBD in pH 6.5 buffer was not adequate to effect an appreciable extent of transamination. For this reason, N-terminal modification was only performed on xER-LBD.

To determine the optimal conditions for transamination of xER-LBD, PLP concentrations between 10 mM and 100 mM, as well as reaction times between 1 and 18 hours were screened. Best results were obtained by combining equal volumes of 200 μM protein solution and 200 mM PLP, both in 25 mM phosphate buffer, pH 6.5 and incubating at 37
Figure 4-14. Modification of kxER and k3ER with PEG-ONH$_2$ to determine extent of C-terminal ketone modification. Upon exposure to an excess of PEG-ONH$_2$, ketone modified protein is converted to the corresponding oxime (top). Conversion of kxER and k3ER to the PEG conjugate increases molecular weight sufficiently to observe by SDS-PAGE, allowing for determination of the extent of original ketone modification of each protein by densitometry (bottom).
°C for 1.5 h (Figure 4-15, a). Upon completion of the reaction, excess PLP was removed by buffer exchange with 25 mM phosphate buffer using a 3 kDa MWCO spin filter.

The extent of N-terminal modification was determined by reaction with an excess of an aminooxy species. In this case, benzoyloxyamine was added to the modified xER solution to a final concentration of 50 mM (Figure 4-15, b). After incubation at ambient temperature for 18 h at pH 6.5, excess small molecule was removed by buffer exchange with 25 mM phosphate buffer using a 3 kDa MWCO spin filter. The resulting mixture was analyzed by mass spectrometry, which indicated that the starting xER-LBD protein was entirely converted to the oxime product (72%), and two side products: PLP adduct (23%) and an oxime plus PLP adduct (5%). These side products have been observed, characterized, and discussed further in previously published work.31

4.5 Synthesis of a suitable aminooxy-containing monomer

Previous examples of hybrids constructed within our lab have employed aminooxy-methacrylamide-co-hydroxypropyl methacrylamide as the polymer component. While this system has proven effective, we were interested in creating and testing other possible polymer systems. Thus, 20 kDa 4-arm PEG 4 was obtained (Creative PEGWorks) for the synthesis of an aminooxy PEG (Figure 4-16). Terminal alcohol groups were protected by reacting with N-hydroxyphthalimide under Mitsunobu conditions. The protected product 5 was then deprotected with hydrazine to give aminooxy terminated species 6. The percent conversion of the –OH groups (4) to –ONH₂ groups (6) was 88%, as quantified by comparison of the number of aromatic hydrogens in intermediate 5 to the number of non-aromatic hydrogens, assuming an average mass of PEG as 20 kDa, and complete deprotection of the phthalimide groups in the following step. Specifically, the ¹H NMR integrations of the PEG signals were calibrated to the theoretical number of ethylene protons in a 20 kDa, 4-arm PEG. Subsequently, the aromatic proton signals were integrated relative to this calibration. The percent conversion was given by dividing the experimentally determined number of aromatic protons by the number of aromatic protons in a 100% functionalized product containing four phthalimide groups. Future work with this monomer will center on attachment to the functionalized protein. Verification of this attachment can be assessed by gel shift assay as well as fluorescent dye labeling experiments.22

4.6 Conclusion

Three different estrogen receptors, ERα-LBD, 3ER-LBD, and xER-LBD, have been designed and expressed as an intein-chitin binding domain fusion. Each was successfully purified by cleavage of the intein in moderate to excellent yields. 3ER-LBD and xER-LBD were further modified at the C-terminus by addition of a piperidone cysteine during cleavage. C-terminal modification levels of each protein were determined by be 75% following oximation with PEG-ONH₂ and gel shift assay. N-terminal modification was successfully achieved on xER-LBD through the use of PLP-mediated transamination.
Figure 4.15. N-terminal modification of xER-LBD. (a) PLP-mediated transamination strategy affords an N-terminal ketone amenable to (b) formation of the benzyl oxime upon exposure to aminoxybenzene. (c) Conversion to the oxime product, as well as xER-PLP adduct and oxime-PLP adduct was determined by quantitative mass spectrometry.
Figure 4-16. Synthesis of 4-arm aminooxy PEG (20 kDa)
Conversion of the N-terminal amine to the corresponding ketone was determined to be complete after oximation using benzyloxyamine and subsequent quantitative mass spectrometry. Finally, a 4-arm aminooxy PEG was synthesized in good yield and characterized for use as a future monomer in a protein-polymer conjugate.

