Brigham Young University
BYU ScholarsArchive

All Theses and Dissertations

2017-03-01

Engineering Cell-free Protein Synthesis Technology for Codon Reassignment, Biotherapeutics Production using Just-add-Water System, and Biosensing Endocrine Disrupting Compounds

Sayed Mohammad Salehi
Brigham Young University

Follow this and additional works at: https://scholarsarchive.byu.edu/etd

Part of the Chemical Engineering Commons

BYU ScholarsArchive Citation

Salehi, Sayed Mohammad, "Engineering Cell-free Protein Synthesis Technology for Codon Reassignment, Biotherapeutics Production using Just-add-Water System, and Biosensing Endocrine Disrupting Compounds" (2017). *All Theses and Dissertations*. 6711.
https://scholarsarchive.byu.edu/etd/6711

This Dissertation is brought to you for free and open access by BYU ScholarsArchive. It has been accepted for inclusion in All Theses and Dissertations by an authorized administrator of BYU ScholarsArchive. For more information, please contact scholarsarchive@byu.edu, ellen_amatangelo@byu.edu.

المنارة للاستشارات

www.manaraa.com

Engineering Cell-Free Protein Synthesis Technology for Codon Reassignment

Biotherapeutics Production Using Just-Add-Water System, and

Biosensing Endocrine Disrupting Compounds

Sayed Mohammad Amin Salehi

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Bradley C. Bundy, Chair
Randy S. Lewis
Thomas A. Knotts
Steven W. Graves
Steven M. Johnson

Department of Chemical Engineering

Brigham Young University

Copyright © 2017 Sayed Mohammad Amin Salehi

All Rights Reserved

المنارة للاستشارات

www.manaraa.com

ABSTRACT

Engineering Cell-Free Protein Synthesis Technology for Codon Reassignment,
Biotherapeutics Production Using Just-Add-Water System, and
Biosensing Endocrine Disrupting Compounds

Sayed Mohammad Amin Salehi
Department of Chemical Engineering, BYU
Doctor of Philosophy

Cell-free protein synthesis is an emerging technology that has many applications. The open nature of this system makes it a compelling technology that can be manipulated to answer many needs that are unavailable in other systems. This dissertation reports on engineering this technology for: 1) sense codon emancipation for incorporation of multiple unnatural amino acids; 2) expressing a hard-to-express anticancer biotherapeutic and introducing a just-add-water system; 3) a biosensing ligand that interacts with nuclear hormone receptors.

Emancipating sense codons toward a minimized genetic code is of significant interest to science and engineering. A promising approach to sense codon emancipation is the targeted in vitro removal of native tRNA. Here we introduce a new in-vitro or "cell-free" approach to emancipate sense codons via efficient and affordable degradation of endogenous tRNA using RNase-coated superparamagnetic beads. The presented method removes greater than 99% of tRNA in cell lysates, while preserving cell-free protein synthesis activity. The resulting tRNA-depleted lysate is compatible with in vitro-transcribed synthetic tRNA for the production of peptides and proteins.

Biotherapeutics have many promising applications, such as anti-cancer treatments, immune suppression, and vaccines. However, due to their biological nature, some biotherapeutics can be challenging to rapidly express and screen for activity through traditional recombinant methods. In this work, we demonstrate the use of cell-free systems for the expression and direct screening of the difficult-to-express cytotoxic protein onconase. Using cell-free systems, onconase can be rapidly expressed in soluble, active form. Furthermore, the open nature of the reaction environment allows for direct and immediate downstream characterization without the need of purification. Also, we report the ability of a "just-add-water" lyophilized cell-fee system to produce onconase.

Here we introduce a Rapid Adaptable Portable In-vitro Detection biosensor platform (RAPID) for detecting ligands that interact with nuclear hormone receptors (NHRs). The biosensor is based on an engineered, allosterically-activated fusion protein, which contains the ligand binding domain from a target NHR. The presented RAPID biosensor platform is significantly faster and less labor intensive than commonly available technologies, making it a promising tool for detecting environmental EDC contamination and screening potential NHR-targeted pharmaceuticals.

Keywords: Sayed Mohammad Amin Salehi, cell-free protein synthesis, codon emancipation, cancer biotherapeutics, endocrine disrupting compounds, nuclear hormone receptors, biosensor

المنارة للاستشارات

www.manaraa.com

ACKNOWLEDGEMENTS

I would like to thank the Department of Chemical Engineering, Brigham Young University, for providing the great opportunity of learning and growing. Being at BYU was one the best times of my life, and was a great experience. Special thanks to my graduate advisor, Dr. Bradley Bundy, for his support, patience and more importantly his friendship. He was a great mentor for me. I am thankful that he gave me the freedom to explore at the same time guidance to find a better path.

I would like to thank my graduate committee members Dr. Lewis, Dr. Knotts, Dr. Graves, and Dr. Johnson for their time, guidance, and their valuable comments. Also, I want to thank Dr. Brent Nielsen, Dr. John Price, Dr. Daniel Simmons, and Dr. David Thomson for they guidance and letting me use their lab. I greatly appreciate their help. I would like to thank Bruce Jackson for helping with mass spectrophotometry, James Armstrong for his technical support anytime we needed, Edwin Jackson for his advice and letting me use his equipment. In addition, I should thank our collaborators at Ohio State University, Dr. David Wood and Dr. Miriam Shakalli-Tang. It was a great collaboration, which expanded my knowledge and view to other fields.

I thank my friends and labmates at the Bundy Lab, Matt Schinn, Mark Smith, Jeffery Wu, Seung-ook Yang, Kristen Wilding, Christina Muhlestein, Conner Earl, Andrew Broadbent, Jeremy Hunt, and Anthony Bennett. They were a great help to my success. Also, I would like to thank the Department of ChemE secretaries, Arlene Cleverley and Serena Jacobson for their kindness, help, and patience. They are among the best people I have ever met.

Finally, I would like to thank my family for their love and support through my life. They are my biggest treasure. In particular, I want to thank my wife; being with her on this journey

المنارة للاستشارات

www.manaraa.com

was my greatest happiness, and now the presence of our daughter, Lena, makes it even more memorable.

Thank you all!

المنارة للاستشارات

www.manaraa.com

TABLE OF CONTENTS

ABSTRACT ii
TABLE OF CONTENTS v
LIST OF TABLES viii
LIST OF FIGURES ix
1 INTRODUCTION 1
1.1 Overview 1
1.2 Scope of the Projects 2
1.2.1 Expanding the Genetic Code 2
1.2.2 Cancer Biotherapeutics Production Using CFPS 4
1.2.3 Biosensing Endocrine Disrupting Compounds 7
1.3 Outline 10
2 Codon Emancipation with a Cell-free Platform: Highly Efficient tRNA Degradation 12
2.1 Introduction 12
2.2 Material and Methods 15
2.2.1 Cell Extract Preparation and Treatment to Deplete Endogenous tRNA 15
2.2.2 Total RNA Purification and qPCR 16
2.2.3 *In vitro* Transcription of tRNA 17
2.2.4 Cell-free Protein Synthesis (CFPS) 17
2.3 Results and Discussion 18
2.3.1 tRNA Depletion of Cell Extract 19
2.3.2 tRNA Depletion Efficiency 22
2.3.3 CFPS of Peptides Using Treated Cell Extract and Synthetic tRNA 24
2.4 Conclusion 26
3 Onconase production and a just-add-water cell-free system 27
3.1 Introduction 27
3.2 Materials and Methods 30
3.2.1 Cell Growth and Extract Preparation 30
3.2.2 In Vivo Expression 30
3.2.3 Cell-free Protein Synthesis 31
3.2.4 Onconase Anticancer Activity Assay 31
3.2.5 tRNA Purification 32

المنارة للاستشارات
www.manaraa.com

3.3 Results 33
3.3.1 Recombinant In Vivo Synthesis of Onconase 33
3.3.2 Cell-free Synthesis of Onconase 37
3.3.3 tRNA-supplemented Cell-free Protein Synthesis 41
3.3.4 Lyophilzed Cell-free System 44
3.3.5 Onconase Activity 44
3.3.6 Protein Synthesis Inhibition Activity 45
3.3.7 Anticancer Activity 48
3.4 Discussion 49
4 A Cell-free Protein Synthesis Approach to Biosensing hTRβ-Specific Endocrine 51
4.1 Introduction 51
4.2 Experimental Section 54
4.2.1 Materials. 54
4.2.2 Biosensor Design and Construction. 54
4.2.3 Cell Extract Preparation 55
4.2.4 Lyophilizing the Biosensor System. 56
4.2.5 Cell-free Protein Synthesis Reaction. 56
4.2.6 Environmental and Human Samples Tested in Cell-Free Protein Synthesis 57
4.2.7 Hormone Biosensor Assay 57
4.2.8 Analysis of Hormone Biosensor Assay Results 58
4.3 Results and Discussion 59
4.3.1 RAPID Biosensor Design and Rationale 59
4.3.2 Cell-free Protein Synthesis of the Reporter Fusion Protein 61
4.3.3 Hormone Biosensor Assay 63
4.3.4 Lyophilized Biosensor 65
4.3.5 CFPS Performance in Different Environmental Samples 66
4.3.6 NHR RAPID Biosensor Performance in an Environmental Sample 68
4.4 Conclusion 69
5 Conclusions and Future Work 71
5.1 Future Work 71
5.1.1 Codon Emancipation 71
5.1.2 Expressing a Hard-to-express Anticancer Biotherapeutics 72
5.1.3 Biosensing Ligand that Interact with Nuclear Hormone Receptors 72

المنارة للاستشارات
www.manaraa.com

References 73

المنارة للاستشارات
www.manaraa.com

LIST OF TABLES

Table 2-1 List of primers used for qPCR of various tRNAs to measure residual tRNA level 17
Table 3-1 Bacterial Growth Regression Curves 35

www.manaraa.com

LIST OF FIGURES

Figure 2-1 Schematic of the in vitro codon emancipation approach demonstrated in this work.. 14
Figure 2-2 a) Schematic diagram of extract treatment to deplete endogenous tRNA 20
Figure 2-3 Normalized protein synthesis of sfGFP under various conditions............................. 21
Figure 2-4 Extent of tRNA-depletion in cell extracts.. 22
Figure 2-5 Synthetic tRNA production.. 24
Figure 2-6 Emancipating codons using a treated cell extract and synthetic tRNAs..................... 25
Figure 3-1 Mass spectrometry analysis of *in-vivo* expressed onconase. 34
Figure 3-2 Onconase In vivo Expression and Solubility. 34
Figure 3-3 Nuclease Assay with In vivo-produced Onconas... 36
Figure 3-4 Cell-free Expression Optimization of ONC 38
Figure 3-5 Green Fluorescent Protein CFPS 40
Figure 3-6 CFPS Reaction Volume Scale up for onconase production....................................... 42
Figure 3-7 CFPS with Commercial and Purified tRNA. 43
Figure 3-8 CFPS ONC Yield costs. 45
Figure 3-9 Lyophilized CFPS Systems.. 46
Figure 3-10 Onconase Activity Assays.. 47
Figure 3-11 Protein Synthesis and Cancer Cell Inhibition Controls.. 48
Figure 3-12 The MCF7 cancer cell line was subjected to. 49
Figure 4-1 (A) The protein construct for the RAPID biosensor 60
Figure 4-2 Scheme of the RAPID biosensor to detect chemicals that target NHRs..................... 62
Figure 4-3 A) Dose-response curve for the hTRβ biosensor in the presence of TRIAC.............. 64
Figure 4-4 Dose-response graph and statistical analysis results for the RAPID biosensor 66
Figure 4-5 Protein production capability of CFPS in the presence of... 67
Figure 4-6 Dose-response graph and statistical analysis results for the RAPID biosensor 69

www.manaraa.com

1 INTRODUCTION

1.1 Overview

Cell-free synthetic biology is an emerging technology that promises to change our lives in ways in the near future. For decades, using living cells to produce useful and varied products from biofuels to cancer drugs has been an essential part of our life [1]; however, *in vivo* synthesis of products has some limitations. In order to survive, each cell must spend energy and use its machinery to maintain its viability, thus limiting the energy and machinery that can be used to produce the desired product. Moreover, in many cases the cell's membrane inhibits the mass transfer of necessary raw materials into the cell or products out of the cell. In addition, some desired products are toxic to the cell and contaminations or viral infections continually threaten cell fermentations. Cell-free synthetic biology is a unique approach that can address these and many other limitations involved in using living cells.

Cell-free synthetic biology is the activation of complex biological processes outside of cells, so there is no cell that needs to be kept alive and energy/machinery can be focused on producing the desired products. This technology was first used to discover the genetic code around 50 years ago. However, only recently have researchers had the knowledge and tools necessary to truly take advantage of the cell-free platform to economically produce complex commercial products [2, 3]. Cell-free synthetic biology has opened new opportunities for new synthesized products. This technology enables direct access to and manipulation of the complex

المنارة للاستشارات
www.manaraa.com

reactions that usually take place inside of living cells [3, 4]. In this way, all reactions can be controlled and optimized to identify bottlenecks and improve product yield [2]. Furthermore, purification of products is simpler from a cell-free environment. These benefits of cell-free synthetic biology make it a powerful tool to produce compounds that are difficult to produce in cells.

1.2 Scope of the Projects

In this work, we aimed to expand applications of the cell-free technology by engineering this system for: 1) expanding the genetic code by emancipating sense codons, 2) producing hard to express cancer biotherapeutics and introducing the Just-add-water system, and 3) biosensing ligands that interact with nuclear hormone receptors.

1.2.1 Expanding the Genetic Code

Life uses a canon of 20 primary amino acids and 64 codons as its code. This is conserved across living organisms and is known as the genetic code - with the exception of some rare species that have slight changes in their genetic code as a response to changes in their environment and energy sources [5]. From the 64 available codons, three codons act as stop codons, TAG (amber), TAA (ochre), and TGA (opal); consequently, the 61 remaining codons are considered as sense codons, and they code for the 20 amino acids. Therefore, some codons code for the same amino acid, and there is promiscuity among them. The idea behind expanding the genetic codes is to reprogram some of the codons to incorporate unnatural amino acids (uNAA). This idea could produce an almost infinite number of new proteins.

www.manaraa.com

The early studies tried to site-specifically incorporate unnatural amino acids into protein by using chemically aminoacylated suppressor tRNA in response to an amber stop codon [6, 7]. Bain et. al. [8] in another study developed a method to incorporate unnatural amino acids by creating a 65th codon-anticodon pair from unnatural nucleoside bases having non-standard hydrogen-bonding patterns. These remarkable efforts were followed by the Schultz lab discovery. Instead of using chemical methods, they introduced orthogonal a tyrosyl tRNA/Synthetase pair from *methanocaldococcus jannaschii* to *E. coli* to incorporate unnatural amino acid in response to the amber stop codon [9, 10]. This tRNA must not be recognized by native *E. coli* aminoacyl-tRNA synthetases and act efficiently to deliver novel amino acids into the translation process. This work introduced a new method to expand the genetic code and add unnatural amino acids.

Following this discovery, researchers could successfully expand the genetic code for Saccharomyces cerevisiae as a eukaryote [11], and mammalian cells [12]. In addition to this method, Anderson et. al. [13] used orthogonal tRNA/aminoacyl-tRNA synthetase pair in response to the quadruplet codon, AGGA, in *E. coli*. These methods made a platform for expanding the genetic code both *in vivo* [12] and *in vitro* [14].

