A Colorimetric Hormone-Dependent Bacterial Biosensor for Early Drug Discovery and Environmental Toxicology

Undergraduate Research Thesis

Presented in partial fulfillment of the requirements for the designation of "Research Distinction" for the degree of Bachelor of Science in Chemical and Biomolecular Engineering in the College of Engineering at The Ohio State University

By

Robert Alexander Law

Undergraduate Program in Engineering

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Thesis Committee:

Dr. David W. Wood, Advisor

Dr. Jeffrey Chalmers

Abstract

Many biosensors that have been developed to sense environmental changes or chemicals rely on change of phenotype assays. These assays often require special equipment or addition of reagents to detect fluorescence and can be both time and cost intensive. Here, a colorimetric *in-vitro* ligand–binding assay is proposed using β-galactosidase (β-gal, from the *lacZ* gene) in bacterially-expressed protein biosensors. The use of β-galactosidase-based assays (based on ONPG hydrolysis) for ligand-binding biosensors presents the advantage that β-galactosidase and the related reagents/equipment necessary are cost-effective, easily reproducible, commercially available, and much faster than traditional assays. The work to be presented was started with an established biosensor that transduces a test hormone-binding activity (estrogen) into a growth-phenotype. To obtain a colorimetric signal for binding rather than a growth phenotype, the growth phenotype reporter gene (thymidylate synthase) was replaced in the biosensor expression vector with the β-galactosidase gene. To verify our vector activity, cells were grown with and without estrogen and colorimetric assays were carried out on cell lysates to determine β-gal activity. Originally, the vector containing β-galactosidase was expressed in BL21(DE3 T7g1) recA $lacZ^+$ strain (BLR) but the chromosomal $lacZ^+$ was contributing background noise to the assay. To eliminate this background signal, the strain ER2566 (ΔlacZ::T7g1) was used for expression studies and three trials of β-gal assays implied

higher β-gal / *lacZ*$^{+}$ activity for cells grown with ligand than cells grown without ligand, suggesting the strain background was no longer contributing to noise. Next, cells were grown in the absence of the estrogen ligand and were lysed as estrogen was added to the lysate with the colorimetric assay reactant *in-vitro* while optimizing the dilution to allow for a longer assay so that the *in-vitro* additions of ligand would have adequate time to bind and potentially increase activity. Despite multiple attempts, no appreciable results were obtained during this step. A new experiment was devised which consisted of purifying the target protein of any proteases and cell debris through affinity chromatography involving binding to an amylose resin column. Estrogen ligand was then added to the purified protein and colorimetric assays were performed to test for increased β-gal activity. The *in-vitro* estrogen ligand additions to the purified protein showed no discernible changes in activity trends between the controls and samples tested. A hypothesis was formed that when cells took up the ligand *in-vivo* during growth, the ligand helped the ligand-binding domain fold properly during translation which produced the desired activity increase. However, it is believed that when the ligand was introduced *in-vitro* after lysis or purification, the ligand binding domain was improperly folded or the active site was buried which caused the suppression of changes in enzyme activity. A series of experiments were then carried out involving the *in-vivo* introduction of a weakly-binding stabilizing molecule (an estrogen antagonist) that would bind and help the ligand-binding domain fold properly during translation with the hope of introducing

estrogen agonists *in-vitro* after lysis or purification that would theoretically compete for binding and cause an increase in enzyme activity. Several experimental variations had been utilized along with many trials, but *in-vitro* estrogen ligand additions with or without antagonist molecules introduced *in-vivo* failed to produce any changes in β-gal enzyme activity. An additional experiment that had been performed concurrently to those presented here involved a similar construct to the human estrogen receptor but the receptor was of the human thyroid (TRβ), which has intrinsically higher signal to noise. The addition of thyroid ligands taken up *in-vivo* showed an identical trend with the thyroid receptor biosensor to that of estrogen additions to the estrogen biosensor. Although increased activity was defined at this step in an analogous manner, the failed progress of the estrogen receptor biosensor led to conclusion that the thyroid biosensor would behave in a similar manner and yield inconclusive *in-vitro* findings. As such, *in-vitro* ligand additions with the thyroid biosensor were never attempted due to the comparable nature of the hypothesized ligand-binding domain folding problem and due to the fact that there are no known thyroid antagonists that could potentially bind *in-vivo* only to get competitively displaced with the addition of binding thyroid agonists *in-vitro*.

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Vita

2008……....	Saint Ignatius High School, Cleveland OH
May 2013……	Late-Stage Pharmaceutical Development Intern Genentech Inc., San Francisco CA
June 2014…….....	B.S. Chemical & Biomolecular Engineering, The Ohio State University
2014…….....	William G. Lowrie Department of Chemical & Biomolecular Engineering "Outstanding Undergraduate Award for Research Excellence" recipient.

Fields of Study

Major Field: Chemical Engineering

Focus: Biomolecular Engineering

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Background and Introduction

The research presented in this work depends fundamentally on the introduction of an interfering protein (intein) into the DNA coding sequence for each of the bacterially expressed protein biosensors. Inteins are biological macromolecules that undergo self-splicing reactions to fuse the ends of the intein together (the N-extein and C-extein, respectively) while excising the middle portion. This unique reaction has a broad range of potential applications in the purification of proteins and as a functioning linker molecule in bacterial biosensors. The main amino acids that participate in the self-splicing reaction are the cysteine, serine, or threonine residues on the N-terminus and the histidine or asparagine residues on the C-terminus. Through genetic manipulation, these residues can be mutated to effectively restrict intein activity at either of the intein-extein bonds to generate an intein with single-site cleavage potential. One of the mutations that had been developed was a C1A mutation at the start of the intein coding sequence which successfully froze the N-terminus from participating in the splicing reaction, but did not inhibit the C-terminus from undergoing cleavage [1, 2].