Recent work has indicated that N-terminal modification of 3ER-LBD at pH 7.5 may be possible by a route other than PLP-mediated transamination. At this point, further optimization and characterization is required to confirm this finding. However, if proven successful, this N-terminal modification strategy will be tested on k3ER-LBD to determine whether this protein can be efficiently modified at both termini. If dual modification is successful, the excellent expression yield of 3ER-LBD will be a great asset in any future material produced. Work is currently underway to integrate this modification technique and test preliminary materials.

4.7 Materials and methods: general methods

Unless otherwise noted, all chemicals and solvents were obtained from commercial sources and used as received. Flash chromatography was carried out using EM silica gel 60 (230-400 mesh). Reaction and chromatography aliquots were analyzed by thin later chromatography using EM Reagent 0.25 mm silica gel 60-F$_{254}$ plates and visualized by ultraviolet (UV) irradiation at 254 nm. Methylene chloride (CH$_2$Cl$_2$) used was distilled under nitrogen from calcium hydride. Solvents used for $R_f$ determination by TLC are the same as the solvents used for flash chromatography. Water (ddH$_2$O) used in biological procedures was deionized using a NANOpure purification system (Barnstead, USA). UV-Vis spectroscopic measurements for OD$_{600}$ determination were made in plastic cuvettes using a Uvikon 933 Double Beam UV/Vis spectrophotometer (Kontron). UV-Vis spectroscopic measurements for $A_{260/280}$ measurements were made from 1.5 μL drops using a NanoDrop spectrophotometer (Thermo). Centrifugations were conducted with a Sorvall RC 5C plus (Sorvall, USA) for samples greater than 50 mL and lysed cell samples after sonication, a Sorvall LEGEND mach 1.6R for samples between 4 and 50 mL, and an Eppendorf Mini Spin plus for samples less than 2 mL (Eppendorf, USA). All images were captured with an Epicchiem3 Darkroom Bioimager (UVP Bioimaging).

4.8 Materials and methods: instrumentation and sample preparation

Mass spectrometry. Analysis and sequencing of tryptic fragments was done with a nanoAcquity ultraperformance liquid chromatograph (UPLC; Waters, Milford, MA) connected in-line with a nanoelectrospray ionization (nanoESI) emitter mounted in the ESI source of a quadrupole time-of-flight mass spectrometer (Q-Tof Premier, Waters). The UPLC was equipped with C18 trapping (180 μm × 20 mm) and analytical (100 μm × 100mm) columns and a 10 μL sample loop.

Analysis of the full protein was done with an Agilent 1200 series liquid chromatograph
(LC; Santa Clara, CA) connected in-line with an LTQ Orbitrap XL hybrid mass spectrometer that is equipped with an Ion Max electrospray ionization source (ESI; Thermo Fisher Scientific, Waltham, MA). The LC is equipped with C8 guard (Poroshell 300SB-C8, 5 um, 12.5 × 2.1 mm, Agilent) and analytical (75 × 0.5 mm) columns and a 100 μL sample loop.

**NMR spectroscopy.** All ¹H NMR spectra were measured with a Bruker AV-300 spectrometer (300 MHz). Chemical shifts are reported as δ in units of parts per million (ppm) relative to chloroform-d (δ 7.26, s). Multiplicities are reported as follows: s (singlet), t (triplet), or m (multiplet). The number of protons (n) for a given resonance is indicated nH, and is based on spectral integration values.

**Gel analyses.** For DNA analysis, agarose gel electrophoresis was carried out with 1% agarose/TAE gels containing 50 ng/mL ethidium bromide and a Mini-Sub Cell from Bio-Rad (Hercules, CA). All DNA samples were mixed with BlueJuice loading buffer (Invitrogen) and run for 20 min at 120 V to give good separation of bands. O’GeneRuler 1 kb DNA ladder (Fermentas) was applied to at least one lane of each gel for assignment of apparent molecular masses. Gel imaging was performed on an EpiChem3 Darkroom system (UVP, USA).

For DNA preparation from agarose gel electrophoresis, sample preparation, electrophoresis, and imaging were conducted as above. Subsequently, gel bands of interest were isolated from the gel by excision with a razor. Then agarose was removed and DNA purified from the slices using a QIAquick Gel Extraction kit (Qiagen).