However, the current technologies for incorporating uNAA into protein suffer from some limitations that have narrowed the applications of uNAA incorporation. These limitations include: 1) inefficient/inaccurate uNAA incorporation due to competition with endogenous (native) components with exogenous (foreign) components (which causes the approximately 30% efficiency commonly observed with current technology), 2) exponential amplification of inefficiencies for incorporation of multiple unnatural amino acids (e.g. less than 1% of product would be correct if 4 uNAA are incorporated), and 3) difficulty in incorporating different types of unnatural amino acids simultaneously [12, 15, 16].

المنارة للاستشارات
www.manaraa.com

This study seeks to address these issues and enable efficient simultaneous incorporation of multiple different unnatural amino acids by using a novel CFPS approach.

1.2.2 Cancer Biotherapeutics Production Using CFPS

From 1991 to 2012 the cancer death rate dropped by 23 percent constituting nearly 1.7 million lives saved [17]. Much of this success could be attributed to the advances in targeted therapies such as monoclonal antibody therapeutics [18, 19] and anticancer peptides [20, 21]. Still, cancer remains the second leading cause of death in the United States and is projected to overtake heart disease in the next few years [22] with a projected 1,685,210 new cancer cases and 595,690 deaths in 2016 [17].

The development of targeted oncological therapies in the field of protein biologics has revolutionized our ability to treat cancer. To date, all FDA-approved anticancer protein biologics have been produced *in vivo* [19] where 69% of those have been produced recombinantly in *E. coli* [19]. As successful as *in vivo* production has been and continues to be, there are still many drawbacks to the closed, transport-limited *in vivo* environment including 1) inability to produce cytotoxic proteins at high yields, 2) transport inhibition of non-natural components, 3) a walled-in environment that complicates direct *in situ* monitoring, control, and dynamic optimization of required reagents (e.g cofactors, redox, translation elements), and 4) a crowded environment that can inhibit the correct folding of complex proteins [2, 4, 23]. The open, non-living environment provided by cell-free technology overcomes these limitations allowing the production of cytotoxic proteins [24, 25], unnatural amino acid (uAA) incorporation [23, 26], and the rapid synthesis of personalized medicines [27].

www.manaraa.com

The open nature and facile manipulation of the cell-free environment allows for greater control, monitoring, and high-throughput screening techniques for improved protein evolution compared to the *in vivo* method [3, 23, 28, 29]. While standard *E. coli*-based cell-free systems lack the ability to perform glycosylation, the potential of using hybrid cell-free lysates and the addition of exogenous components has the promise of overcoming this limitation [30, 31]. All of these aspects combined with the high toxicity tolerance of CFPS makes this system a compelling platform for rapidly developing, screening, and producing difficult to express anticancer biotherapeutic proteins.

One emerging application in cancer research where cell-free technology is vital is the synthesis of personalized vaccines to more quickly and efficiently treat certain types of cancers. Previous work reported that vaccine proteins for anti-cancer therapeutics could be produced rapidly in *E. coli*-based cell-free systems [32]. In that study, complex fusion of Granulocyte macrophage colony-stimulating factor (GM-CSF) and B-lymphocyte Id scFv with different arrangements were produced successfully with CFPS [27]. Importantly cell-free reagents can be stockpiled and then scaled for reliable consistent production at the microliter, milliliter, or liter scale. Thus the major limitation becomes the time required for synthesizing the DNA that templates of CFPS and custom DNA can now be economically synthesized in as little as a day. While the implementation of customized immune therapy soon after diagnosis on a large scale requires further research and streamlining; simple, rapid and economical production of personalized anti-cancer therapeutics may soon be practicable using cell-free technology [27].

While many proteins of oncological value are produced recombinantly *in vivo* [19], cell-free technologies could contribute to improving the effectiveness and economics of these and future therapeutics. For example, the cell-free technologies of protein evolution with ribosome

المنارة للاستشارات
www.manaraa.com

display can improve the solubility and activity of protein therapeutics [3, 28, 33]. Another important technology in oncological therapies is that of anticancer peptides. Anticancer peptides in many cases have higher target specificity, lower intrinsic toxicity, and greater ease of modification compared to full-length proteins used in chemotherapy [21, 34, 35]. Peptides can also be fused to cell penetrating moieties to better target and treat cancer [34]. Some of these modification schemes include attaching radionuclides, hormones, vaccines, or other drugs to a nascent peptide [36]. One great challenge in the development of clinically viable therapeutic anticancer peptides is improving delivery, minimizing non-specific toxic effects, and a greater understanding of pharmacokinetic properties [20].

While peptides are traditionally produced by chemical synthesis or *in vivo* via nucleotide sequences, these methods have significant drawbacks in time scale and cost [37]. Cell-free technologies offer a compelling platform that could be explored to rapidly and more efficiently study, screen, and produce similar peptides on an industrial level [4, 23, 37]. For example, Lee et. al. developed a strategy for rapid cell-free expression and recovery of multiple peptide molecules [37]. They optimized peptide production by developing a DNA construct with an enhanced sequence for improved translation efficiency, protease resistance, purification recovery, and cleavage efficiency. Continued engineering of cell-free technologies such as these demonstrates how cell-free systems could be utilized for the automated, rapid production of clinically viable pure proteins and peptides that have significant oncological value.

Taking advantage of these benefits, we engineered CFPS to produce a hard to express anticancer protein biotherapeutics, onconase. We expanded our lyophilized CFPS and introduced the just-add-water system. The presented method showed huge advantages over *in vivo*

المنارة للاستشارات
www.manaraa.com

production of onconase by reducing the expression and screening time of onconase from several weeks to a few hours.

1.2.3 Biosensing Endocrine Disrupting Compounds

Nuclear hormone receptors (NHRs) are involved in vital functions of the cells including: metabolism, homeostasis, differentiation, development, and reproduction [38, 39]. NHRs interact with many natural and synthetic ligands and about 4% of all currently marketed therapeutics interfere with the activity of one or more NHR [40]. Understanding effects and interactions between these therapeutics and NHRs is critical to developing new drugs and improving available ones. For instance, many NHRs have multiple subtypes and isoforms with a high degree of homology but different functionalities [41]. Many diseases are the result of one of these subtype's or isoform's disorder, but many drugs have agonist or antagonist activity against the whole family or multiple subtypes of a NHR, which can cause significant side effects. The demand for subtype-selective therapeutics, in addition to difficulties in their development require screening of them, which in turn brings about the necessity of a fast, inexpensive, and robust screening method against NHRs [41, 42].

NHRs can also be targeted by endocrine disrupting chemicals (EDCs), which have become a public safety concern due to their ability to interfere with naturally occurring endocrine control. EDCs have been found in common dietary, environmental, and household chemicals and have been linked to diverse diseases and disorders, including multiple cancers, developmental disorders, and other epigenetic dysfunction [43, 44]. Due to the risk EDCs pose to the endocrine systems in humans and other animals, in 1996 the Environmental Protection Agency (EPA) listed approximately 10,000 chemicals of concern and established a two-tier screening system in

المنارة للاستشارات
www.manaraa.com

which the first tier is simply the assay of a compound's endocrine disrupting activity. According to the EPA, as of May 2014, only 109 chemicals (~1% of EPA list) have been actively screened as potential EDCs [45]. Despite the ability of currently available assays to generate substantial data on suspected endocrine disruptors, they remain low throughput, expensive, and require lengthy laboratory analysis. These complications thus limit current EDC assays to a laboratory setting with extensive capital and consumable costs. Due to the sheer enormity in number, most chemicals and mixtures in commerce worldwide, therefore, remain largely uncharacterized for endocrine disrupting activity. Many of the EDCs implicated in these trends affect the endocrine system in human and animals, and commonly have an affinity for specific NHRs. This important class of EDCs interacts with human and animal NHRs through their ligand-binding domains by mimicking natural hormones [46]. Examples of these include medical and industrial xenoestrogens, such as diethylstilbestrol (DES) and bisphenol-A (BPA), as well as naturally occurring phytoestrogens, such and genistein and daidzein [47-49].

For these reasons, numerous *in vivo* and *in vitro* ECD assays have been developed, each with their advantages and disadvantages [45, 50-52]. Mammalian cell and live animal assays are generally the most reliable for the detection and prediction of the biological effects of a test chemical [53]. However, animal and most mammalian cell assays are expensive, time-consuming and labor-intensive, making them unrealistic for high-throughput screening. In addition, live animal data can be profoundly affected by very subtle confounders, including feed nutrient and phytoestrogen levels, maternal stress, intrauterine position, seasonal changes, dosing levels and timing, cage materials, housing strategies, strains used, differences in measurement techniques, and selection of endpoints, among others [54-57]. A final limitation of animal assays is that they

المنارة للاستشارات
www.manaraa.com

cannot indicate the specific target and mechanism of a particular endocrine-active chemical, particularly if gross morphology or metabolic effects are observed as endpoints.

Specific determinations of target and activity, however, can be made by direct ligand-binding assays *in vitro*, which are fast and have the potential to screen large libraries in a high-throughput manner [58, 59]. These assays typically evaluate the binding of test ligands to a purified ligand binding domain, or whole receptor, by quantifying the displacement of a labeled ligand standard [60]. The primary drawback to these methods is that they require the receptor ligand binding domains to be expressed and/or purified in a stable and active form, and remain stable during the testing procedure. These assays also require isolation and labeling of the relevant NHR proteins and/or ligands.

Engineered yeast-based transcriptional assays are another type of assay which provide a good compromise between the simplicity of direct binding assays and the complexity of native cellar assays [61]. Yeast systems based on engineered intracellular ligand-binding-domain-reporter-protein fusions are also simple and relatively inexpensive. However, they are still time-consuming, and a significant drawback to the yeast system is the apparent ability of yeast to actively export many classes of small molecules. It has been suggested that the efflux of chemicals is a mechanism of general drug resistance, and has led to problems in detecting and classifying some known endocrine disruptor chemicals by yeast-based methods [62].

Considering the available assays, there remains a need for a robust, inexpensive, rapid, high-throughput-capable assay, which can both detect various chemicals that target NHRs in diverse environments and be easily adopted for the broad range of human and animal NHRs. In this work, we introduce a new class of biosensor for detecting ligand that interact with nuclear hormone receptors. This CFPS-based biosensor is significantly faster and less labor intensive

المنارة للاستشارات
www.manaraa.com

than commonly available technologies, making it a promising tool for detecting environmental EDC contamination and screening potential NHR-targeted pharmaceuticals.

1.3 Outline

The bulk of this dissertation is based on publications where I was the primary author and led this work. However, I also received significant help from colleagues and collaborators. Colleagues and collaborators who assisted in the work described are listed at the start of each chapter.

Chapter 2: Codon Emancipation with A Cell-Free Platform: Highly Efficient tRNA Degradation Coupled with Custom tRNA Synthesis

This chapter is an adaptation of the article entitled "Efficient tRNA Degradation and Quantification in Escherichia Coli Cell Extract Using RNase-Coated Magnetic Beads: A Key Step Towards Codon Emancipation" submitted for publication in March 2017 to *Biotechnology Progress*.

Chapter 3: Onconase Production and a Just-Add-Water Cell-Free System

This chapter is an adaptation of an article entitled "Cell-free protein synthesis of a cytotoxic cancer therapeutics: Onconase production and a just-add-water cell-free system" published on September 2015 in *Biotechnology Journal*, and "Escherichia coli-based cell-free extract development for protein-based cancer therapeutic production" published on April 2016 in *International Journal of Development Biology*.

Chapter 4: A Cell-free Protein Synthesis Approach to Biosensing hTRβ-Specific Endocrine Disruptors

www.manaraa.com

This chapter is an adaptation of a research paper entitled “Cell-Free Protein Synthesis Approach to Biosensing hTRβ-Specific Endocrine Disruptors” published on February 2017 in *Analytical Chemistry, ACS Publication.*

Chapter 5: Conclusion and Future Work

In this chapter, I propose some directions to expand these projects as part of future work.

المنارة للاستشارات
www.manaraa.com

2 CODON EMANCIPATION WITH A CELL-FREE PLATFORM: HIGHLY EFFICIENT tRNA DEGRADATION COUPLED WITH CUSTOM TRNA SYNTHESIS

This chapter is adaptation of a paper entitled "Efficient tRNA Degradation and Quantification in Escherichia Coli Cell Extract Using RNase-Coated Magnetic Beads: A Key Step Towards Codon Emancipation" submitted for publication in March. This work was developed and led by myself with the help of Mark T. Smith, Song-Min Schinn, Jeremy M. Hunt, Christina Muhlestein, Joann Diray-Arc, Dr. Brent Nielsen, and Dr. Bradley Bundy.

Emancipating sense codons toward a minimized genetic code is of significant interest to science and engineering. A promising approach to sense codon emancipation is the targeted *in vitro* removal of native tRNA. However, challenges remain such as the insufficient depletion of tRNA in lysate-based *in vitro* systems and the high cost of the purified components system (PURE). Here we introduce a new *in vitro* or "cell-free" approach to emancipate sense codons via efficient and affordable degradation of endogenous tRNA using RNase-coated superparamagnetic beads. The presented method removes greater than 99% of tRNA in cell lysates, while preserving cell-free protein synthesis activity. The resulting tRNA-depleted lysate is compatible with *in vitro*-transcribed synthetic tRNA for the production of peptides and proteins.

2.1 Introduction

A minimized genetic code promises to expand the available proteomic toolset with implications in many fields, including biotherapeutics [63, 64], biocatalysis [65], protein labeling [66], and minimal cells [67]. Such a rewriting of the genetic code requires the decoupling and reengineering of the codon-tRNA-amino acid relationships, which define the genetic coding of proteins by nucleic acids [68]. These relationships, while extremely functional and adaptive, are also redundant, promiscuous and complex [69, 70]. In theory, the great redundancy of codon-tRNA-amino acid relations has the potential to be exploited for incorporation of unnatural amino

المنارة للاستشارات
www.manaraa.com

acids. Indeed, promising efforts have expanded the genetic code using stop codons or 4-base codons [9, 71]. Unfortunately, these methods are constrained by a limited number of available codons for emancipation, or by competition from native tRNA and release factors. The emancipation of sense codons has the potential to free up high numbers of codons for efficient unnatural amino acid incorporation. However, sense codon reassignment has been met with many challenges such as low fidelity or poor rates of incorporation [72-75]. Furthermore, the extensive reassignment of sense codons throughout the genome would necessitate massive mutagenesis or complete genome replacement to maintain the production of active endogenous proteins and maintain cell viability. Here we present an efficient and cost-effective alternative strategy where the codon-tRNA-amino acid relationship is reengineered in an open and highly customizable *in vitro* environment.

In vitro or "cell-free" protein synthesis systems provide a compelling platform to decouple the codon-tRNA-amino acid relationship for multiple reasons: 1) The system utilizes harvested biological machinery (e.g. ribosomes, elongation factors, tRNA synthetases), thus genome-wide mutagenesis replacing sense codons is not necessary to maintain the activity of transcription/translation enzymes. 2) The system is "dead", allowing for manipulations that would devastate *in vivo* systems such as the complete removal or degradation of tRNA. 3) The system is open, facilitating direct addition of heterologous machinery and exquisite control of reaction conditions such as pH, osmolarity, and reagent concentration [23, 76]. Based on these features, the genomic code could be minimized and manipulated by complete or near-complete destruction of the native tRNA followed by repopulation with a synthetic minimal set of tRNA (Figure 2-1).