The enzyme thymidylate synthase (TS) has been used as a valuable tool to study the effects of genetic mutations on inteins. TS is a phenotype growth reporter that when actively expressed in TS-knockout bacterial strains allows the cells to grow in the absence of thymine. When fused to the N-terminus of the intein, TS has been shown to be

inactive, but TS activity has been restored when linked to the cleavable C-terminus. The exact reason for this difference in activity is unknown, but it has been speculated that upon cleavage, TS forms a dimer which renders it more active, thus, when linked to the C-terminus of the intein, TS can aid in mutant selection on thymineless media which can be quantified by analyzing the growth phenotype of cells [1].

Genetic mutations using TS as a growth and activity modulator have produced mutant inteins that are susceptible to both temperature and pH. When a maltose binding domain had been added to the frozen N-terminus of the intein, a purification platform had been developed which would allow the maltose binding protein to bind to the resin in an amylose affinity column and upon changing the reaction temperature or pH of the buffer, the cleavage of the intein could be influenced to yield a relatively pure product protein. Another interesting development had been discovered when the ligand-binding domain of a human estrogen nuclear hormone receptor had been linked to the central domain of a genetically modified intein to yield a three-part protein consisting of maltose binding protein (MBP), an intein with an estrogen receptor (ERβ), and TS. The intein and maltose binding protein increased the solubility and stability of the estrogen receptor, but cells were still unable to grow with the new chimeric fusion protein until a genetic mutation was introduced to generate mutants that demonstrated an increase of growth in the presence of an estrogen ligand on thymine-less media in the TS-deficient *E. coli* strain

D1210Δ*thyA*. Effectively, a bacterial biosensor was created that showed hormone dependent activity which was expressed via changes in phenotype growth in selective media [1-5].

Nuclear hormone receptors are an important part of the endocrine system and their involvement has been linked to a variety of diseases which makes them an important point of interest as drug targets. As such, it was desired to extrapolate the results of the estrogen receptor (ERβ) to include another receptor, namely the human thyroid receptor (TRβ). When the nuclear hormone receptor was successfully swapped out, the cells containing the new TRβ mutants could only grow in thymine-less media in the presence of the natural thyroid hormone (T3) but had no differences in effect when an estrogen was added, which demonstrated ligand-receptor specificity. When the intein was removed from the sequence altogether, growth was significantly reduced which showed that the intein played a vital role in the function of the biosensor. It was necessary to test each biosensor with a variety of compounds to demonstrate their functionality as tools for drug discovery. An assortment of agonists was tested for the TRβ receptor along with a variety of agonists and antagonists tested with the ERβ receptor. The results illustrated that only estrogen agonists/antagonists had any effect on the ERβ biosensor with an analogous effect correlated to the TRβ biosensor using thyroid agonists. The general outcome was that compounds with higher binding affinities to the ligand-binding domain caused

greater changes in growth [1, 3-5]. Thus, the format of these assays could be converted to a 96-well plate format to yield a high throughput assay which could essentially detect potency of each small molecule ligand through the use of a plate reader. The successes of these experiments generated a novel tool that could be used for early drug screening as well as for analyzing environmental toxicity of molecular entities [3-5].

Though this was a significant step towards finding the solution to an important problem, there are some limitations. The main drawback for these assays is the dependence on time. *E. coli* cells need to be grown overnight before being converted to a 96-well plate and then incubated for several more hours after transfer. The work presented here aims to improve this constraint through the use of a different target protein other than thymidylate synthase that could have the same effect through a much faster route. It was also desirable to extrapolate the results from *in-vivo* ligand additions to *in-vitro* ligand additions so that the protein moiety could be extracted and purified from the cells in adequate quantities to additionally cut back on time constraints for reproducible high throughput results.

Methodology

The protein that was selected to expand upon the previous experiments is an enzyme known as β-galactosidase (β-gal) which has several applications in microbiology as a reporter protein. β-gal is 1,024 amino acids long and forms homologous tetramers. It is encoded by the *LacZ* gene and is recognized for the hydrolysis of β-galactosides into monosaccharide subunits. One of the more convenient substrates used for β-gal assays is ortho-nitrophenyl-β-galactoside (ONPG) for the reason that after hydrolysis, the ortho-nitrophenol product turns yellow and the amount of hydrolyzed product can then be quantified with a standard spectrophotometer.

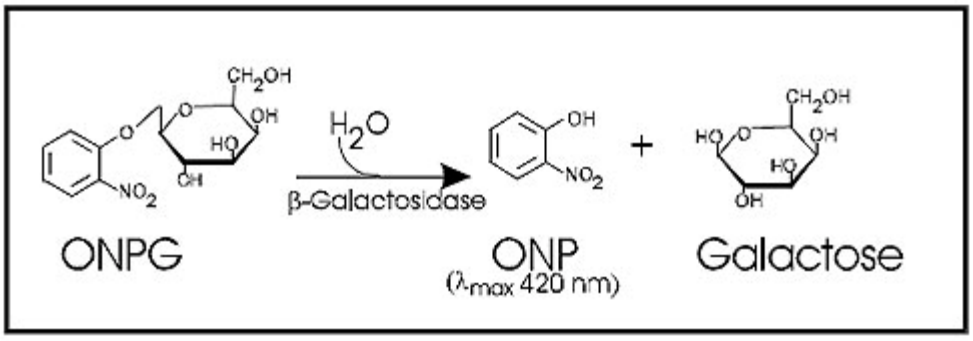


Figure 1: β-galactosidase reaction with ortho-nitrophenyl-β-galactoside

Upon successful replacement of thymidylate synthase with β-galactosidase, the assay format could be converted from a multi-hour growth phenotype assay to a colorimetric assay that takes less than five minutes to obtain data.