For protein analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 10-20% gradient gels and a Mini-Protean apparatus from Bio-Rad (Hercules, CA), according to the general protocol of Laemmli. All protein electrophoresis samples were heated for 5-10 min at 100 °C in the presence of SDS and 1,4-dithiothreitol (DTT) to thoroughly denature the proteins. Gels were run for 30-50 min at 220 V to give good separation of bands. Commercially available markers (Bio-Rad) were applied to at least one lane of each gel for assignment of apparent molecular masses. Gel imaging was performed on an EpiChem3 Darkroom system (UVP, USA).

**Size exclusion chromatography (SEC).** SEC was performed using a Waters system including Waters 515 pump, a Waters 717 autosampler, a Waters 996 Photodiode Array detector (210-600 nm), and a Waters 2414 differential refractive index (RI) detector. SEC was performed on two SDV Linear S (5 mm) columns (Polymer Standards Service, 300 x 8 mm) at 1.0 mL/min using DMF with 0.2% LiBr as the mobile phase and linear PEG (4,200-478,000 MW) as the calibration standards. The columns were kept at 70 °C.

### 4.9 Materials and methods: synthesis of modified cysteine for cleavage and C-terminal ketone installation

**Synthesis of protected piperidone cysteine (8).** Compound 8 was synthesized based
Figure 4-17. Synthesis and deprotection of piperidone cysteine 1 for C-terminal functionalization.
on a previously described procedure. Briefly, to a 50 mL round bottom flask containing 10 mL of DMF was added N-Boc-S-trityl cysteine (7) (Nova BioChem) (1.0 g, 2.1 mmol) and carbonyldiimidazole (CDI) (0.43 g, 2.7 mmol). The resulting solution was stirred magnetically at rt for 30 min while CO₂ evolved. To this mixture was added 4-piperidone hydrochloride monohydrate (0.44 g, 2.9 mmol). The resulting suspension was stirred overnight at ambient temperature, at which point 4-piperidone hydrochloride monohydrate had completely dissolved. Significant conversion to product 8 was confirmed by TLC analysis. DMF was removed by rotary evaporation and the resulting yellow oil was dissolved in 20 mL of EtOAc. The solution was washed with three 50 mL portions of water, followed by two 50 mL portions of a 0.1 M NaHSO₄ solution, 30 mL of saturated NaHCO₃ solution, and 50 mL of brine. The organic layer was dried with MgSO₄. Purification by flash chromatography (CH₂Cl₂: 5% MeOH), followed by rotary evaporation of volatile components at reduced pressure afforded 0.62 g of 8 as an off-white solid (52% yield): TLC: (CH₂Cl₂: 5% MeOH) Rf = 0.67. ¹H NMR spectral data were consistent with published data.

Synthesis of deprotected piperidone cysteine (1). Deprotection of compound 8 was done immediately prior to use as described by a previously published procedure. Briefly, to a 20 mL scintillation vial containing 8 (0.16 g, 0.30 mmol), was added 0.9 mL of trifluoroacetic acid (TFA), giving an amber-brown solution, 0.05 mL of H₂O, and 0.05 mL of triisopropylsilane (TIPS), giving a white precipitate suspended in clear solution. A stream of N₂ was then bubbled through the solution to evaporate the liquid components. To the remaining, dried solid was added an additional 2.0 mL of TFA, which was then similarly evaporated. The dried material was resuspended in 20 mL of dH₂O and passed through a 0.2 um syringe filter to remove any remaining solids. Dilute, aqueous NaOH was used to adjust the pH to 8.0. The resulting solution was immediately used for subsequent protein functionalization.