المنارة للاستشارات
www.manaraa.com

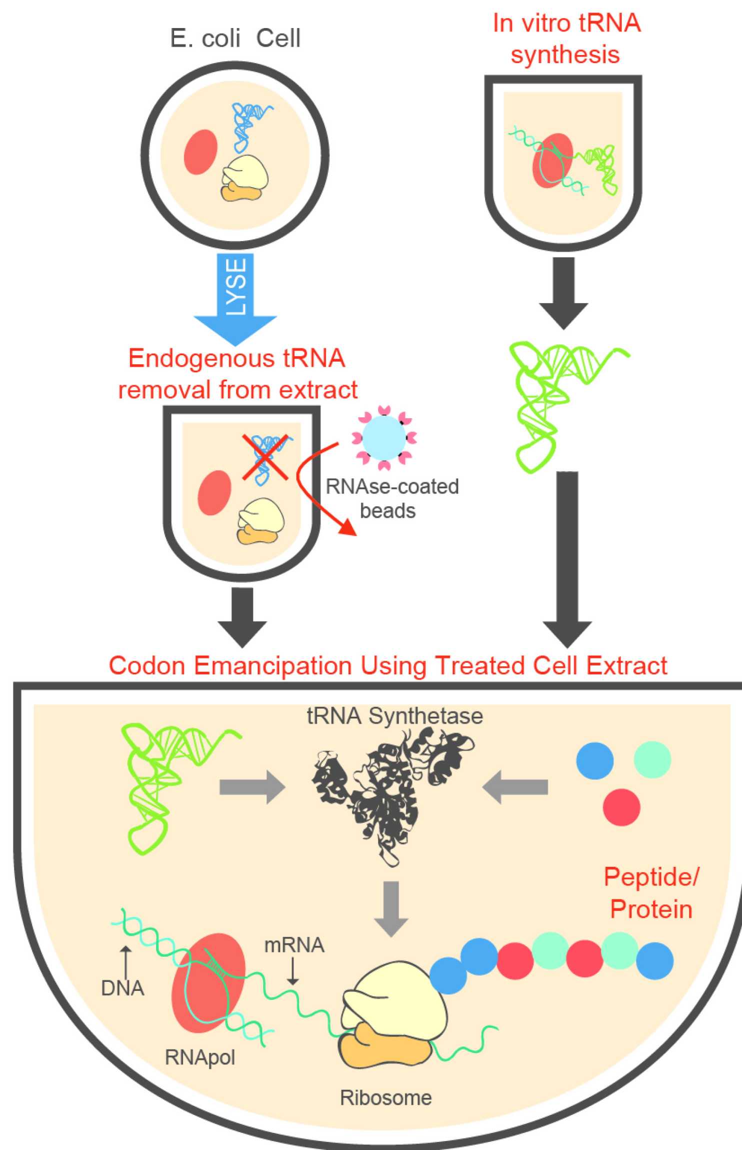


Figure 2-1 Schematic of the in vitro codon emancipation approach demonstrated in this work. The cell extract is treated using RNase A-coated magnetic beads to efficiently degrade endogenous tRNA. Synthetic tRNA, produced using in vitro transcription, is used to repopulate the cell-free system with only the desired tRNA, resulting in codon emancipation.

A recent study illustrated the potential of a cell-free protein synthesis system as a platform to reengineer the codon-tRNA-amino acid relationship by demonstrating the decoding of 61 sense codons by synthetic tRNAs at various efficiencies [77, 78]. The complete

المنارة للاستشارات
www.manaraa.com

emancipation of sense codons, however, was limited due to insufficient removal of native tRNA by chromatography, at an average of 62% of a specific tRNA removed [77, 78]. The PURE system, where individually purified transcription/translation components are combined, also has the potential to emancipate codons, but the monetary high cost severely limits its utility [77, 78]. Here we report a new approach for near-complete depletion of native tRNA from *E. coli* cell extracts as a cost-effective platform to emancipate sense codons *in vitro*. Such efficient tRNA depletion is essential to reduce the competition between the native and synthetic coding elements for high fidelity codon reassignment. The presented method is compatible with various approaches of synthetic tRNA production, including *in vitro* transcription of T7-promoted tRNA, T7-promoted hammerhead Ribozyme-tRNA, and flexizyme [77, 79, 80]. Additionally, for the first time we directly measured residual tRNA using quantitative real-time PCR and report the inaccuracy of assuming tRNA removal based solely on the indirect method of protein expression levels.

2.2 Material and Methods

2.2.1 Cell Extract Preparation and Treatment to Deplete Endogenous tRNA

Cell extract preparation was performed as previously described [81] using *E. coli* BL21 Star™ (DE3) strain. To treat the cell extract and remove endogenous tRNA, RNase A from bovine pancreas (Sigma) was covalently attached to epoxy superparamagnetic beads (Dynabeads® M-270 Epoxy, Invitrogen) according to the manufacturer protocol. Briefly, the reaction buffer containing 0.02M monosodium phosphate, 0.08M disodium phosphate, 3M ammonium sulfate, and 6mg/ml RNase A was prepared. 60 mg of epoxy beads were resuspended in 2ml of dimethylformamide (DMF) to a final bead concentration of $4x10^9$ beads per mL. Beads

المنارة للاستشارات
www.manaraa.com

were washed two times with PBS/Tween 20 (0.5%Vol) buffer and one time with PBS buffer. Each time beads were separated from the buffer by magnetic force using DynaMag™- Spin Magnet (Life Technology). After washing the beads, an equivalent volume of reaction buffer was added to the beads which were then incubated at 37ºC for 24h with end-over-end shaking. After the reaction, beads were washed extensively with PBS/Tween and PBS, in order to remove all unattached RNase A, and were stored in PBS buffer. For cell extract treatment, the extract was first incubated at room temperature with 0.5mM phenylmethylsulfonyl fluoride (PMSF) for 5 min to decrease protease activity of the extract before adding RNase A beads. After PMSA treatment, the cell extract was treated with RNase A on beads at a final concentration of 17.5 beads per ml extract and incubated at room temperature with end-over-end shaking. Treated extract was separated by DynaMag™- Spin Magnet and transferred to a new microcentrifuge tube (Figure 2-2) and stored at -80ºC.

2.2.2 Total RNA Purification and qPCR

To investigate the tRNA cleavage efficiency, tRNA was first purified from RNase A treated extract and non-treated extract using Trizol reagent (ThermoFisher Scientific) and isopropanol in the form of total RNA. Briefly, 1 equivalent volume of Trizol reagent and 0.2 equivalent volumes of chloroform were added to the cell extracts followed by centrifugation at 15000 RCF for 15 min. The top layers containing the RNA were transferred to new microcentrifuge tubes and RNA was precipitated using 1.45 equivalent volumes of isopropanol and 0.45 equivalent volumes of 8 M ammonium acetate. The mixtures were incubated at -20ºC for 20 min and centrifuged at 15000 RCF for 15 min. The pellets were washed with 75% ethanol and centrifuged for 5 min at 15000 RCF. RNA pellets were resuspended in RNAse free water.

المنارة للاستشارات
www.manaraa.com

For qPCR, reverse complementary DNAs were polymerized using ThermoScript™ Reverse Transcriptase (ThermoFisher Scientific) and reverse primers (Table 2-1) according to the manufacturer protocol. The products of this step were subjected to real time quantitative PCR using KAPA SYBR® FAST qPCR Kits (Kapa Biosystems).

Table 2-1 List of primers used for qPCR of various tRNAs to measure residual tRNA level in treated cell extracts compared to untreated cell extract

Residue	Anti-Codon (5` to 3`)	Codon (5` to 3`)	Reverse primer (5' to 3')	Forward Primer (5' to 3')
Ala	GGC	GCC	GCT GAC CTC TTG CAT GCC AT	GGG GCT ATA GCT CAG CTG G
Arg	CCG	CGG	CCT GAG ACC TCT GCC TCC GGA	GCG CCC GTA GCT CAG CTG GAT A
Glu	UCC	GAA	CCC CTG TTA CCG CCG TGA AAG G	GTC CCC TTC GTC TAG AGG CCC AG
Ser	CGA	UCG	GTA GAG TTG CCC CTA CTC CGG T	GGA GAG ATG CCG GAG CGG
Val	UAC	GUA	CGC CGA CCC CCT CCT TGT AAG	GGG TGA TTA GCT CAG CTG GGA GAG C

2.2.3 *In vitro* Transcription of tRNA

To produce synthetic tRNA, forward and reverse complementary Ultramer® DNA oligonucleotides (Integrated DNA Technology, forward sequences reported in Figure 2-5,c) were combined and amplified using a PCR technique. *In vitro* transcription of tRNA was performed at 37°C for 2 hr with shaking at 150 RPM using the following reaction conditions: 50mM Tris-Cl pH 7.5, 30 mM $MgCl_2$, 5 mM DTT, 5.5 mM spermidine, 2 mM putrescine, 2 mM NTPs, 2 mg/ml Poly(vinylsulfonic acid, sodium salt) (PVSA), 0.1 mg/ml T7 RNA polymerase, and 10 ug/ml DNA template.

2.2.4 Cell-free Protein Synthesis (CFPS)

Cell-free protein synthesis (CFPS) was performed in a PANOxSP system as described in previous studies [82], with the crucial modification of not adding *E. coli* tRNA mixture. The

المنارة للاستشارات
www.manaraa.com

reaction contained 33.33mM phosphoenolpyruvate (PEP; Roche Molecular Biochemicals), 10mM magnesium glutamate, 10mM ammonium glutamate, 175mM potassium glutamate, 2.7mM potassium oxalate, 1mM diaminobutane, 1.5mM spermidine, 0.33mM nicotinamide adenine dinucleotide (NAD), 0.27mM coenzyme A (CoA), 1.2mM ATP, 0.86mM CTP, 0.86mM GTP, 0.86mM UTP, 0.17mM folinic Acid, 2mM concentrations of 19 canonical amino acids (excluding glutamate), and 12 nM DNA. All components were obtained from Sigma Aldrich (St. Louis, MO), unless explicitly stated otherwise.

pY71sfGFP vector [15], prepared using Qiagen Maxi-Prep kits, templated the sfGFP cell-free reactions. The sfGFP yield was determined by fluorescence with a Synergy MX microplate reader (BioTek Instruments, Winooski, VT, USA). The DNA 1 construct shown in Figure 2-6,a was synthesized by GenScript and cloned into vector pET-9a under a T7 promoter. 5 µM radiolabled-^{14}C valine (PerkinElmer Inc., Waltham, MA) was added to the CFPS reaction and the protein yield was measured using scintillation counting based on total and washed sample, as previously described [83].

2.3 Results and Discussion

To emancipate sense codons and reassign them, the following two steps were performed: 1) native tRNA depletion, where codon-tRNA-amino acid relationships are decoupled, and 2) repopulation of the system with a synthetic set of tRNA, where codon-tRNA-amino acid relationships are reorganized according to a minimal genetic code (Figure 2-1). In this work, we present a method of near-complete tRNA depletion in cell extracts and directly measured the residual native tRNA. The treated extract was capable of producing various proteins and peptides using purified native tRNA or synthetic tRNA.

www.manaraa.com

2.3.1 tRNA Depletion of Cell Extract

Inspired by a previous study where tRNA was degraded with RNase A, RNase A was assessed for its ability to degrade tRNA from our system (Figure 2-2) [84]. To optimize RNase exposure for both tRNA depletion and preservation of translation-essential rRNAs, RNase A molecules were covalently immobilized to epoxy-functionalized superparamagnetic beads. RNase A-coated beads enable: 1) control of RNase A concentration by adjusting bead concentrations, 2) control of treatment time, 3) rapid removal of RNase A by magnetic force, and 4) facile homogenous mixing of RNAse A to achieve uniform treatment of cell extracts. Bead concentration, treatment time, washing and cleaning steps were fine-tuned to maximize tRNA degradation and rRNA preservation, as discussed below.

RNase A-bead treatment for 15 min with 17.5 μl RNase A-beads per ml cell extract (3.5×10^7 beads/ml) was sufficient to inhibit protein synthesis (Figure 2-3,a, 1st and 2nd bars). However, after the RNase-coated beads were removed from the cell-extract and 2 mg/ml bulk-purified tRNA was supplied, less than 10% of its original protein synthesis capability was retained (Figure 2-3,a, 4th bar). The possibility of residual RNase A in the system, which would degrade translation-essential mRNA, was assessed by adding RNase Inhibitor (New England BioLabs, MA) to the cell-free system following the removal of the RNase-coated beads. The result was a greater than 70% recovery of initial protein synthesis capability after adding bulk-purified tRNA (Figure 2-3,a, 5th and 3rd bars).

المنارة للاستشارات
www.manaraa.com

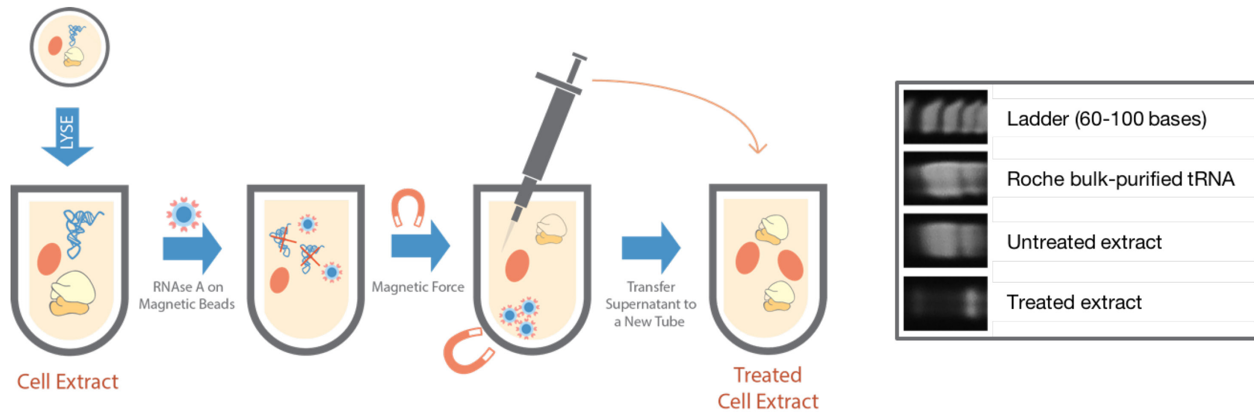


Figure 2-2 a) Schematic diagram of extract treatment to deplete endogenous tRNA. Ribonuclease A is covalently attached to superparamagnetic beads. After extensively washing steps to remove the unattached enzyme, the beads were added to the PMSF-treated extract. Later the treated extract is separated from the RNase beads and transferred to a new microcentrifuge tube. b) Gel electrophoresis of purified tRNA from the untreated extract, treated extract for 15min, and Roche purified-bulk tRNA. The gels showed that the treatment significantly decreases the tRNA level.

Leaching of covalently immobilized RNase A by latent proteases in the extract was hypothesized to be the cause of RNase A found after removal of the magnetic beads. This hypothesis was tested by adding a serine-protease inhibitor, phenylmethanesulfonylfluoride (PMSF), to the cell extract prior to treatment with RNase-coated beads. Adding PMSF (0.5 mM final concentration) 5 minutes prior to RNase had a similar effect of adding RNase Inhibitor with greater than 70% recovery of initial protein synthesis capability after adding bulk-purified tRNA (Figure 2-3,b). The ability to degrade tRNA while maintaining rRNA activity is attributed to a partial shielding effect of ribosomal proteins [85], and the partial loss of protein synthesis capability following RNase treatment could be attributed to loss of rRNA function due to RNase A cleavage. Indeed, longer RNase treatments (30 min, 60 min) resulted in the retention of 58% and 20%, respectively, of the initial protein synthesis capabilities compared to over 70%

المنارة للاستشارات
www.manaraa.com

retention with 15 min of treatment (Figure 2-4,a). However, significant protein synthesis capabilities of ~300 μg/ml were obtained even with the 60 min treatment.