The DNA coding for wild type β-galactosidase was obtained and isolated from a stored vector (pET-E11-I-βgal). The desired endonuclease restriction sites for proper cutting and removal of βgal were Xba1 and Bsrg1. The vector containing the thymidylate synthase (pMIT::ERβ) contained both restriction sites, but the vector containing β-gal was missing the Xba1 site. Primers were designed to add an Xba1 site through a polymerase chain reaction (PCR) to successfully swap the target proteins. After DNA digestion and ligation of the vector, the desired protein sequence was obtained yielding maltose binding protein, an intein with an ERβ ligand binding domain, and β-galactosidase (referred to collectively as MIβ-gal::ERβ). The sequence was confirmed through the use of a whole-cell PCR and a DNA agarose gel.

After successful confirmation of the desired protein sequence, the new MIβ-gal::ERβ vector was transfected into a BLR strain of *E. coli*. The cells were grown in liquid growth media (Luria-Bertani) overnight and then transferred to over-day cultures in the presence of an ERβ ligand (Estradiol). An identical experiment was performed in the same fashion simultaneously with dimethyl sulfoxide (DMSO) added instead of the estrogen ligand to serve as a negative control. Both groups were grown until an optical density of ~1.0 was

obtained at 600 nm. The cells were then re-suspended in assay buffer (Z-buffer) and the OD_{600} was measured of the re-suspended cells. It was necessary to extract the protein from the cells via lysing and centrifugation after which the protein dilution was optimized and found to be 1:10 in assay buffer to yield a reaction time of 3.5 minutes. The ONPG substrate mixture was added to the protein and the time of reaction was recorded precisely. After the reaction was sufficiently allowed to continue for the allotted time, the reaction was quenched with 1M Na_2CO_3 which raises the pH to 11 and ultimately terminates the reaction. The resulting mixture was yellow in color and the optical density was subsequently recorded at 420 nm (OD_{420}) and 550 nm (OD_{550}). The activity of β-gal was then measured with Eq. (1) which quantified the activity in Miller Units.

$$\text{Miller Units} = \frac{????(?\,?\,???-?.???\,?\,???)}{??\;?*????\,?\;?*?\,?\,???} \qquad (1)$$

The results for the initial experiment can be found in Table 1 below.

Table 1: *In-vivo* β-gal activity assay results for MIβ-gal::ERβ in presence and absence of estrogen ligand in BLR strain of *E. coli*.

Plasmid (Ligand, Strain)	Activity (Miller Units)
MIβ-gal::ERβ (Estradiol, BLR)	1143.2
MIβ-gal::ERβ (DMSO, BLR)	984.662

At first glance, the biosensor appeared to function as predicted with the addition of ligand causing an increase in β-gal activity while the addition of pure DMSO caused a lesser amount of activity. The experiment was repeated under identical conditions and the results of the replicate can be found tabulated in Table 2 below.

Table 2: *In-vivo* β-gal activity assay results (repeated) for MIβ-gal::ERβ in presence and absence of estrogen ligand in BLR strain of *E. coli*.

Plasmid (Ligand, Strain)	Activity (Miller Units)
MIβ-gal::ERβ (Estradiol, BLR)	1198.62
MIβ-gal::ERβ (DMSO, BLR)	1461.65

Upon further investigation and data analysis, the calculated data from both trials was discovered to have a very low signal to noise ratio and the results of the assays were found to be inconclusive. It was then brought to attention that the BLR strain of *E. coli* that was used contained endogenous chromosomal β-galactosidase which was interfering with the results of the β-gal assays and producing the noise. The entire MIβ-gal::ERβ plasmid sequence was then transformed into a *LacZ* operon knockout strain of *E. coli* known as ER2566 in an effort to minimize interference from β-galactosidase not originating from the plasmid sequence of interest. The preliminary *in-vivo* experiments

and β-gal assays were then repeated in the ER2566 strain and a much stronger signal was obtained. The results were then repeated once in duplicate and the activity can be seen below in Table 3 and Table 4.

Table 3: *In-vivo* β-gal activity assay results for MIβ-gal::ERβ in presence and absence of estrogen ligand in ER2566 strain of *E. coli*.

Plasmid (Ligand, Strain)	Activity (Miller Units)
MIβ-gal::ERβ (Estradiol, ER2566)	3539.11
MIβ-gal::ERβ (DMSO, ER2566)	2281.26
MIT::ERβ (no β-gal, Estradiol, ER2566)	151.03

Table 4: *In-vivo* β-gal activity assay results (repeated in duplicate) for MIβ-gal::ERβ in presence and absence of estrogen ligand in ER2566 strain of *E. coli*.