4.10 Materials and methods: synthesis of aminooxy-4-arm PEG

Synthesis of N-hydroxypthalimide-4-arm PEG (5). N-hydroxypthalimide-4-arm PEG 5 was prepared based upon similar, previously published procedures. To a 100 mL round bottom flask was added 4 (Creative PEGWorks; SEC in DMF: Mn = 14013, Mw = 17478, PDI = 1.25) (1.0 g, 0.050 mmol), N-hydroxyphthalimide (NHP) (0.50 g, 3.1 mmol), and triphenylphosphine (0.87 g, 3.3 mmol). The flask was then sealed with a rubber septum and purged with N₂. Subsequently, 15 mL of dry methylene chloride was added through the septum by syringe, followed by stirring under a N₂ atmosphere for 0.5 h to dissolve the solids. To the mixture was then added diisopropyl azodicarboxylate (DIAD) (0.70 mL, 3.6 mmol) in a dropwise fashion. The mixture was stirred for 18 h under a nitrogen atmosphere, yielding a clear, yellow solution. The reaction mixture was then added dropwise to 500 mL of ice-cold ether in a Teflon beaker, immediately forming a white precipitate, and stirred for 1 h at 0 °C. The suspended precipitate was then filtered by gravity, washed with 35 mL of cold ether, and allowed to dry. The product was then washed through the filter paper into a flask with two 35 mL portions of methylene chloride, and the solvent removed by rotary evaporation. Conversion of the –OH groups
to phthalimide groups was calculated as 48%, so the reaction was repeated under the same conditions with the isolated product. The combined processes afforded 0.59 g (59% yield, 88% conversion) of 5, a fine white powder. SEC in DMF: $M_n = 15573$, $M_w = 18031$, PDI = 1.16. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$: 7.84-7.81 (m, 2H, C$_6$H$_4$), 7.75-7.72 (m, 2H, C$_6$H$_4$), 4.34 (t, 2H, CH$_2$ON), 3.88-3.38 (m, PEG signals).

Synthesis of aminooxy-4-arm PEG (6). Primarily following a previously published procedure,$^{35}$ to a 50 mL round bottom flask containing 5 (0.55 g, 0.028 mmol) was added hydrazine hydrate (40 μL, 0.8 mmol) and 15 mL of methylene chloride and stirred overnight at rt. The resulting cloudy solution was filtered over glass wool to remove the solids, which were washed with two additional 5 mL portions of methylene chloride. The filtered liquid was then dripped into 500 mL of cold ether in a Teflon beaker, giving a white precipitate, and purified in a manner consistent with that described in the previous synthetic step. 0.36 g (65% yield) of 6 was afforded as a white powder. SEC in DMF: $M_n = 13653$, $M_w = 16622$, PDI = 1.22. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$: 3.88-3.37 (m, PEG signals).

4.11 Materials and methods: construction, expression, and purification of estrogen receptors

Construction of ERα-LBD-pTYB1 plasmid. The ERα-LBD domain gene was obtained in a pUC57 vector (Genscript). The ERα-LBD was amplified from the ERα-LBD-pUC57 vector using the following primers:

Forward: 5’-AAG AAG GAG ATA TAC ATA TGG CAA AAG CGA AGA AAA ACA GC-3’
Reverse: 5’-TGG CAA AGC AGC TCT TCC GCA TTT ACC CGG GGC-3’

These primers placed a SapI restriction site at the C-terminus. The PCR product was digested sequentially with SapI and NdeI and ligated into the pTYB1 vector (NEB), which contained an ampicillin resistance gene. NEB-5α sub-cloning grade cells were transformed with 2 μL of the ligation mixture by a standard heat-shock protocol and spread onto a Luria Broth (LB)-agar plate containing 100 μg/mL ampicillin, and then grown overnight at 37 °C. DNA from resulting colonies was purified for subsequent use with a QIAPrep Spin Miniprep Kit (Qiagen).

Construction of 3ER-LBD-pTYB1 plasmid. Using the ERα-LBD-pTYB1 plasmid as a template, sequential site-directed mutagenesis$^{29}$ was performed using the following overlapping primers:

C381S
Forward: 5’-GCT GGA AAG CGC ATG GCT GGA AAT CCT GAT GAT TGG-3’
Reverse: 5’-GCC ATG CGC TTT CCA GCA GAT GCA CCT GAT CAT GCA GGG-3’
C417S
Forward: 5’-GGG CAA AAG CGT TGA AGG CAT GGT TGA AAT TTT TGA TAT GC-3’
Reverse: 5’-CCT TCA ACG CTT TTG CCC TGG TTA CGA TCC AGC-3’

C530S
Forward: 5’-GCA TGA AAA GCA AAA ATG TTG TTC CGC TGT ATG ATC TGC TGC TGG-3’
Reverse: 5’-CAT TTT TGC TTT TCA TGC TAT ACA GAT GTT CCA TGC C-3’

After successful mutagenesis, the 3ER-LBD-pTYB1 plasmid was transformed into NEB-5α sub-cloning cells in the same manner as ERα-LBD-pTYB1.