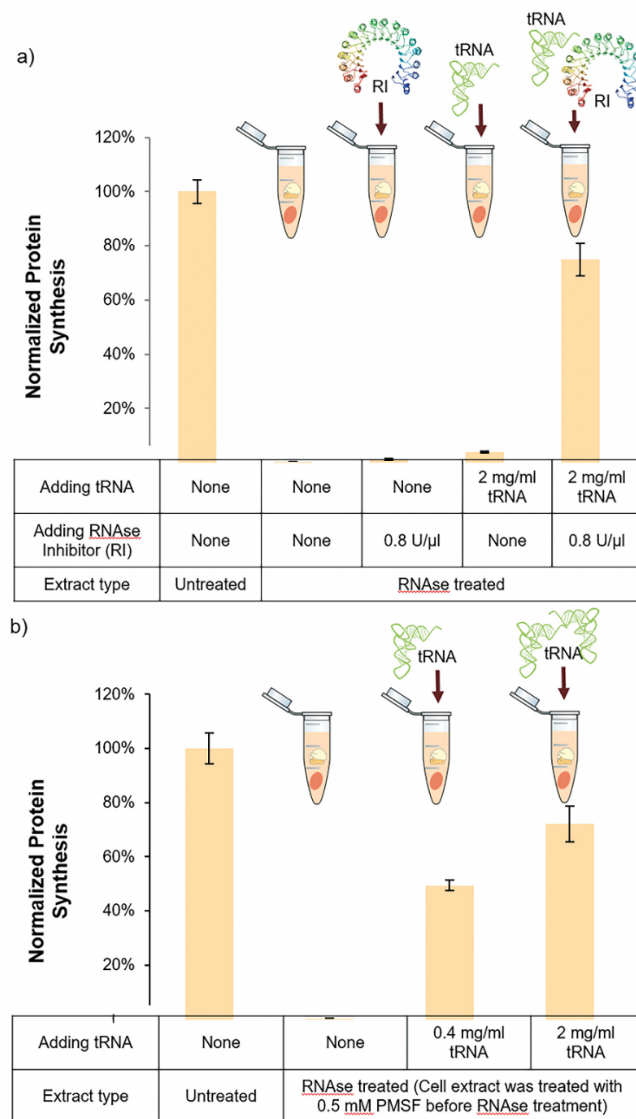


Figure 2-3 Normalized protein synthesis of sfGFP under various conditions, normalized to the 1.4 mg/ml yield of the control reaction (untreated extract). a) RNase inhibitor addition to the cell-free protein synthesis at 0.8U/μl reaction restores most of the activity to the 15 min RNase A-bead treated extract. b) Cell extract incubation with PMSF for 5 min before treatment with RNAse A beads restores most of the activity to the 15 min RNase A-bead treated extract. PMSF treated extract does not require RNase inhibitor, which reduces the cost of cell-free protein synthesis. The error bars represent one standard deviation for n=3.

للاستشارات
www.manaraa.com

2.3.2 tRNA Depletion Efficiency

The tRNA concentrations were further quantified in both treated and untreated extracts using a qPCR technique via tRNA-specific primers (Table 3-1). Quantification results revealed that at short treatment times (15 min), significant residual tRNA remained (6-32%), even though detectable protein synthesis activity had been eliminated (Figure 2-4,b, Figure 2-3). Such residual tRNA represents inadequate decoupling of the codon-tRNA-amino acid relationship, as they can compete with heterologous synthetic tRNA resulting in incomplete emancipation and reassignment.

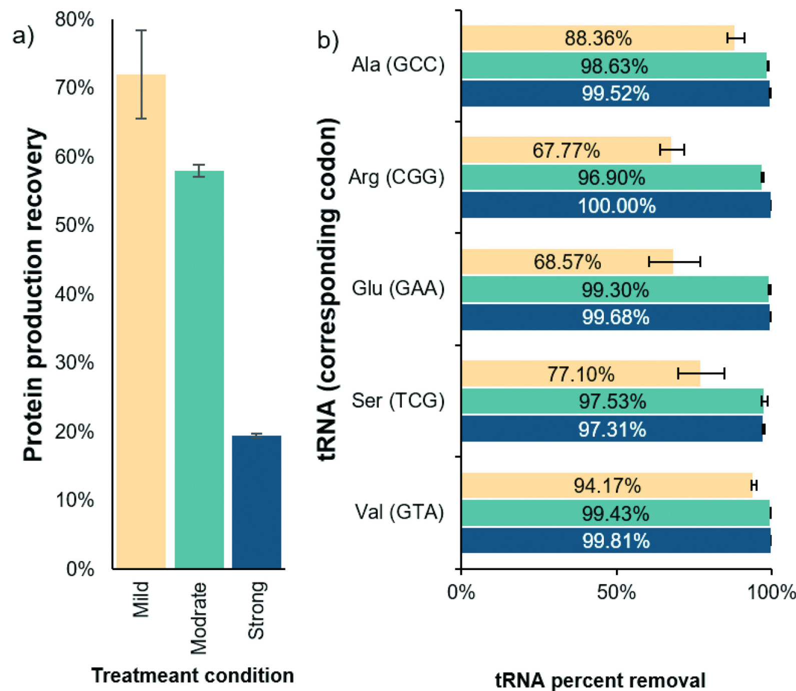


Figure 2-4 Extent of tRNA-depletion in cell extracts. Colors from light to dark represent mild, moderate, and strong pretreatment conditions, respectively, throughout. a) Effect of different treatment severity on protein synthesis capability of the cell extract. Cell extracts were treated for 15, 30, and 60 min for mild, moderate, and strong treatments, respectively. b) Analysis of tRNA residue of different treated cell extracts with the qPCR technique. The error bars represent one standard deviation for n=3.

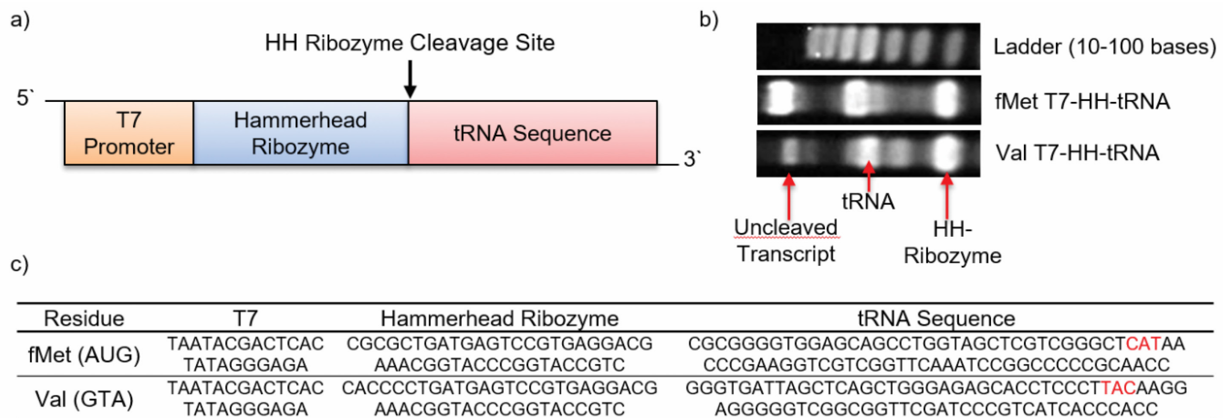
المنارة للاستشارات
www.manaraa.com

Increasing the treatment time substantially decreased the residual tRNA. For instance, 30 min treatment of cell extract resulted in a drop of 97% or more in tRNA concentrations for all targeted tRNAs assessed – Ala(GCC), Arg(CGG), Glu(GAA), Ser(TCG), and Val(GTA). A 60 min RNase A treatment resulted in an average of 99.3% of tRNA degraded for all tRNA assessed, with greater than 99.5% degradation for Ala(GCC), Arg(CGG), Glu(GAA), and Val(GTA) tRNA. Previous methods to remove tRNA involving ethanol-Sepharose matrix achieved lower depletion efficiencies that varied widely for different tRNAs, with an average of 62% depletion and standard deviation of 28% [77, 84]. In addition, the reported depletion efficiencies were measured indirectly via protein synthesis activity. Given that cell extracts without detectable protein synthesis activities can harbor significant residual tRNA (discussed above), the actual depletion efficiencies could be significantly lower than reported.

The presented method is a significant improvement in consistency and efficacy. Such an improvement is essential for codon emancipation and reassignment. For example, if 10 codons are reassigned in a protein 98% of produced product would be correct if only 0.2% of the native tRNA remained, which is the level of removal reported with Val(GTA) tRNA. However, if 38% of native tRNA remained and competed for the 10 reassigned codons, less than 1% of produced protein would be correct. Even at 90% depletion efficiency, the 10% remaining would result in more than 65% of the product being incorrect for a protein with 10 reassigned codons. Thus, the high tRNA removal efficiency reported in this paper is essential for facilitating true codon emancipation for reassignment. Equally important, the reported tRNA emancipated cell-free system is capable of producing protein at yields (~300 μg/ml) and costs (~$5/mg) that are reasonable for widespread commercial and research applications [86]. For example, these results show that the presented method can approximate the near tRNA-free environment of a

المنارة للاستشارات
www.manaraa.com

customized PURE system [78], but with higher yields and at a 1,000 fold reduction in cost [86, 87]. In conclusion, quantification of tRNA removal is essential to verifying near 100% tRNA removal and such an optimized cell-free protein synthesis platform holds great promise for widespread use of codon reassigned systems.



Residue	T7	Hammerhead Ribozyme	tRNA Sequence
fMet (AUG)	TAATACGACTCAC TATAGGGAGA	CGCGCTGATGAGTCCGTGAGGACG AAACGGTACCCGGTACCGTC	CGCGGGGTGGAGCAGCCTGGTAGCTCGTCGGGCTCATAA CCCGAAGGTCGTCGGTTCAAATCCGGCCCCCGCAACC
Val (GTA)	TAATACGACTCAC TATAGGGAGA	CACCCCTGATGAGTCCGTGAGGACG AAACGGTACCCGGTACCGTC	GGGTGATTAGCTCAGCTGGGAGAGCACCTCCCTTACAAGG AGGGGGTCGGCGGTTCGATCCCGTCATCACCCACC

Figure 2-5 Synthetic tRNA production. a) T7-Hammerhead ribozyme-tRNA DNA construct for making fMet and Val tRNA. Hammerhead ribozyme cleaves itself exactly at the targeted position, resulting in the correct tRNA sequence. b) TBE-Urea gel electrophoresis of the *in vitro* transcription reactions to produce tRNAs. c) The DNA sequence used for producing fMet and Val tRNA.

2.3.3 CFPS of Peptides Using Treated Cell Extract and Synthetic tRNA

Previously Cui et al. have demonstrated that tRNA can be synthesized using *in vitro* transcription of a T7-promoted tRNA platform [77]. Additionally, Iwane et al. demonstrated the use of flexizyme to produce functional tRNA, which was used at higher concentrations in the tRNA depleted PURE system [78]. Here, we briefly demonstrate that synthetic tRNAs are also compatible with the RNase-treated extract reported above for protein synthesis. To this end, synthetic tRNAs for fMet and Val were produced with a preceding hammerhead ribozyme to

المنارة للاستشارات
www.manaraa.com

yield precise tRNA sequences (Figure 2-5,a) [14, 80]. Synthetic tRNA for fMet and Val were added at optimized concentrations to produce the designer peptide containing the formylmethionine and valine (Figure 2-6,a). The synthetic tRNA produced protein at the same yields as bulk purified tRNA (Figure 2-6,b), demonstrating successful codon emancipation and use of the system with synthetic tRNA.

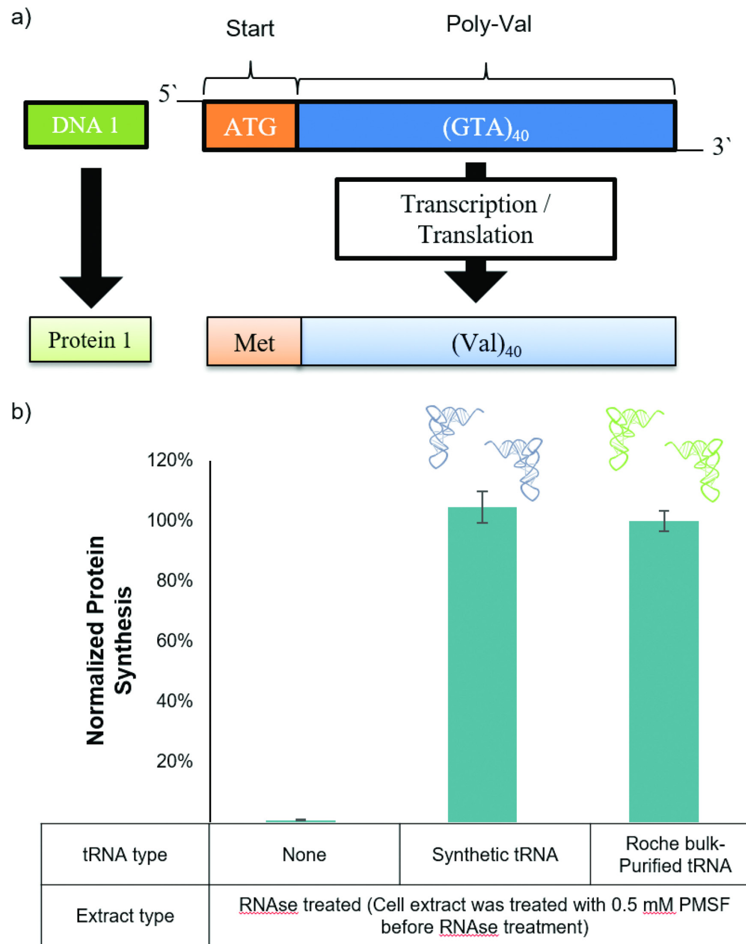


Figure 2-6 Emancipating codons using a treated cell extract and synthetic tRNAs. a) The peptide was designed to contain fMet as starting sequence for the translation process followed by 40 Val residues, with protein synthesis yield determined using C^{14}-labled Val. b) Cell-free protein synthesis of the peptide using the 30 min RNase A treated extract. The protein synthesis yields were normalized based on the control reaction with Roche bulk-purified tRNA (yield of 170 µg/ml). *In vitro* transcribed synthetic tRNA was added to the cell-free protein synthesis system at 100 µg/ml fMet and 500 µg/ml Val, while the bulk-purified tRNA from Roche was added at 2 mg/ml. The error bars represent one standard deviation for n=3.

المنارة للاستشارات
www.manaraa.com

2.4 Conclusion

In this study, we developed a robust method to emancipate codons at greater than 99% efficiencies. This was done by efficiently and affordably degrading native tRNAs of *E. coli* cell extracts, which previously had been a key technical challenge. The presented approach resulted in 2000-fold decreases in native tRNA level compared to untreated extract, while also preserving protein synthesis activity of the cell extract. Most importantly, the approach maintains high level protein production (~300 mg/ml) at low cost (~$5/mg) for widespread use. We also demonstrated that the treated extract can be used with synthetic tRNAs for protein synthesis. Overall, codon emancipation is an essential and typically limiting step in codon-tRNA-amino acid reassignment and holds great promise in expanding proteomic toolset.