Plasmid (Ligand, Strain)	Activity (Miller Units)
MIβ-gal::ERβ (Estradiol, ER2566)	3539.07
MIβ-gal::ERβ (DMSO, ER2566)	2853.36
MIT::ERβ (no β-gal, Estradiol, ER2566)	136.05
(Duplicate)	
MIβ-gal::ERβ (Estradiol, ER2566)	3715.06
MIβ-gal::ERβ (DMSO, ER2566)	2908.25
MIT::ERβ (no β-gal, Estradiol, ER2566)	142.86

In Table 3 and Table 4, the original construct MIT::ERβ containing the original reporter protein thymidylate synthase was introduced to serve as a second negative control. Since neither the vector nor the strain contained β-gal, the activity signal was observed to be extremely low, as expected for this control. Additionally, the activity for the MIβ-gal::ERβ constructs was on average ~900 Miller Units higher when estrogen ligand was introduced during over-day growth than when pure DMSO was added. The hypothesis for the observed activity increase is that ligand-induced cleaving allows the target protein to oligomerize (β-gal potentially forms a tetramer, TS potentially forms a dimer) which renders the reporter protein more active. This is historically consistent with previous results and is in good agreement with the data presented here, but attempts to further prove this hypothesis were not explored any deeper in this work.

Following the success of obtaining a greater β-gal activity signal for *in-vivo* ligand additions, a modified experiment was then devised very similar to the previous experiment with the exception that the ligand was to be added post-translationally *in-vitro* after the protein had already been expressed and the cells had already been lysed. The reaction time was also extended to 30 minutes to allow for sufficient ligand-receptor binding time after cell-lysis, but the data that was obtained had a very low signal to noise ratio and nothing conclusive was determined after repeated attempts. The raw β-gal activity data for these trials can be seen in Table 5 and Table 6.

Table 5: *In-vitro* β-gal activity assay results for MIβ-gal::ERβ in the presence of estrogen ligands and DMSO added after cell-lysis in ER2566 strain of *E. coli*

Plasmid (Ligand, Strain)	Activity (Miller Units)
MIβ-gal::ERβ (diethylstilbestrol, ER2566)	1627.74
MIβ-gal::ERβ (estradiol, ER2566)	1787.41
MIβ-gal::ERβ (bisphenol a, ER2566)	2255.21
MIβ-gal::ERβ (DMSO, ER2566)	1937.85

Table 6: *In-vitro* β-gal activity assay results (repeated) for MIβ-gal::ERβ in the presence estrogen ligands and DMSO added after cell-lysis in ER2566 strain of *E. coli*

Plasmid (Ligand, Strain)	Activity (Miller Units)
MIβ-gal::ERβ (diethylstilbestrol, ER2566)	1700.62
MIβ-gal::ERβ (estradiol, ER2566)	1653.38
MIβ-gal::ERβ (bisphenol a, ER2566)	1747.08
MIβ-gal::ERβ (DMSO, ER2566)	1841.71

It was expected that the β-gal activity would show a positive correlation with the binding affinity of each estrogen ligand with the human estrogen β receptor, but no such trend could be deduced from the preceding data. Additionally, it was expected that the negative control (DMSO) would show the least amount of β-gal activity analogous to the

preliminary *in-vivo* experiments, but this observation was not apparent for these *in-vitro* whole-lysate ligand addition experiments. It was hypothesized that some of the other cellular components such as proteases were interfering with or destroying the protein of interest before sufficient binding, cleavage, and activity could be quantified. Thus, it was necessary to purify the protein to rid it of these components in an effort to generate meaningful data.

In the next set of experiments, the cells were grown in the absence of ligand and were expressed at 37C. After lysis and centrifugation, the resulting protein was purified through an amylose column, ligand was introduced to the purified protein along with negative controls in separate experiments, and the resulting mixtures were incubated. Rather than measure enzyme activity at this step, the protein was run on an SDS-PAGE protein gel with a molecular weight standard to quantify the components of the mixture. There was no difference in cleavage products between the control sample and the ligand sample which led to a hypothesis that the ligand-binding domain was improperly folding in the absence of its substrate and that the active site was getting buried rendering ligand additions post-translation as ineffective. Thus, adding ligands post-translationally either in the lysate or to purified protein gave inconclusive results.

Traditionally, the human TRβ receptor has given a much higher signal to noise ratio than the corresponding ERβ receptor. The TRβ version of the above protein was then designed

and obtained in a very similar manner to that of the ERβ receptor through molecular cloning to give MIβ-gal::TRβ. MIT::TRβ was used as the vector backbone while MIβ-gal::ERβ was used as the insert departure point to swap thymidylate synthase for β-gal in the backbone vector using the same restriction sites as before (Xba I and Bsrg I) to give the new MIβ-gal::TRβ clone. A colony PCR and digest check were performed and the new clones were found to contain the proper insert (β-gal). The clones were then transformed into the same *LacZ* knockout strain of *E. coli* (ER2566) and the initial ERβ experiments were performed on the new TRβ clones using the appropriate thyroid ligand that fit the new receptor. Here, triiodothyroacetic acid (triac) and triiodothyronine (T3) were chosen as the thyroid ligands of interest for the new human thyroid receptor β. The ligands were introduced separately during over-day cell growth along with negative controls of estradiol (estrogen ligand) and DMSO. The cells were then centrifuged, lysed, and assayed using the same β-gal assay with the activity quantified in Miller Units. The activity results can be viewed in Table 7.

Table 7: *In-vivo* β-gal activity assay results for MIβ-gal::TRβ in presence and absence of thyroid ligands T3 and triac with E2 (negative control) and DMSO (negative control) in ER2566 strain of *E. coli*.