**Construction of xER-LBD-pTYB1.** The amino acid and DNA sequences of xER-LBD and the xER-LBD gene construct, bearing three cysteine to serine mutations, are given as follows:

AGGTAEQILISALMEAEAPIVYSEHDSTKPLSEASMMTLTTNLADRELVHM
INWAKRVPGFVDLTLHDQVHLLESAWLEILMVGLIWRSEVHPGKLFSAPN
LLLDRNQGRSVEGLVEIFDMVLVTATFRMMRLRGEIFICLKSIIILLNSG
VYTFLSSTLESLEDTDILHIIDTLVHFMASKGLSLQQQQRRLAQL
LLILSHIRHSMNKGMEHLSMSKKNVPLYDLLEMLDAHRIHTPKDKTT
TQEEDSRSPPTTTTVNGASPCLQPYYNTTEEVSLQSTVPQ

and

GGTGGTCATATGGCCGGGTGTGACCGCAGAACAGCTGATCTCCCGCTCTCATG
GGAGGCGAAGCGCCGATTTGTATACTCCGAGCAGACTCCACAGAACGCGC
TGAGCGAAGCGGTCTATGATGACGCTGCTGACCAACCTGAGCCAGCCGTAA
CTGGTTGCATATGATTAACCTGGCTAAACAGTGTGCGGGGTTTTCTGCTG
GACCGCTGCGACCCAGCCTGCTCTGCCTGAGACGCCGCTCTGCCTGAT
TGATGTTGCCCTGATCTGCGACGCTGAGAAACCCGGGTAACCTCTCTT
TTTGCCCCGAACCTGCTGCTGAGACTGTAATCAAGGCCGCTCTGCTGAGA
GCTGGTTGAAATCTTGACATGCTGTTACCCCGCAACTCCGCTCCGC
TGATGCGTCTGCGTGGCGCAAGAATTTATCTGCTGAAACGACATCATCCTG
CTGACACAGCGGTGGTTTACACGTTCCTGCTTCTCTACTCCTGAGAGCCTGGA
GGACACCGACCACCTGATCCCATATCATTTCTGCTGAAATATCATGACTACTCTGG
TCGCATTTATGCTACCAATTCGGCCCTGTCTCTCTGCAACAGACGACGCTGCT
CTGAGCAGCCAGGCTGCTGCTGAGACTGTAATCAAGGCCGCTCTGCTGAGA
AGGTATGGAACACCTGTACTCCATGAAAATGTAGATCTGCTGCTGCTG
TCGACTTATGCTACCAATTCGGCCCTGTCTCTCTGCAACAGACGACGCTGCT
CTGAGCAGCCAGGCTGCTGCTGAGACTGTAATCAAGGCCGCTCTGCTGAGA
AGGTATGGAACACCTGTACTCCATGAAAATGTAGATCTGCTGCTGCTGCTG
TCGACTTATGCTACCAATTCGGCCCTGTCTCTCTGCAACAGACGACGCTGCT
CTGAGCAGCCAGGCTGCTGCTGAGACTGTAATCAAGGCCGCTCTGCTGAGA
AGGTATGGAACACCTGTACTCCATGAAAATGTAGATCTGCTGCTGCTGCTG
TCGACTTATGCTACCAATTCGGCCCTGTCTCTCTGCAACAGACGACGCTGCT
CTGAGCAGCCAGGCTGCTGCTGAGACTGTAATCAAGGCCGCTCTGCTGAGA
AGGTATGGAACACCTGTACTCCATGAAAATGTAGATCTGCTGCTGCTGCTG
TCGACTTATGCTACCAATTCGGCCCTGTCTCTCTGCAACAGACGACGCTGCT
CTGAGCAGCCAGGCTGCTGCTGAGACTGTAATCAAGGCCGCTCTGCTGAGA
AGGTATGGAACACCTGTACTCCATGAAAATGTAGATCTGCTGCTGCTGCTG
TCGACTTATGCTACCAATTCGGCCCTGTCTCTCTGCAACAGACGACGCTGCT
CTGAGCAGCCAGGCTGCTGCTGAGACTGTAATCAAGGCCGCTCTGCTGAGA
AGGTATGGAACACCTGTACTCCATGAAAATGTAGATCTGCTGCTGCTGCTG
TCGACTTATGCTACCAATTCGGCCCTGTCTCTCTGCAACAGACGACGCTGCT
CTGAGCAGCCAGGCTGCTGCTGAGACTGTAATCAAGGCCGCTCTGCTGAGA
AGGTATGGAACACCTGTACTCCATGAAAATGTAGATCTGCTGCTGCTGCTG
TCGACTTATGCTACCAATTCGGCCCTGTCTCTCTGCAACAGACGACGCTGCT
CTGAGCAGCCAGGCTGCTGCTGAGACTGTAATCAAGGCCGCTCTGCTGAGA
AGGTATGGAACACCTGTACTCCATGAAAATGTAGATCTGCTGCTGCTGCTG
TCGACTTATGCTACCAATTCGGCCCTGTCTCTCTGCAACAGACGACGCTGCT
CTGAGCAGCCAGGCTGCTGCTGAGACTGTAATCAAGGCCGCTCTGCTGAGA
AGGTATGGAACACCTGTACTCCATGAAAATGTAGATCTGCTGCTGCTGCTG
The xER-LBD gene was synthesized by OE-PCR according to a previously published procedure\textsuperscript{30} using the following overlapping primers:

\begin{verbatim}
R0      ACCactgcctgggagta
F0      tactcccaggcagtGGTGGTCATATGGCCGGTG
R17     CAGCTGTTCTCGGCTCCACCGCCATATGACC
F33     GTACCGCAGAACAGCTGATCTCCGCTCTGCTCA
R50     ATCGGCGCTTCGGCCTCCATCAGAGCGGAGAT
F67     GGCCGAAGCGCCGATTGTGTACTCCGAGCAGC
R82     GCGGCTTCGTGGAGTCTCAGTCTCGGAATACACA
F99     ACTCCACGAAGCCCTGTAAGCGAAGCGTCTATG
R114    TGGTCAGCAGCGTCATCATAGCAGCGTCTCC
F132    TGACGCTGCTGGAGAGTCCTCAAGCTGGTTAAC
R147    CAGTTAATCATGTGACCAGTTCACGGTCTGCCAGGT
F162    AACTGGTTCACATGATTAACTGGGCTAAACGTGTGCCG
R184    GTCAGGTCACCCCGCAGCAGGTCGGCTGGTAAC
F200    GGTTTCGTGGACCTGACCCTGCACGACCAGGT
R217    GCTCTCCAGCAGCAGCTCCGAGCTGCGAGGCA
F232    GCATCTGCTGAGAGCGCCCGTCTTCTGCTAGCA
R248    CAGATCGAGCGCAACACGATAATTTCCAGCGCCACGC
F265    GATGTTGGGCCTGATCTGGCGTGCAGCCAGGTAACA
R283    AAAGACAGGTTTACCCGGGTGTTCTACGCTGCG
F298    CCCGGGTAAATCTGCTTGGCCACCAAGGTAAC
R316    GGCTTTGATTACGGTCCACGAGCGTGTCCGGGGCA
F332    CTGGACCGTAATCAAGGCGCTCTGTCAGAGGTGCTTG
R351    CAGCATGTCAGCGCACTCCGCGACCTCCGAGACGC
F369    TGGGAAATCTTGGAGCATAGGCTCCTGAGCAGCG
R389    AGACGCATCATGCGGAAGCGAGTTGCGGTGGTAAC
F407    TTTCCGATGATGCGTCTGCGTGGCGAAGAATTTAACT
R424    CAGGATGATGCTCTTCTAGCTTCCACCACCCCACTCGC
F444    GCCCTGAAAGACGAACTATCCTTGCTGAAACCAGCGGTGGTTAC
R464    CCAGGTCAGCGCAAGCGAGCGCTGCTGCTCAGCAG
F484    GTTCCTGTCTTCTACCTGAGAGGGCTGGTTCAGC
R504    TCCGAAATGATGTAGATGCTCCCTGCCTGCTCCAGGCC
F521    GACCTGATCCATATCATCATTCTGGAATAAATCATCGATACTTCTAGTGC
R544    GCCGGAATTGACCCATAAAGTGCAAAGGATTCGATGATTTTTA
F567    ACTTTGATGATGATTGTCGTTGCTCTGGATAACAGCA
R587    GCCAGCAGCGAGCCTGCTGCTGCTGCTGCTGCTGC
F604    GCAGGCTCCTGCGCAAGCAGCTGCTGCTGCTGCTGCT
R619    TGACGGAATGATGCGTCCAGATCGACAGCAGCAGCTG
F636    TGAGCCACATCCGCTCAGTCTGCTGCTGCTGCTGCTG
R652    TGCTTTTCAATGGAATGATGCTTACACCTTTGTTAGACATG
F675    ACCTGTACTCCATGAAAAGCAAATTTGAGTCTGGTTCAGCAGAC
R696    CAGCATTTTCCGACGAGGTCATACAGTGGAACACTACATTT
F719    CTGGCTGCTGGAAATGCTGGATGCCCATCGATCCACA
\end{verbatim}
In the above primers, the preceding F and R correspond to forward and reverse, respectively, while the following number corresponds to the position in the xER-LBD gene to which the first base in the primer corresponds. All above primers are listed from 5’-3’.