المنارة للاستشارات
www.manaraa.com

3 ONCONASE PRODUCTION AND A JUST-ADD-WATER CELL-FREE SYSTEM

This chapter is an adaptation of the paper entitled "Cell-free protein synthesis of a cytotoxic cancer therapeutics: Onconase production and a just-add-water cell-free system" published September 2015 in *Biotechnology Journal[25]*, and "Escherichia coli-based cell-free extract development for protein-based cancer therapeutic production" published April 2016 in *International Journal of Developmental Biology[88]*. These works were developed and led by myself with the help of Mark T. Smith, Anthony M. Bennett, Jacob B. Williams, Conner C. Earl, Christina Muhlestein, Dr. William Pitt, and Dr. Bradley Bundy.

Biotherapeutics have many promising applications, such as anti-cancer treatments, immune suppression, and vaccines. However, due to their biological nature, some biotherapeutics can be challenging to rapidly express and then screen for activity through traditional recombinant methods. For example, difficult-to-express proteins may be cytotoxic or form inclusion bodies during expression, increasing the time, labor, and difficulty of purification and downstream characterization. One potential pathway to simplify the expression and screening of such therapeutics is to utilize cell-free protein synthesis. Cell-free systems offer a compelling alternative to *in vivo* production, due to their open and malleable reaction environments. In this work, we demonstrate the use of cell-free systems for the expression and direct screening of the difficult-to-express cytotoxic protein onconase. Using cell-free systems, onconase can be rapidly expressed in soluble, active form. Furthermore, the open nature of the reaction environment allows for direct and immediate downstream characterization without the need of purification. Also, we report the ability of a "just-add-water" lyophilized cell-fee system to produce onconase. This lyophilized system remains viable after being stored above freezing for up to one year. The beneficial features of these cell-free systems make them compelling candidates for future biotherapeutic screening and production.

3.1 Introduction

Biopharmaceuticals is a burgeoning 140 billion USD industry and continues to grow [89]. The industry has the potential to improve and save lives by tapping into the vast evolved diversity and function that nature has to offer. This is epitomized by the success of biological therapeutics, as 7 of the top 10 grossing prescription drugs are recombinant antibodies or fusion

www.manaraa.com

proteins [90]. Production and screening systems for recombinant antibodies are considerably streamlined and continually improving [91]. However, as new non-antibody biotherapeutics are investigated, production and screening technologies will need to adapt accordingly.

Cancer is the second leading cause of death in the United States, presenting a compelling target for current and future biologics (CDC, *Deaths: Final Data for 2013*) [92]. Non-prophylactic anticancer treatments are by definition cytotoxic. Cytotoxic anticancer biologics may prove difficult to overexpress, scale-up, or screen due to their intrinsically toxic properties. For example, onconase (ONC) is a known ribonuclease that is in clinical trials for treatment of malignant mesothelioma and has demonstrated potency against multiple other forms of cancer [93-95]. However, ONC is derived from the oocytes of the Northern Leopard Frog, which are time and labor intensive to harvest and culture [96, 97]. Furthermore, downstream evaluation and screening of ONC requires purification including anion exchange, cation exchange, and finally size-exclusion chromatography [93].

Attempts at alternative recombinant expression methods for ONC have had limited success. Producing ONC in the most widely used recombinant expression system, *Escherichia coli,* results in the formation of inclusion bodies, requiring laborious purification and refolding procedures taking three or more days [98]. Although strides have been made to recombinantly express and secrete ONC in yeasts, these systems also require laborious purification and concentration steps prior to downstream evaluation and screening [99].

In an effort to circumvent the production limitations of cell-based systems and enable the rapid contiguous evaluation of the protein, we propose cell-free protein synthesis (CFPS) of ONC. Cell-free systems feature open reaction environments that can be readily manipulated and monitored for optimal reaction conditions [2, 100]. Furthermore, CFPS systems are generally

المنارة للاستشارات
www.manaraa.com

less sensitive to cytotoxic elements, as there are no cells to keep alive [101]. Recently, the lyophilization of CFPS reagents has enabled long-term storage at non-ideal temperatures (up to 90 days above freezing), creating a robust "just-add-water" expression system [102]. These traits make CFPS a compelling alternative for the production and screening of difficult-to-express biomolecules, such as cytotoxic, insoluble, and membrane proteins [103, 104].

E. coli-based cell-free systems are particularly robust recombinant expression platforms with reported yields as high as 2.3 mg per mL reaction [105]. These systems have been utilized to produce diverse products, such as cytotoxic viral proteins, oxygen-sensitive Fe-Fe enzymes, and therapeutic peptides [101, 106, 107]. Other applications of *E. coli*-based CFPS have significantly improved protein solubility and avoided the difficulties associated with inclusion body formation [108, 109].

Here we demonstrate the robust production of active ONC in *E. coli*-based CFPS. The cell-free produced ONC (ONC_{CFPS}) is highly soluble during and after overexpression. ONC_{CFPS} exhibits similar protein synthesis inhibition and improved anticancer activities compared to *in vivo* produced ONC (ONC_{invivo}). Finally, we show how ONC_{CFPS} could be produced on-demand by stable and portable lyophilized CFPS systems. We also report the ability of "just-add-water" lyophilized cell-free systems to be active for up to one year when stored above -80 ºC. In short, cell-free protein synthesis is a robust alternative to produce and screen the difficult-to-express protein ONC. The ability to rapidly produce soluble, active proteins has implications in engineering, screening, and producing current and future biotherapeutics. Such technologies will be essential as medicine becomes more advanced, personal and on-demand.

المنارة للاستشارات
www.manaraa.com

3.2 Materials and Methods

3.2.1 Cell Growth and Extract Preparation

Cell extracts were prepared as previously described with the following specifications [109]. *Escherichia coli* BL21-Star™ (DE3) (Invitrogen, Carlsbad, CA) were grown serially from glycerol stocks at 37 ºC and 280 rpm as follows: 5 mL LB media overnight, 100 mL LB media until an OD_{600} of 2.0, and 1 L 2YT media. Growths were induced by addition of 1 mL 1 M isopropyl-1-thio-β-D-galactopyranoside (IPTG) at OD_{600} 0.4-0.7 and monitored until the end of log-phase growth. Cells were harvested by centrifugation at 6000 xg, 4 ºC for 15 mins and subsequently washed with Buffer A (10 mM Tris-acetate, 14 mM magnesium acetate, 60 mM potassium glutamate, 1 mM dithiothreitol, pH 8.2). Cells were again centrifuged as before, resuspended in 1 mL Buffer A per gram wet cells, and lysed as previously described [109]. Lysates were centrifuged for 30 mins at 16000 xg, 4 ºC. The supernatant was removed, aliquotted, flash frozen with liquid nitrogen, and stored at -80 ºC until use. Lyophilized extracts were prepared as previously described [102].

3.2.2 In Vivo Expression

Recombinant onconase gene (NCBI Accession# 1PU3_A) was codon optimized and custom synthesized (DNA 2.0, Menlo Park, CA) for improved expression in *Escherichia coli*. The optimized gene was cloned into the T7-promoted expression plasmid pJ411-KanR with a Q2E mutation and C-terminal 6xHis tag, resulting in plasmid pJ411-Onc.

Escherichia coli BL21-Star™ (DE3) was transformed with pJ411-Onc. Cells were grown serially as described above with the following specifications. The final stage of cell growth was

www.manaraa.com

in 1 L Terrific Broth media. Growths were induced at OD_{600} 0.4-0.7 by addition of 1 mL 1 M IPTG and grown overnight. Cells were harvested, washed, and lysed as above. ONC_{invivo} was purified from inclusion bodies in the cell lysate pellet and refolded as previously described [110]. To verify the molecular mass, ONC_{invivo} was desalted and analyzed using an Agilent Technologies 6230 TOF LC/MS (Agilent, Palo Alto, CA).

3.2.3 Cell-free Protein Synthesis

Cell-free protein synthesis (CFPS) ONC production was performed using the PANOxSP system, as previously described [102]. Reactions were performed for 3 hours 15 mins at 37 ºC. Production yields were calculated by incorporation of radiolabeled C^{14}-Leucine, as previously described [83]. Yields from CFPS reactions diluted during the reaction incubation were calculated based on the initial reaction volume (IRV). CFPS reactions for protein synthesis inhibition assays were performed without dilution. Reaction volumes were scaled between 50 µL and 1 mL with no observable change in yield.

3.2.4 Onconase Anticancer Activity Assay

To assay the anticancer properties of ONC, we measured the cell viability of breast cancer cells (MCF7) using an MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide)] assay [111]. MCF7 cells were grown in Dublecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Once confluent, cells were trypsinized (trypsin-EDTA 0.05%) and plated into a clear culture-grade 96-well plate at 3000 cells per well and grown for 24 hours in 100 µL fresh media. Before treatment, the growth media was replaced with 100 µL fresh media, treatment sample, and up to 50 µL PBS (pH 8.0) for a total well volume of 150 µL. After 48 hours incubation, treatment solution was

المنارة للاستشارات
www.manaraa.com

aspirated, wells were washed with 200 µL PBS, and finally filled with 90 µL fresh media and 10 µL of 5 mg per mL MTT in PBS. After 4 hours incubation, MTT solution was aspirated and replaced with 75 µL DMSO. The plate was incubated for 15 minutes at room temperature and absorbance was measured at 540 nm with background absorbance of 720 nm subtracted.

3.2.5 tRNA Purification

Total RNA was purified from BL21-Star™ (DE3) extract using two rounds of acid phenol:chloroform:isoamyl alcohol (Ph:Chl:IA) 125:24:1 extraction as follows. Ph:Chl:IA was added to one volume of extract, vigorously vortexed, and centrifuged at >16,000 xg, 4 ºC for 30 mins. After centrifugation and transferring the aqueous phase to a new tube, a mix of sample:isopropyl alcohol:8M ammonium acetate 100:145:45 by volume was used to precipitate total RNA, and the pellet was washed with cold 95% and 70% ethanol. The alcohol precipitation and wash were repeated two times. The resulting pellet was dissolved in a mixture of 0.73 M LiCl, 10 mM sodium acetate pH 4.5 to precipitate large RNA. The tRNA remained in the supernatant, and was precipitated and washed using the above described alcohol protocol. The pellet was resuspended in RNase-free water and purity was checked using 260/280 nm absorbance ratio and TBE-Urea PAGE gel. In the case of using TRIzol® (Life Technologies), Trizol was used instead of phenol:chloroform:isoamyl alcohol.

Reagent cost calculations were based on previous calculations and on reagent prices obtained from the 2015 online catalogues of Roche Applied Sciences, Sigma-Aldrich, VWR, Zymo Scientific, and Life Technologies [86]. Cost of labor was not included.

المنارة للاستشارات
www.manaraa.com

3.3 Results

The protein onconase (ONC) of the RNase A superfamily is a promising biotherapeutic to target multiple forms of cancer. ONC, like other RNases, is difficult to overexpress recombinantly and poses difficulties for overexpression in the endogenous cell-line. Some of the difficulties include cytotoxic activity, ribonuclease activity, and the formation of inclusion bodies. We compare *E. coli*-based *in-vivo* produced ONC (ONC_{invivo}) to CFPS produced ONC (ONC_{CFPS}) in terms of solubility and activity. We demonstrate that CFPS is a valuable platform to rapidly produce and directly screen this biotherapeutic.

3.3.1 Recombinant In Vivo Synthesis of Onconase

The ONC gene was designed for optimal T7-promoted *Escherichia coli* expression. The resulting pJ411-Onc plasmid was transformed into BL21-Star™ (DE3) cells. Cells were cultured and induced with 1 mM IPTG 2.5 hours after inoculation (OD_{600} 0.4-0.7, Figure 3-2, A). The *in-vivo*-produced onconase was verified using mass spectrophotometry (Figure 3-1).

Control cells harboring the non-toxic pJ411-MbRS (*Methanosarcina barkeri* pyrrolysyl-synthetase) were cultured identically. MbRS was chosen as a control, as it is also known to form inclusion bodies. Induction with IPTG resulted in a premature transition out of exponential phase for both growth types (Figure 3-2, A). The transition is expected, as energy is diverted from cell growth to protein overexpression and IPTG is a known toxin for *Escherichia coli* [112]. The growth of cells harboring the ONC gene slowed beyond that of the nontoxic MbRS gene (p-value = 0.0227), implying the toxic action of ONC may inhibit growth until the toxic protein precipitates or is otherwise inhibited.

المنارة للاستشارات
www.manaraa.com

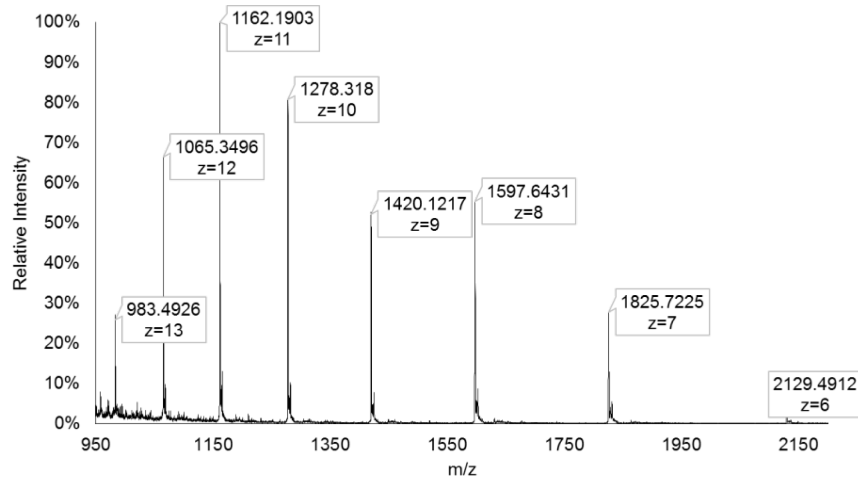


Figure 3-1 Mass spectrometry analysis of *in-vivo* expressed onconase. *In vivo* produced, purified, and refolded onconase was prepared for mass spec using C-18 desalting columns. Onconase was analyzed using an Agilent 6200 Series TOF LC/MS. The observed peaks correspond to the expected molecular weight of onconase (12772 Da).