Plasmid (Ligand, Strain)	Activity (Miller Units)
MIβ-gal::TRβ (triac, ER2566)	4572.25
MIβ-gal::TRβ (T3, ER2566)	4430.69

MIβ-gal::TRβ (estradiol (E2), ER2566)	2766.85
MIβ-gal::TRβ (DMSO, ER2566)	2550.53

As expected, a very good signal was obtained for the ligand samples and the difference in activity was much more significant for the TRβ receptor when comparing the negative controls with the ligand samples. The results were repeated again in duplicate to ensure that the data was meaningful. Table 8 and 9 show the repetition results of this experiment.

Table 8: *In-vivo* β-gal activity assay results (repeated) for MIβ-gal::TRβ in presence and absence of thyroid ligands T3 and triac and DMSO (negative control) in ER2566 strain of *E. coli*.

Plasmid (Ligand, Strain)	Activity (Miller Units)
MIβ-gal::TRβ (triac, ER2566)	3482.0
MIβ-gal::TRβ (T3, ER2566)	4067.34
MIβ-gal::TRβ (DMSO, ER2566)	1748.48

Table 9: *In-vivo* β-gal activity assay results (duplicate) for MIβ-gal::TRβ in presence and absence of thyroid ligands T3 and triac and DMSO (negative control) in ER2566 strain of *E. coli*.

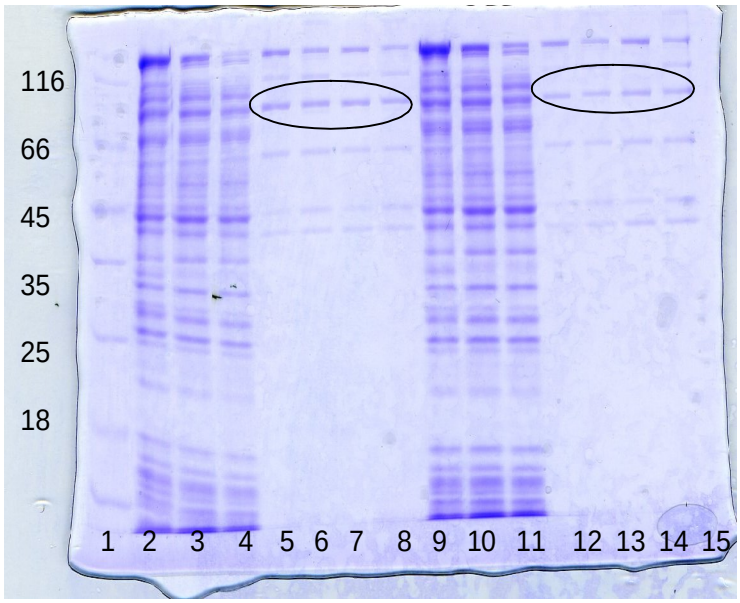
Plasmid (Ligand, Strain)	Activity (Miller Units)
MIβ-gal::TRβ (triac, ER2566)	3221.53

MIβ-gal::TRβ (T3, ER2566)	3838.26
MIβ-gal::TRβ (DMSO, ER2566)	1528.30

For the data obtained in Tables 8 and 9, the E2 (estradiol) negative control had been omitted for convenience and since ligand-receptor specificity had already been demonstrated historically in previous work, in the ERβ *in-vivo* trials, and in the first *in-vivo* trial of this TRβ experiment. From Tables 8 and 9, it is also apparent that the overall trend is conserved and that both the ERβ and now the TRβ clones exhibit greater increase in β-gal activity with a β-gal assay when the cells are grown with the appropriate ligands. The ERβ clones, however, had failed at producing relevant data when adding ligands to either the lysate or the purified protein *in-vitro* and it was assumed that the TRβ clones would give similar results. Thus, since the ERβ clones had failed with the *in-vitro* experiments at the same time that the TRβ clones were being made, the TRβ clones were not tested further.

In order to study the human ERβ receptor in more depth, MIβ-gal::ERβ cells were grown and over-expressed with IPTG at 20 °C for 3 hours. Estradiol (ERβ ligand) was added during over-expression along with the IPTG in order to allow the ERβ ligand-binding domain to fold properly during protein translation. In addition, one sample received DMSO to serve as the negative control. Both the ligand and no ligand samples were then

lysyed and purified on an amylose column at pH 7.0 and pH 7.5. The fractions were then run on an SDS-PAGE protein gel to quantify expression and cleavage products. It was hypothesized that the samples that received ligand expression would show cleavage products consistent with historical data while the DMSO sample would show no cleavage products and the lanes would consist of mostly precursor protein. As can be seen below in Figure 2, each of the ligand and DMSO purification products showed identical cleavage products at this expression temperature and time.



1.) Marker (kDa)
2.) Whole Lysate (+ estradiol)
3.) Clarified Lysate (+)
4.) Flow Through (+)
5.) Elute (+) pH 7.5
6.) Elute (+) pH 7.5 (+2 days at 4°C)
7.) Elute (+) pH 7.0
8.) Elute (+) pH 7.0 (+2 days at 4°C)
9.) Whole Lysate (- estradiol)
10.) Clarified Lysate (-)
11.) Flow Through (-)
12.) Elute (-) pH 7.5
13.) Elute (-) pH 7.5 (+2 days at 4°C)
14.) Elute (-) pH 7.0
15.) Elute (-) pH 7.0 (+2 days at 4°C)

Figure 2: SDS-PAGE protein gel of MIβ-gal::ERβ over-expressed with IPTG with and without estradiol for 3 h at 20°C and purified on amylose column