After construction of the xER-LBD gene, it was amplified using the following primers:

Forward: 5’- TAC TCC CAG GCA GTG GTG GTC ATA TGG CCG GTG GTA CCG C-3’
Reverse: 5’- GCT ACA GAA GTG CTA AGT AGG TGG TAG CTC TTC CGC ATT G-3’

Following amplification of the xER-LBD gene, the resulting PCR reaction was purified by column using a QIAPrep Spin Miniprep Kit (Qiagen).

Expression of ERα-LBD, 3ER-LBD, and xER-LBD. T7 Expression Cells (NEB) were transformed with the ERα-LBD-pTYB1, 3ER-LBD-pTYB1, or xER-LBD-pTYB1 vector by a standard heat-shock protocol, spread onto a LB-agar plate containing 100 ug/mLampicillin, and grown at 37 °C overnight. Cells were picked from the plate and grown at 37 °C in 1 L of LB containing 5% sucrose, 100 ug/mL ampicillin, and 10 μM estradiol (E2) until OD₆₀₀ reached 0.4-0.6. At this point protein expression was induced through the addition of 0.5 mM IPTG and the E2 concentration was increased to 20 μM. Cells were then allowed to continue growth for 4 hours at 25 °C, at which point they were removed to a centrifuge bottle and pelleted by centrifugation at 5000 xg for 10 min at 4 °C. Excess broth was discarded and cells were resuspended in 35 mL of lysis buffer (0.02 M Tris-Cl, 2 M urea, 10 μM E2, 0.2 M NaCl, 1 mM EDTA, pH 8.5) by a combination of vigorous pipetting and vortexing. The cells were lysed by sonication using a Branson Digital Sonifier (VWR Scientific) for 5 min (2 s on, 6 s off, 91% amplitude) with a blunt ended tip. Cell debris was removed by centrifugation at 17,600 xg for 40 min to give a translucent yellow solution.

Purification and C-terminal modification of ERα-LBD, 3ER-LBD, and xER-LBD. Estrogen receptor purification was adapted from a previously published protocol.³⁷ Lysate was first diluted by the addition of 105 mL (3 volumes) of wash buffer (0.02 M Tris-Cl,
0.5 M urea, 10 μM E2, 0.2 M NaCl, 1 mM EDTA, pH 8.5) to decrease the total urea concentration. To the lysate was then added 5 mL of chitin resin (NEB) that had been previously equilibrated with 50 mL (10 CV) of wash buffer utilizing a 25 mL fritted column. The lysate-chitin suspension was then stirred at 4 °C for 30 min. This suspension was returned to the fritted column and allowed to flow through by gravity. The resulting resin-bound protein was washed with 50 mL of wash buffer. Binding to the column was confirmed by SDS-PAGE analysis of the eluent and that of the resulting chitin. A 15 mL solution containing 50 mM MESNa or a solution of 50 mM MESNa and 5 mM 3 in wash buffer (adjusted pH to 8.5) was flowed over the resin bound protein with mild suction. The column bed was allowed to stand in this solution at rt for 18 h. Protein was eluted from the column with addition of 15 mL of wash buffer. Purified protein was then buffer exchanged into 25 mM phosphate buffer, pH 6.5, to remove free salts and 1, using Amicon Ultra 15 mL 10,000 MWCO (Millipore) spin concentrators.
4.12 References