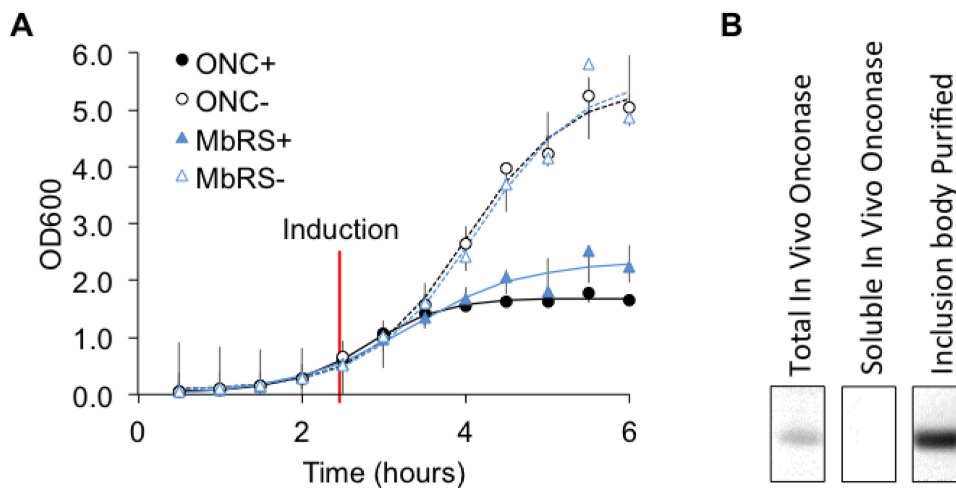


Figure 3-2 Onconase In vivo Expression and Solubility. A) Growth Rate of Escherichia coli harboring plasmids pJ411-ONC or pJ411-MbRS, both genes known to produce inclusion bodies. Growths were induced with IPTG (+) or not induced (-). Graphed curves represent growth regression curves (Table 3-1). After induction (ONC+ and MbRS+), the growth rate slowed. ONC+ growth rate became nearly static 1 hr after induction, which is consistent with the cytostatic/cytotoxic nature of ONC. B) Electrophoretic analysis of total, soluble, and inclusion body fractions of ONC+ growth. The overexpression band is visible only in the total and inclusion body fractions. No soluble ONC was detected. (error bars represent 95% confidence interval of regression).

المنارة للاستشارات
www.manaraa.com

Table 3-1 contains the constants for the Verhulst-Pearl Equation after fitting this equation to our data. This equation is commonly used to model cell population growth and is below

$$P(t) = \frac{kP_o e^{rt}}{k+P_o(e^{rt}-1)} \quad (3\text{-}1)$$

Table 3-1 Bacterial Growth Regression Curves

	Onconase		*M. barkeri* Pyryl-Synthetase (MbRS)	
	IPTG Induced	Not Induced	IPTG Induced	Not Induced
P_0	0.007	0.013	0.022	0.013
k	1.70	5.45	2.35	5.69
r	2.01	1.52	1.41	1.48
R^2	0.996	0.993	0.978	0.979
Adjusted R^2	0.995	0.992	0.973	0.974

In this case, P_0 was unknown due to the nature of the growth. Therefore, we included P_0 as a constant to be fitted. We calculated adjusted R^2 (coefficient of determination) which are displayed in the table above. ANOVA analysis of the induced growth curves reveals a statistically significant difference between the MbRS and ONC growths ($p\text{-value} < 0.022$).

Previous works report the formation of inclusion bodies during recombinant bacterial expression of ONC_{invivo} [98]. To assess expression and formation of inclusion bodies, crude and

المنارة للاستشارات
www.manaraa.com

centrifugation-clarified lysates were run on SDS-PAGE (Figure 3-2, B). Protein overexpression bands appear in the crude lysate and not in the clarified lysate, indicating the low solubility of ONC_{invivo}. Indeed, densitometric analysis of the expression bands indicate that less than 1% of the ONC_{invivo} remains soluble (Figure 3-2,B).

ONC_{invivo} protein with potent nuclease activity was produced only after steps of inclusion body purification, washing and refolding (a 3+ day process, Figure 3-3). While the purity of the ONC_{invivo} was greater than 90%, the post-fermentation purification steps are time consuming, expensive, and challenging to scale-up, thus restricting the feasibility of high-throughput screening and inexpensive production scale-up. To overcome the difficulties of *in vivo* production of ONC, we assessed the effectiveness of a cell-free approach.

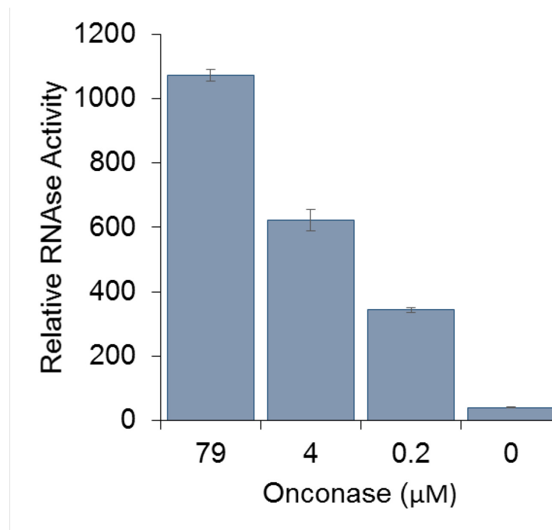


Figure 3-3 Nuclease Assay with In vivo-produced Onconas. Onconase nuclease activity was assayed directly using RNAseAlert® substrate. Sample was added to 40 nM substrate in a 96-well plate with a final well volume of 100 µL. Reactions were incubated for 1 hour at room temperature. The cleaving of the substrate was measured by observing fluorescence at 480/520 nm excitation/emission.

المنارة للاستشارات
www.manaraa.com

3.3.2 Cell-free Synthesis of Onconase

ONC was produced in cell-free protein synthesis (CFPS) from the identical plasmid as *in vivo* and assayed for yield and solubility (Figure 3-4,A). Initial yields from a standard CFPS reaction were less than 3% of the reference protein Green Fluorescent Protein (GFP) yields (0.03 mg per mL ONC_{CFPS} versus 1.45 mg per mL GFP_{CFPS}). However, and notably, greater than 80% of the ONC_{CFPS} yield was soluble, representing a greater than 80-fold increase of percent soluble yield over *in vivo* expression. If the soluble protein is active, its production would unleash the protein's nuclease activity, likely degrading the machinery used to produce it and leading to lower overall yields. ONC predominantly targets tRNA and can also degrade mRNA and rRNA [113]. We hypothesized that if the soluble product was active, low yields would be due in part to tRNA degradation.

In principle, the inhibition of ONC_{CFPS} during production would improve overall yields by protecting essential RNA machinery for protein synthesis. However, one of the features of RNase A-like ONC is its ability to resist inhibition by RNase A inhibitors, specifically at physiological salt concentrations [114]. This resistance is thought to provide ONC its beneficial anticancer properties, allowing the RNase to function after endocytosis despite the potential presence of RNase inhibitors [110, 115]. Accordingly, when we added a potent RNase A inhibitor (RNase Inhibitor, Murine; New England Biolabs, MA) to our CFPS productions for ONC_{CFPS}, we observed no improvement in overall yields compared to standard CFPS (Figure 3-4,B, RNase Inhibitor).

المنارة للاستشارات
www.manaraa.com

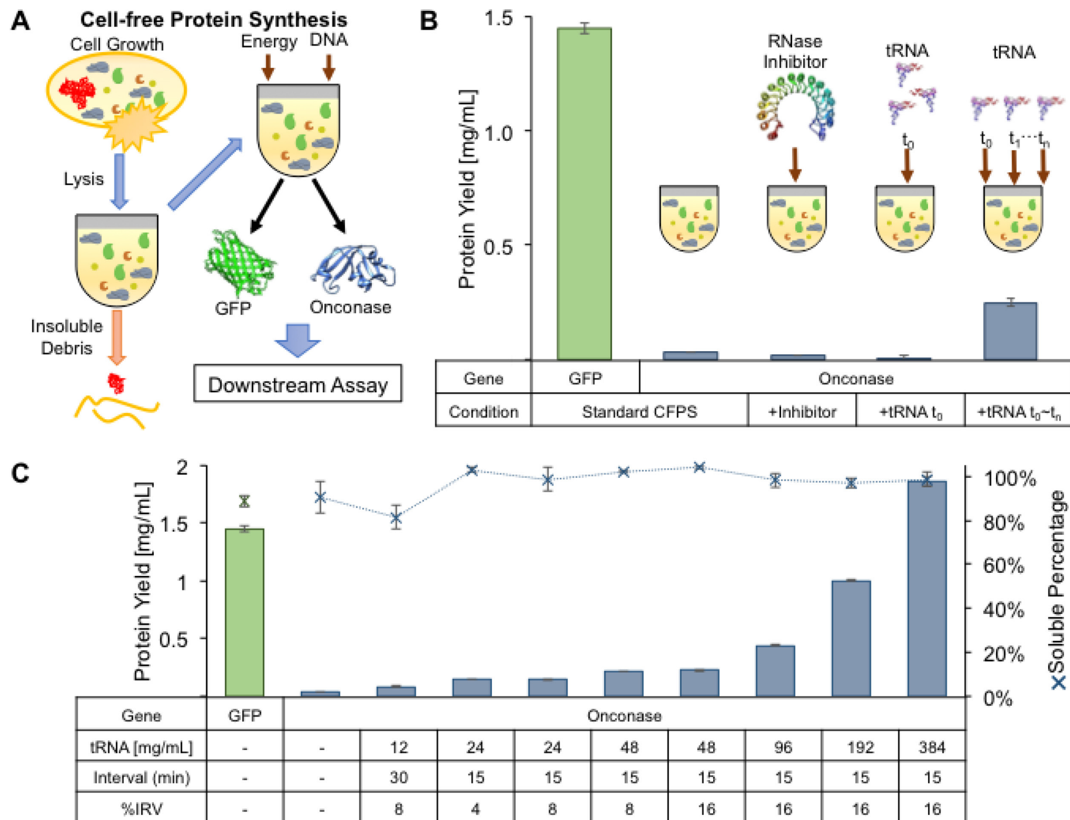


Figure 3-4 Cell-free Expression Optimization of ONC. A) Abbreviated scheme of CFPS production for direct downstream applications. B) Modified CFPS methods in attempts to improve ONC_{CFPS} yields by RNase inhibition and tRNA addition. tRNA was added as a single bolus (+tRNA t_0) or dosed throughout the reaction at specific intervals (+tRNA t_0-t_n). C) CFPS optimization of ONC_{CFPS} yields through the addition of tRNA at specified intervals and volumes by percent of initial reaction volume (%IRV). ONC_{CFPS} yields were directly correlated to total tRNA addition per mL of initial reaction volume (tRNA [mg/mL]). Soluble yield percentage (X) were >80% for all ONC_{CFPS}. (n>=3, error bars represent 1 standard deviation).

We hypothesized that the replacement or supplementation of tRNA would improve overall ONC_{CFPS} yields by mitigating tRNA-degradation effects. The open nature of CFPS makes possible the addition of excess tRNA without need to modify the expression organism. Harnessing this feature, CFPS was performed while providing additional purified tRNA. Addition of an initial bolus of tRNA (96 mg tRNA per mL initial CFPS, dilution with 1.92

المنارة للاستشارات
www.manaraa.com

volumes) effectively eliminated ONC_{CFPS} production (Figure 3-4,B, +tRNA t_0). One possible cause of this inhibitory effect may be due to excessive deacylated tRNA. Previous work has reported that excessive deacylated tRNA can inhibit binding of amino acyl-tRNA at the ribosome P site [116]. In the case of adding excess tRNA at the start of the reaction, it can be postulated that a majority of tRNA remain non-acylated due to the limited supply of amino acids and aminoacyl synthetases.

As an alternative approach, the same amount of tRNA was added to the CFPS reaction at 15 minute time intervals throughout the reaction, resulting in a >7-fold yield improvement (Figure 3-4,B, +tRNA t_0-t_n). To further optimize yields, the system was modified by adding a range of 12-384 mg total tRNA per mL initial CFPS. Stock concentrations of tRNA were 25, 50, 100, or 200 mg per mL in water. Time intervals were every 15 or 30 minutes. Volumes added at each time interval were 4, 8 or 16 percent of the initial reaction volume (%IRV). We observed ONC_{CFPS} yield was directly proportional to the amount of tRNA added to the reaction and not effected by dilution within the employed range of %IRV (4-16 %IRV at each interval) (Figure 3-4,C). For example, yields were statistically indistinguishable when a total of 48 mg per mL CFPS was added, regardless of %IRV.

In the best case, ONC_{CFPS} production increased greater than 56-fold (from standard CFPS of 0.03 to 1.86 mg per ml for the conditions 384 mg total tRNA per mL CFPS, 15 min tRNA addition interval, 16% IRV). This increase in total yield was matched in increased soluble yield. In all cases when reactions were supplemented with tRNA at 15 min intervals, the solubility of ONC_{CFPS} remained above 95% of total yield.

The best ONC_{CFPS} yield outperformed our reference protein (GFP) in standard CFPS by 28%. The addition of supplemental tRNA to CFPS producing onconase linearly improved yields.

المنارة للاستشارات
www.manaraa.com

To verify this effect was not due to the lack of tRNA in CFPS, we performed CFPS of an expression-optimized GFP gene. tRNA was added at multiple concentrations at the beginning of CFPS reaction without dilution (Figure 3-5, A). The addition of tRNA at the initiation of CFPS appears to slightly improve yields, however the improvement is not statistically significant and fails to reject the null hypothesis of no difference in mean yield (p-value = 0.87). Periodic addition of tRNA to CFPS (15 min interval, 16% initial CFPS reaction volume [IRV], 48 mg tRNA per mL IRV) caused GFP yields to drop by >50% (Figure 3-5, B). One possible cause of this protein synthesis inhibition is the inundation of the reaction environment with deacylated tRNA, which can compete for the ribosomes' p-site (i.e. entry site of tRNA into the translation process) [116]. The competition between acylated- and deacylated-tRNA would potentially be eliminated or mitigated in CFPS of ONC, due to the degradation of excess tRNA by ONC_{CFPS}.

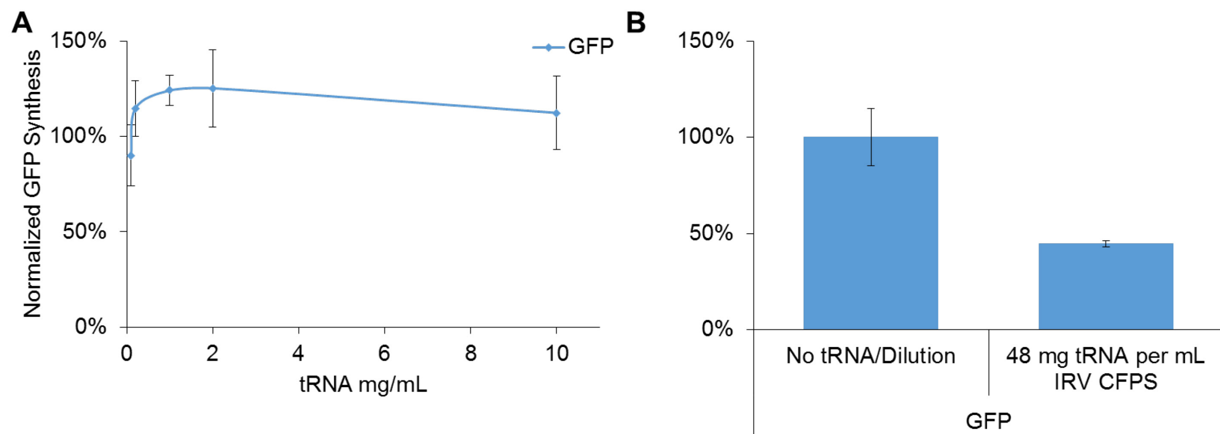


Figure 3-5 Green Fluorescent Protein CFPS. A) The addition of tRNA to the CFPS reactions of GFP doesn't produce significant different in final protein production level. B) The addition of large amount of tRNA at the beginning of the reaction inhibit the CFPS of GFP.