The target protein MIβ-gal::ERβ is 204 kilo-Daltons (kDa) and can be seen in the whole lysates, clarified lysates, and elute fractions as the very top dark band above the 116 kDa marker in Figure 2. The reason it is not present in the flow through wells is because this is the point where the protein is bound to the amylose column before becoming displaced with maltose in lanes 5-8 and 12-15. In these wells, we see cleavage around 88 kDa in both ligand and DMSO samples at both measured values of pH. This cleavage corresponds to the molecular weight of M-I::ERβ fraction (88 kDa) with a cleaved and washed β-gal fragment (116 kDa). This is the expected result for the protein samples expressed with ligand, but for the DMSO samples, this was somewhat of an anomaly. The protein was expected to have an improperly folded ligand-binding domain in the absence of a stabilizing molecule such as a ligand, which should have produced no cleavage products. The result would have been nothing but precursor band at the top of the gel in lanes 12-15 in Figure 2 above, but this was not the case and premature cleavage occurred independent of ligand-receptor interaction.

The same experiment above was repeated but with an expression temperature of 37°C and with an additional elution fraction taken at pH 6.5. The results can be seen in Figure 3.

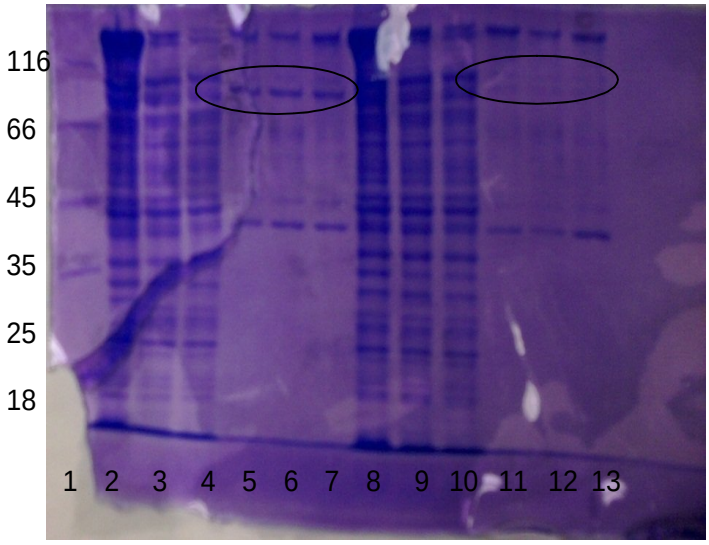


Figure 3: SDS-PAGE protein gel of MIβ-gal::ERβ over-expressed with IPTG with and without estradiol for 3 h at 37°C and purified on amylose column

In Figure 3, the protein gel had ripped during handling, but the end result had agreed with the expected result considerably. The protein over-expressed in the presence of ligand had cleavage products around 88 kDa (lanes 5-7) while the protein expressed in DMSO had nothing but precursor (lanes 11-13). This result is very significant because it is the strongest evidence to support that ligand-receptor interactions cause both cleavage and an activity increase when the ligand is added during growth or over expression. The idea is that the ligand helps the ligand-binding domain fold properly during translation which ultimately is suspected to cause cleavage and renders the free β-gal fragment more active

which is what caused the signal increase when running β-gal assays. Though this hasn't been proven directly, this is the best explanation for the phenomenon observed here.

The goal, however, was to develop an *in-vitro* colorimetric assay. As such, attempts were made to purify the protein and add ligands after purification so that the target protein could be purified in quantity which would make the assay much faster and presumably more sensitive since there would be fewer molecules affecting the assay after purification. For the next experiment, MIβ-gal::ERβ was grown and expressed at 37°C and pH 7.0 for 3 hours in the absence of ligand. Ligand (estradiol) was then added and incubated for an additional 3 hours with a negative control sample (DMSO). Time points were then taken at 0.5, 1, 2, and 3 hours of incubation. Each data point was then run on a n SDS-PAGE protein gel and the results can be seen below in Figure 4.

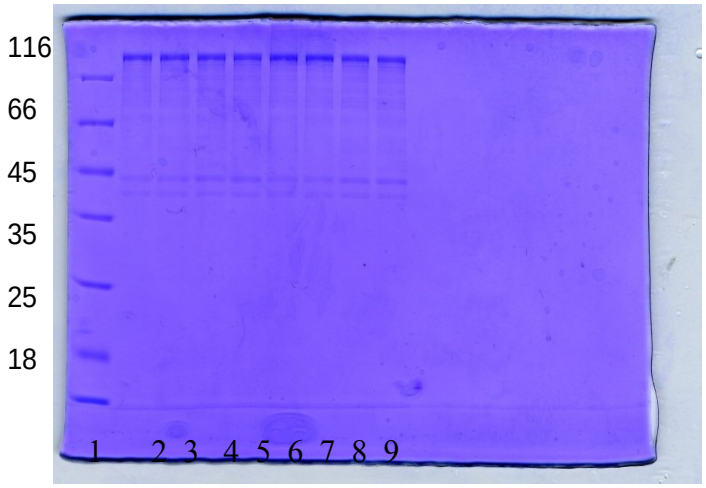
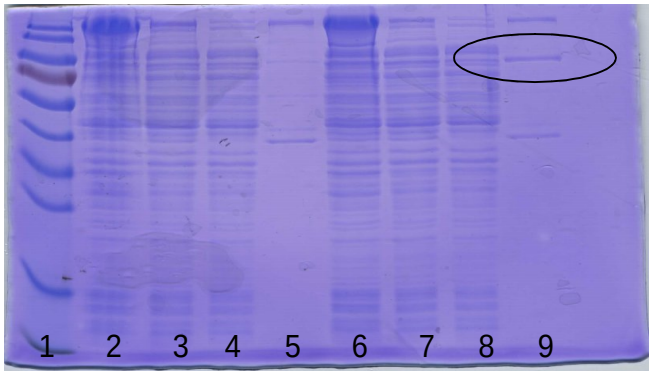


Figure 4: SDS-PAGE gel of purified MIβ-gal::ERβ expressed at 37°C for 3 hours with no ligand and purified at pH 7.0 with ligand and no ligand added after purification.