المنارة للاستشارات
www.manaraa.com

The open nature of CFPS provides the control required to modify the translation environment for ONC_{CFPS} production. The periodic addition of tRNA secures sufficient tRNA for translation processes essential during ONC_{CFPS} expression. Moreover, excess tRNA may act as sacrificial RNA and provide fodder for endogenous *E. coli* RNases and ONC_{CFPS} present in the reaction, aiding in the preservation of essential translational RNA (mRNA encoding ONC and rRNA). Furthermore, adding doses of tRNA throughout the CFPS reaction mitigates any inhibition that excessive deacylated tRNA may have on the ribosome. These effects make our modified CFPS a propitious system for rapid ONC expression.

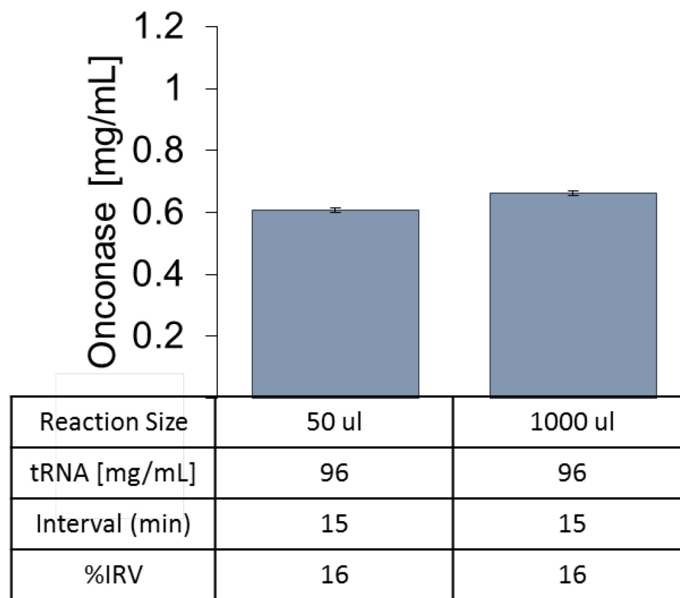
One key component of biotherapeutics is the ability to scale-up production. Our and other's previous work have reported that CFPS is readily scaled, from as little as 15 μL to 100 L [23, 117]. To verify that our modified CFPS setup would also scale, we produced ONC_{CFPS} with initial reactions volumes of 50 and 1000 μL. To maintain consistent heat and mass transfer, total reaction volume never exceeded 3 mL in 50 mL conical centrifuge tubes and the tubes were shaken at 280 RPMs. Small and large scale reactions produced ONC_{CFPS} equally well (Figure 3-6), indicating our modified CFPS system may work for larger scale as long as reaction engineering principles are considered. Scaling from 50 to 1000 μL increased yield by 10%, indicating this 20-fold scale-up was not deleterious to the modified CFPS.

3.3.3 tRNA-supplemented Cell-free Protein Synthesis

While the production of ONC_{CFPS} can be increased in CFPS systems by adding supplementary tRNA, this improvement is beneficial particularly if overall costs per yield decrease. We have previously described the costs of our standard cell-free system [86]. To analyze the impact of tRNA costs due to addition of tRNA, we considered 3 methods of

المنارة للاستشارات
www.manaraa.com

procuring tRNA: 1) commercially purchased tRNA (Roche, MRE600), 2) tRNA purified with a standard commercial kit (TRIzol®, Life Technologies), and 3) tRNA purified with an in-house phenol-chloroform extraction method, detailed in Materials and Methods. Purification costs included all reagents' costs (cost of kit components, cost of consumable materials for cell growth, lysis, and purification). Purified tRNA were verified to have similar or better activity per mg in the CFPS reaction than the commercially purchased material (Figure 3-7). The tri-reagent purified tRNA performed at about 80% of the commercial tRNA. The in-house purified tRNA allowed for an increase in total yields of about 15% of the commercial tRNA.



Reaction Size	50 ul	1000 ul
tRNA [mg/mL]	96	96
Interval (min)	15	15
%IRV	16	16

Figure 3-6 CFPS Reaction Volume Scale up for onconase production.

Although convenient and effective, the expense of commercially purchased tRNA outweighed the benefit (Figure 3-8, B). Indeed, the use of this tRNA increased the costs per mg ONC_{CFPS} by an average of 110% over standard CFPS. Alternatively, the commercially purchased tri-reagent was effective at providing tRNA at about 45% the expense of the commercially purchased tRNA. This price represented a break-even price-point: the average cost per mg

المنارة للاستشارات
www.manaraa.com

ONC$_{CFPS}$ remained nearly level, regardless of tRNA addition. While there is not a yield cost reduction associated with adding commercial or tri-reagent purified tRNA, their addition to the CFPS reactions did significantly improve the yield of ONC per volume, which simplifies and decreases the costs of downstream assays without further treatment.

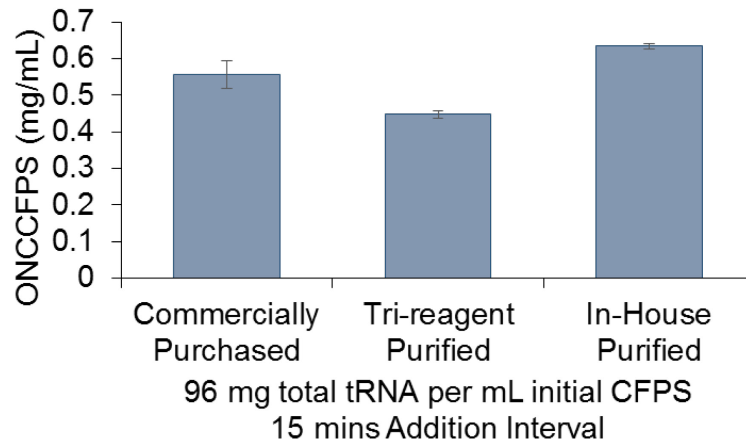


Figure 3-7 CFPS with Commercial and Purified tRNA.

To further reduce the cost per mg ONC$_{CFPS}$ produced, we developed an in-house phenol-chloroform extraction procedure based on the same principles as the commercial tri-reagent. The optimized procedure was effective at reducing the cost of tRNA by greater than 92% compared to the commercially purchased tRNA. The 13-fold reduction in tRNA cost caused overall yield costs to decrease asymptotically from ~115 USD towards ~20 USD per mg ONC$_{CFPS}$ produced (Figure 3-8, A). This 5-fold reduction in ONC$_{CFPS}$ production costs and 56-fold increase in yield was achieved by 1) the direct replenishment of tRNA afforded by the cell-free system and 2) the development of an in-house optimized tRNA purification method.

المنارة للاستشارات
www.manaraa.com

3.3.4 Lyophilzed Cell-free System

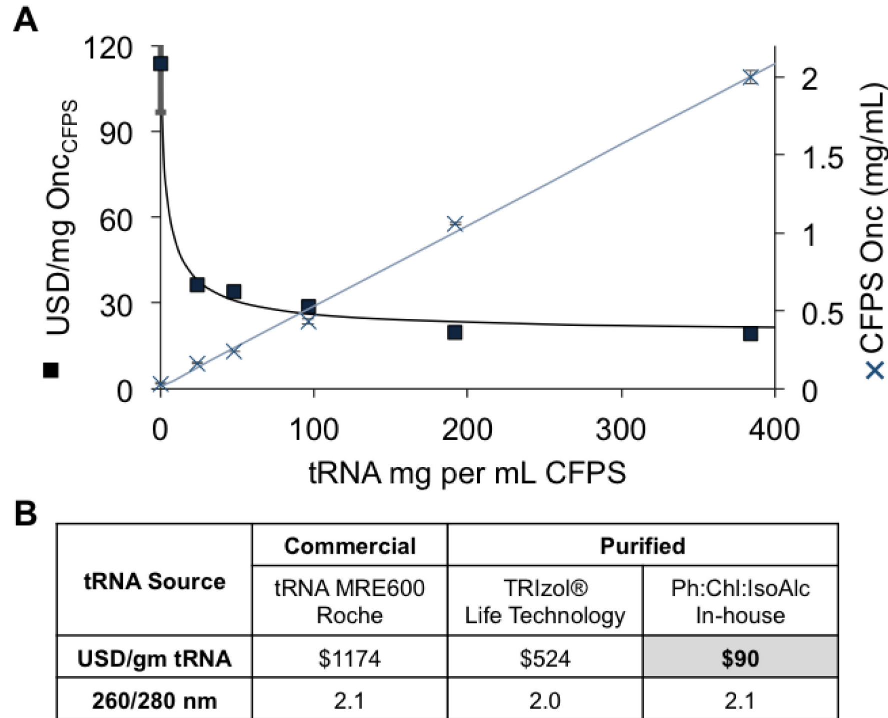
We have previously demonstrated the ability of CFPS to be stabilized up to 90 days by lyophilization, as outlined in the scheme of Figure 3-9,A [102]. Utilizing this lyophilized CFPS (lyo-CFPS) to produce biotherapeutics is a promising method for rapid, mobile and on-demand therapeutics. The robust stability of lyo-CFPS is characterized by its ability to outperform standard aqueous extracts after storage at temperatures above -80 °C (Figure 3-9).

Our previous report included stability data up to 90 days in storage. Figure 3-9,C reports for the first time the viability of lyo-CFPS stored for 1 year. The standard extract lost all observable protein synthesis viability under all storage conditions except at -80 °C. In stark contrast, the lyo-CFPS retained protein synthesis viability under all storage conditions except after 1 year at 25 °C. We tested our lyo-CFPS system against the standard CFPS to produce ONC_{CFPS}. The lyo-CFPS performed equally well or better under standard CFPS and upon addition of tRNA (Figure 3-9,B). The robust stability of lyo-CFPS lays the fundamental framework for applications in stabile, mobile, and "just-add-water" biotherapeutic expression.

3.3.5 Onconase Activity

The methods we describe for CFPS production of ONC can reduce costs while providing for direct expression of soluble, accessible proteins ready for downstream characterization and applications. To demonstrate the direct downstream assessment, we analyzed our ONC by 1) cell-free protein synthesis inhibition assay and 2) cancer cell inhibition assay.

www.manaraa.com



tRNA Source	Commercial	Purified	
	tRNA MRE600 Roche	TRIzol® Life Technology	Ph:Chl:IsoAlc In-house
USD/gm tRNA	$1174	$524	**$90**
260/280 nm	2.1	2.0	2.1

Figure 3-8 CFPS ONC Yield costs. A) ONC_{CFPS} was produced with increasingly concentrated doses of tRNA. Yields (x) were directly proportional to the amount of tRNA added. The yield cost (US dollars per mg ONC_{CFPS}, ■) is calculated based on the in-house produced tRNA. B) Table of prices and purities of tRNA sources.

3.3.6 Protein Synthesis Inhibition Activity

Protein synthesis inhibition is an indirect measurement of the nuclease activity of ONC and likely plays a key role in the anticancer action. Nuclease activity of cell-free produced ONC_{CFPS} was evident by the increase in protein yield when dosed with tRNA over time. To confirm this effect in controlled conditions, we produced GFP in the presence and absence of ONC. ONC was added at the initiation of CFPS reactions. The addition of ONC_{invivo} resulted in an inhibitory concentration of 50% of protein synthesis (IC_{50}) at 5.3 ± 1.1 µM (Figure 3-10 A, B). The addition of ONC_{CFPS} resulted in an IC_{50} of 11.2 ± 3.9 µM , while negative CFPS controls

www.manaraa.com

without ONC did not inhibit protein synthesis as severely (Figure 3-11). CFPS allowed for immediate downstream analysis of ONCCFPS protein synthesis inhibition.

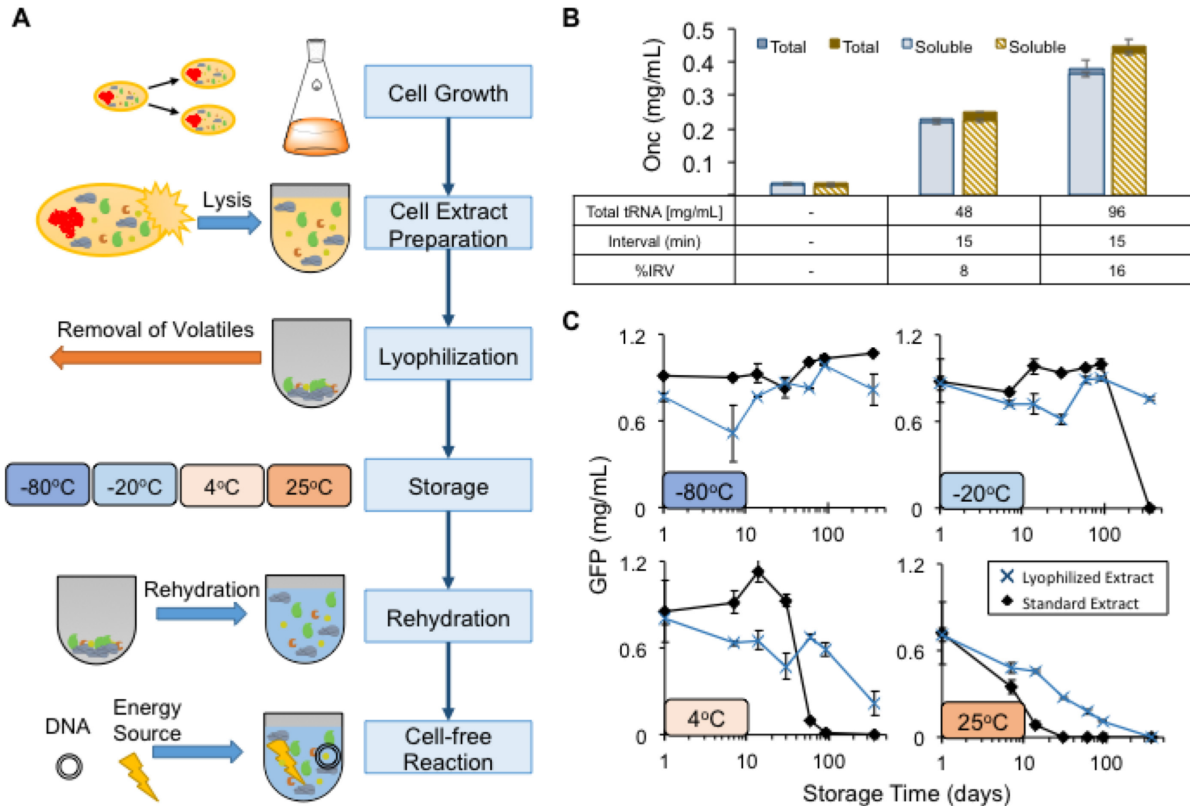
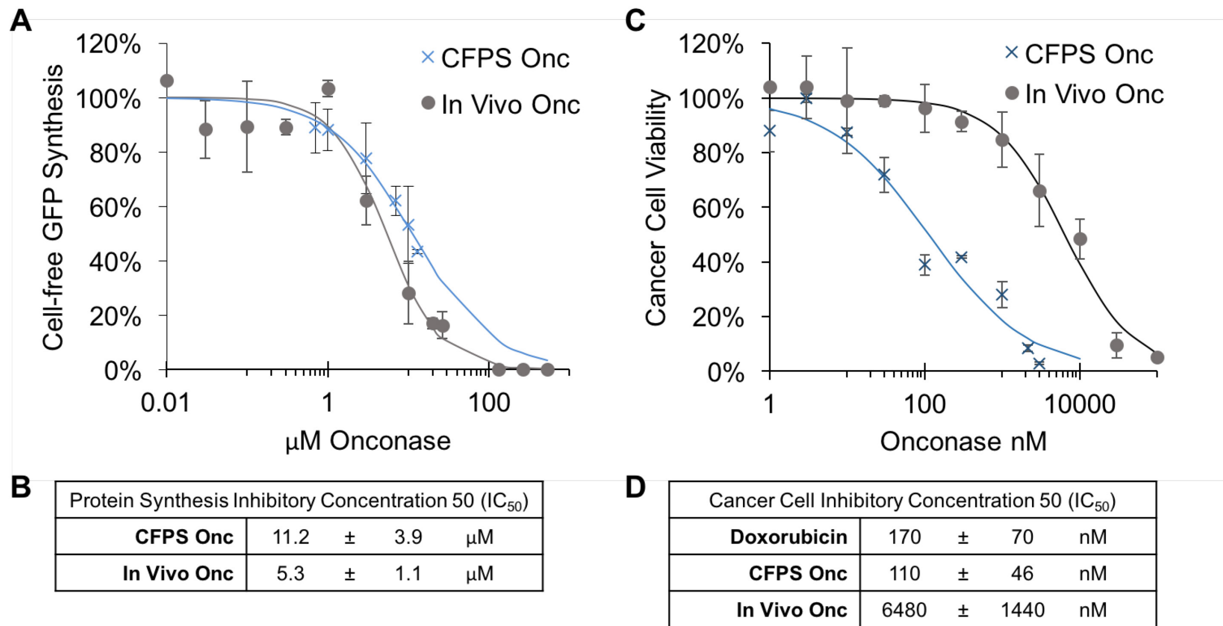


Figure 3-9 Lyophilized CFPS Systems. A) Scheme of lyophilized CFPS system. B) ONC yield comparison between standard aqueous extracts and lyophilized extracts. C) Extract stability data for extracts stored for up to 1 year under specified temperature conditions. Data up to 90 days was reported previously (Smith et al. 2014a). The cell-extract utilized for lyophlization was not initially as active as the cell-extract used in Figure 3-4, which accounts for the difference in baseline GFP expression levels. (n=3, error bars represent 1 standard deviation).