As seen in Figure 4, ligand additions after purification produced no change in cleavable products, again, because of the hypothesized improper folding of the ligand binding domain in the absence of a stabilizing molecule (ligand) during translation. β-gal assays were performed on each sample (data not shown), and the activity between ligand and DMSO samples was nearly identical, but the activity also decreased in both samples as the incubation time increased.

Since *in-vitro* ligand additions appeared to have no effect on the protein of interest, the idea of using another stabilizer molecule that would bind to the receptor during translation but not cause cleavage was implemented into the experiments. The molecule of interest was an estrogen antagonist called raloxifene and historically, this molecule did not cause cleavage of the C-extein portion of the intein. Once raloxifene binded to the receptor weakly (higher dissociation constant), a tightly-binding agonist could potentially displace the antagonist *in-vitro* and cause cleavage and the desired activity increase. The next set of experiments consisted of expressing the MIβ-gal::ERβ protein in the presence of the antagonist raloxifene and in the presence of both raloxifene and estradiol (agonist) and then purifying each sample in an effort to determine binding affinity of raloxifene and to see if any cleavage products would form. The SDS-PAGE results can be seen in Figure 5.

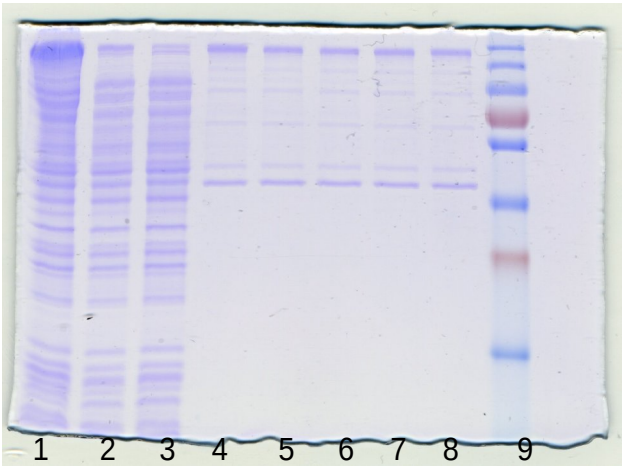


1.) Marker 9kDa)
2.) WL Raloxifene (ant.)
3.) CL Raloxifene (ant.)
4.) FT Raloxifene (ant.)
5.) Purified protein (Ralox.)
6.) WL Ralox. + E2 (ant. + agon.)
7.) CL Ralox. + E2 (ant. + agon.)
8.) FT Ralox. + E2 (ant. + agon.)
9.) Purified protein (Ralox. + E2)
All at pH 7.5

Figure 5: Purification of MIβ-gal::ERβ at 37°C at pH 7.5 expressed with antagonist (raloxifene) and antagonist + agonist (estradiol)

In Figure 5, it is clear that the sample expressed in both agonist and antagonist exhibits the same cleavage band around 88 kDa which is analagous to previous results involving expression of the target protein in the presence of a tightly binding ligand. By contrast, the sample expressed in only the antagonist produces no cleavable products near the 88 kDa marker as expected. However, from this experiment, it is impossible to tell if the raloxifene antagonist was binding to the target protein at all in either sample. It is possible that in the antagonist sample, the raloxifene was not binding at all which would still give the same result of no cleavage products on the SDS-PAGE gel. In the combination sample, if the raloxifene antagonist wasn't binding, the same result of the cleavage band at 88 kDa in lane 9 would have been obtained.

As a result, another experiment was designed to investigate if the estrogen antagonist was binding. Here, the antagonist was still introduced during expression, but a variety of estrogen agonists with varying binding affinities were added in an effort to displace the raloxifene antagonist and cause a β-gal activity increase. The ligand agonists that were added after purification were diethylstilbestrol (DES), estradiol (E2), and bisphenol a (BPA) with binding affinities in that order (tight to weak). Figure 6 displays the results of the SDS-PAGE gel.



1.) WL Raloxifene (ant.)
2.) CL Raloxifene (ant.)
3.) FT Raloxifene (ant.)
4.) Purified protein (Ralox.)
5.) purified + DES
6.) purified + E2
7.) purified + BPA
8.) purified + DMSO
9.) Marker (kDa)
All at pH 7.5 incubated for 12 h overnight at RT

Figure 6: Purification of MIβ-gal::ERβ at 37°C at pH 7.5 expressed with antagonist (raloxifene) with a variety of agonists added after purification

Again, there were no cleavage bands visibile near the 88 kDa marker (thick red band). Possible explanations for this are that the raloxifene wasn't binding during expression so

that the ligand binding domain was still improperly folding or that the ligand binding domain was folding around the molecule but the active site was still getting buried upon release of the antagonist. It is very difficult to prove either hypothesis directly, but another experiment was designed which involved trying to displace the antagonist before purification after cell lysis. The rationale was that the purification process was dialyzing the stabilizing antagonist molecule out which was then causing the active site to become mis-folded or buried.

For this experiment, the target protein MIβ-gal::ERβ was expressed at 37°C with estradiol (agonist, positive control), DMSO (negative control), and raloxifene (target sample of interest). Diethylstilbestrol (strongest agonist in the lab) was then added to each sample after cell lysis but before purification and incubated for 18 hours at 37°C, 4°C, and room temperature. The DMSO sample was expected to have an improperly folded ligand-binding domain and hence no cleavage and the lowest activity. The E2 sample was expected to exhibit cleavage and an activity increase as in the previous experiments. Finally, the raloxifene sample was expected to be displaced and have activity and cleavage products similar to that of the positive control (E2) sample. The results can be seen in Table 10.

Table 10: β-gal activity assay results of MIβ-gal::ERβ expressed with E2, DMSO, and Raloxifene with DES agonist added to cell lysates.

Temperature/OD405 reading	Expression Molecule		
37C	DMSO	E2	Raloxifene
OD450	0.705	0.696	0.642
4C			
OD450	1.048	0.972	0.847
RT			
OD450	1.02	0.964	0.844

In Table 10, the activity was compared using the OD_{405} rather than the full Miller Units equation for convenience and because the OD_{405} was the governing value of the equation. Table 10 also shows a different trend as expected because the negative control (DMSO) has higher activity at all three measured temperatures than either the target sample or the positive control (E2). It was observed that there was significant protein aggregation at 37°C so the experiment was repeated at only room temperature. The results were expected to be the same with no discernible trend, but Table 11 and 12 show the new trend that was discovered.

Table 11: β-gal activity assay results of MIβ-gal::ERβ expressed with E2, DMSO, and Raloxifene with DES agonist added to cell lysates at room temperature (suspensions)

Suspensions			
Temperature/OD405 reading	Expression Molecule		
RT	DMSO	E2	Raloxifene
OD450	0.6	0.886	0.756
RT (duplicate)			
OD450	0.76	0.966	0.913

Table 12: β-gal activity assay results of MIβ-gal::ERβ expressed with E2, DMSO, and Raloxifene with DES agonist added to cell lysates at room temperature (centrifuged)

Centrifuged			
Temperature/OD405 reading	Expression Molecule		
RT	DMSO	E2	Raloxifene
OD450	0.366	0.52	0.552
RT (duplicate)			
OD450	0.382	0.581	0.561

In Tables 11 and 12, the protein had aggregated slightly even at room temperature so the activity and cleavage products were analyzed for both the suspensions and centrifuged products of each sample. The trend was surprisingly close to what was expected with the negative control containing the lowest activity while the positive control and target sample contained higher activity. Upon purification of the clarified samples, the trend was still conserved, but there was no difference in cleavage products for any sample on

an SDS-PAGE gel (data not shown) so the difference in activity was rendered as noise. If the diethylstilbestrol ligand was displacing the raloxifene, it would be expected to cause cleavage for the activity increase, but this was not observed. It is possible that the protein was undergoing proteolysis and nonspecific degradation, but this has not been proven and the results from Table 11 and 12 are inconclusive. With the exhaustive work obtaining inconclusive data and with time quickly running out, the project was terminated at this point and no further work was completed on this project.

Summary of Results

In-vivo additions of appropriate ligands produced noticeable changes in cleavage products consistent with intein C-extein cleavage along with increased β-gal activity in both the MIβ-gal::ERβ clone and the MIβ-gal::TRβ clone. The assay time was greatly minimized with this proposed platform from other platforms, but ligand specificity was lost and there was no apparent correlation with ligand-binding affinity and signal strength. Additions of ligand agonists *in-vitro* to the estrogen receptor (ERβ) protein both before and after purification also produced no signal. Several attempts were then made involving adding a weakly binding antagonist (raloxifene) to potentially get displaced with a tightly binding agonist to obtain the desired activity change. These attempts failed when expressing the protein of interest with the antagonist and trying to displace it both before and after purification. No meaningful data was obtained after the initial *in-vivo* ligand addition experiments and the project was terminated.

Conclusions

The work presented here was based primarily on the original phenotype growth biosensor platform developed by David Wood [1-5]. The new MIβ-gal::ERβ/TRβ clones were experimentally confirmed to contain the β-gal substitution for TS. The MIβ-gal::ERβ/TRβ clones exhibited significantly increased β-gal activity when the appropriate ligand was added *in-vivo* during overday growth or expression and the protein also cleaved at what is presumed to be the C-extein which was observed on an SDS-PAGE gel. The mechanism of action is still unknown, but *in-vivo* ligand additions correlate very strongly with cleaved products. For the MIβ-gal::ERβ clone, however, no cleavage or difference in activity was observed when ligands were added to the lysate or to the purified product either with or without the antagonist added during expression. The results presented in the Thesis support both the hypothesis that the ligand-binding domain was misfolding along with the hypothesis that the intein cleavage reaction had something to do with the increase in β-gal activity that was observed. *In-vitro* activity increase or cleavage had never been observed with either the TS platform or the β-gal platform presented in this Thesis. Other attempts were made in the lab to translate the protein *in-vitro* with the help of an *in-vitro* translation kit in an effort to help the ligand-binding domain fold properly outside of the cell so that ligand-induced cleavage could take place, but these attempts generated no meaningful data. As such, this project has answered some

important questions, but has ultimately failed to meet the goal of developing an *in-vitro* colorimetric ligand-binding affinity assay. The *in-vivo* work produced some good data, but this semi-functional assay platform has proven to be mostly analogous to other platforms both in this lab and in the field and there are no real added benefits.

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