To verify that the CFPS solution was not responsible for the observed protein synthesis inhibition or cancer cell inhibition, CFPS reactions devoid of genes (CFPS NEG) and CFPS with non-toxic gene Q[beta] coat protein (CFPS Neg (Qbeta)) were added at identical dilutions to

المنارة للاستشارات
www.manaraa.com

each assay as reported in Figure 3-11. CFPS NEG generally enhanced the viability rather than inhibiting. CFPS NEG (Qbeta) did inhibit the synthesis of GFP, which inhibition may be due to ATP:ADP ratio changes caused by the addition of relatively large volumes of used CFPS reaction solution. Importantly, CFPS solution containing ONC_{CFPS} was distinguishably more active at inhibiting GFP synthesis than CFPS NEG (Qbeta). In the cases when CFPS Neg inhibited, such as with the MTT assay for 2150 and 3000 nM, the exclusion of these data points for calculating the IC_{50} of ONC_{CFPS} actually lowered the calculated value. Thus, these data were included in the analysis of the anticancer IC_{50} of ONC_{CFPS}.



Protein Synthesis Inhibitory Concentration 50 (IC_{50})				
CFPS Onc	11.2	±	3.9	µM
In Vivo Onc	5.3	±	1.1	µM

Cancer Cell Inhibitory Concentration 50 (IC_{50})				
Doxorubicin	170	±	70	nM
CFPS Onc	110	±	46	nM
In Vivo Onc	6480	±	1440	nM

Figure 3-10 Onconase Activity Assays. A) ONC was added to CFPS of GFP to assay its ability to inhibit protein synthesis. B) Protein Synthesis IC_{50} of ONC_{CFPS} and ONC_{invivo}. C) Breast cancer cells (MCF-7) were treated ONC to assay its ability to inhibit and kill cells. D) Cancer cell viability IC_{50} under treatment with control doxorubicin, ONC_{CFPS}, and ONC_{invivo}. (n=3, error bars represent 1 standard deviation, IC_{50} ranges represent 95% confidence interval).

المنارة للاستشارات
www.manaraa.com

3.3.7 Anticancer Activity

One of the promising characteristics of ONC is its potential as an anticancer therapeutic. To validate our ONC was active against cancer cells, we tested our CFPS and *in vivo* produced protein against the breast cancer cell line MCF-7 using an MTT cell viability assay, which quantifies the mitochondrial activity after a specified treatment period. As a baseline comparison, we performed the assay with doxorubicin and observed an IC_{50} = 0.17 ± 0.07 µM DOX, consistent with previously published results (Figure 3-12) [111].

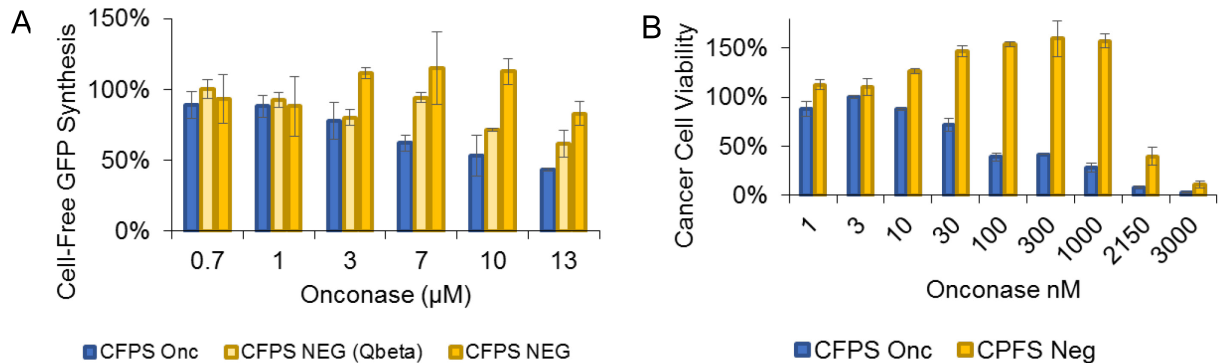


Figure 3-11 Protein Synthesis and Cancer Cell Inhibition Controls. A) Effect of different positive and negative controls on CFPS of GFP.; B) effect of positive and negative controls on cancer cell viability.

The treatment of the cancer cells with ONC reduced cell viability by upwards of 95%. The IC_{50} of ONC_{invivo} was consistent with previously published results at 6.48 ± 1.44 µM (Figure 3-10, C, D) [118]. Of considerable note, the IC_{50} of ONC_{CFPS} was about 60 times lower than ONC_{invivo} at 0.11 ± 0.046 µM, while the addition of CFPS negative control reaction generally maintained or improved the overall viability of the cells (Figure 3-11). The lowered IC_{50} of

المنارة للاستشارات
www.manaraa.com

ONC_{CFPS} suggests it may be more potent than ONC_{invivo} or the CFPS reaction mixture may act as an adjuvant for the endocytosis of ONC.

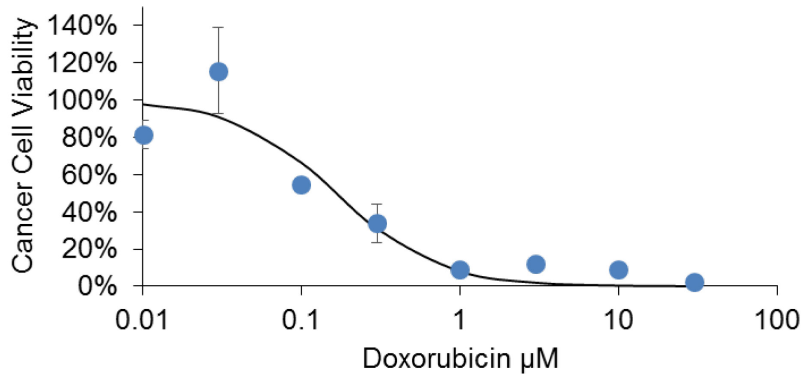


Figure 3-12 The MCF7 cancer cell line was subjected to a doxorubicin control for an MTT cell-viability assay. The resulting IC_{50} at 48 hours was 0.17 ± 0.07 µM. This is consistent with previously published IC_{50} at 72 hours of 0.1 µM [111].

3.4 Discussion

CFPS production directly to downstream assay (protein synthesis inhibition and anticancer assay) without purification or refolding dramatically reduces the time from gene to meaningful data. This system, together with the ability of CFPS to express genes directly from PCR-produced linear expression templates, lays the foundation for high-throughput technologies to screen large libraries of characterized and uncharacterized proteins for anticancer properties. Such a high-throughput method may reduce research investment of time and money towards future cancer therapies and, in turn, reduce cancer treatment expense to the patient. These CFPS techniques have the potential to also be adapted for other difficult-to-express proteins and biological complexes, such as DNA-protein fusions.

المنارة للاستشارات
www.manaraa.com

We demonstrated the cell-free protein synthesis system's ability to produce a cytotoxic anti-cancer therapeutic, ONC. We have shown that with inherent open nature of cell-free system, we can freely modify, replace, and supplement the system towards making the final product in a highly soluble and active form without the need of specialized cell lines or complex mutagenesis. With our system, the ONC yield can be increased by 56-fold compared to standard CFPS with greater than 95% solubility. This system can open unique opportunities for making other difficult-to-express biotherapeutics. In addition, CFPS is a promising platform for biopharma high-throughput that can save time and expense, and it can be easily scaled for making commercial or personalized drugs, however cost-effectiveness comparing scaled CFPS to traditional *in vivo* scale-up would need to be analyzed for each individual biotherapeutics produced.

The robust and stable nature of lyophilized cell-free systems provides further benefit to the production of biotherapeutics. We demonstrated that lyophilized cell-free system was comparable to aqueous CFPS at producing ONC. Furthermore, Lyo-CFPS retains its viability longer under non-ideal storage conditions. Thus, lyophilized extract can enable CFPS to become a platform for mobile biopharma applications, such as lab-on-a-chip, by just adding water to the system.

المنارة للاستشارات
www.manaraa.com

4 A CELL-FREE PROTEIN SYNTHESIS APPROACH TO BIOSENSING HTRβ-SPECIFIC ENDOCRINE DISRUPTORS

This chapter is an adaptation of the paper entitled "Cell-Free Protein Synthesis Approach to Biosensing hTRβ-Specific Endocrine Disruptors" published February 2017 in *Analytical Chemistry, ACS Publication[119]*. This work was developed and led by myself, Miriam J. Shakalli Tang, and Mark T. Smith with the help of Jeremy M. Hunt, Robert A. Law, Dr. David Wood, and Dr. Bradley Bundy. This work was a collaboration between our lab and Dr. Wood's lab at Ohio State University (OSU). Miriam Shakalli, Robert Law, and Dr. David Wood are affiliated with OSU.

Here we introduce a Rapid Adaptable Portable In-vitro Detection biosensor platform (RAPID) for detecting ligands that interact with nuclear hormone receptors (NHRs). The RAPID platform can be adapted for field use, allowing rapid evaluation of endocrine disrupting chemicals' (EDCs) presence or absence in environmental samples, and could also be applied to drug screening. The biosensor is based on an engineered, allosterically-activated fusion protein, which contains the ligand binding domain from a target NHR (human thyroid receptor β in this work). *In vitr*o expression of this protein using cell-free protein synthesis (CFPS) technology in the presence of an EDC leads to activation of a reporter enzyme, reported through a straightforward colorimetric assay output. In this work, we demonstrate the potential of this biosensor platform to be used in a portable "just-add-sample" format for near real-time detection. We also demonstrate the robust nature of the cell-free protein synthesis component in the presence of a variety of environmental and human samples, including sewage, blood, and urine. The presented RAPID biosensor platform is significantly faster and less labor intensive than commonly available technologies, making it a promising tool for detecting environmental EDC contamination and screening potential NHR-targeted pharmaceuticals.

4.1 Introduction

Biosensors can be life-changing devices, with uses ranging from daily glucose monitoring for diabetes patients to the rapid detection of toxins in the environment[120, 121]. When biosensors provide the required degree of specificity and sensitivity in combination with more rapid assay times, they are excellent alternatives to traditional detection methods[122].

المنارة للاستشارات
www.manaraa.com

Biosensing systems are available in various formats, from cell-based systems with complex metabolic pathways to less complex *in vitro* systems. Cell-based systems can have a broader spectrum of detection capabilities, however, they are hindered by transmembrane transport limitations, the need to maintain cell viability and stability, time-consuming preparation, and protracted assay times[123, 124]. In contrast, *in vitro* methods are commonly faster, more straightforward, simpler to store, and less expensive. Here we present a versatile, near-real time *in vitro* biosensor for detecting ligands that bind nuclear hormone receptors (NHRs).

NHRs help regulate vital functions of the cells and organisms, such as metabolism, homeostasis, differentiation, development, and reproduction[38, 39, 47]. NHRs interact with many natural and synthetic ligands and about 4% of all currently marketed therapeutics interfere with the activity of one or more NHRs[40]. NHRs also can interact with environmental endocrine disrupting chemicals (EDCs), which have become a public safety concern due to their ability to disrupt naturally occurring endocrine control. EDCs affect the endocrine system in humans and animals, commonly by mimicking natural hormones and binding to specific NHR ligand binding domains[46]. Examples of these include medical and industrial xenoestrogens, such as diethylstilbestrol and bisphenol-A, as well as naturally occurring phytoestrogens, such and genistein and daidzein[47-49]. EDCs have been found in common dietary, environmental, and household chemicals and have been linked to diverse diseases and disorders, including multiple cancers, developmental disorders, and other epigenetic dysfunction[43, 44]. Unfortunately, due to their large numbers, most chemicals and mixtures in commerce worldwide remain largely uncharacterized for endocrine disrupting activity[45].

In order to deliver faster detection of NHR-ligand interactions, we previously developed an EDC biosensor platform where the presence of an EDC is reported through a change in

المنارة للاستشارات
www.manaraa.com

growth phenotype of an engineered *Escherichia coli* strain[125, 126]. This platform relies on a multi-domain engineered allosteric fusion protein, which reports ligand binding to a given NHR through the activation of a fused thymidylate synthase reporter enzyme (Figure 4-1,A). In practice, the biosensor protein is constitutively expressed in an engineered *E. coli* thymidine-auxotroph strain, leading the growth phenotype of the strain to be dependent on the presence of an NHR-targeting ligand. Binding of the ligand to the NHR ligand binding domain activates the thymidylate synthase reporter enzyme and enables cell growth, allowing the presence and activity of a specific NHR ligand to be readily ascertained by a simple turbidity measurement after overnight incubation. An inserted mini-intein splicing domain has also been included to stabilize the fold of the NHR, and provide a conduit for the ligand-binding signal to the reporter protein. A critical aspect of this multi-domain biosensor protein is that it is modular, potentially allowing new biosensors based on alternate human and animal NHRs to be generated by swapping NHR ligand binding domains embedded in the intein domain scaffold[127-129]. However, the system still relies on bacterial growth phenotypes for activity quantification, and thus requires a minimum overnight incubation to produce a sufficient signal. Also, this assay and other cell-based assays (i.e. bacterial, yeast, and mammalian) for detecting NHR-binding ligands can be affected by the presence of cytotoxic chemicals in samples and poor cellular uptake rates.

In this work, we introduce the *R*apid *A*daptable *P*ortable *In-vitro D*etection biosensor (RAPID). This assay system combines our existing multi-domain biosensor protein design with rapid and efficient CFPS technology to overcome specific limitations of both *in vitro* and cell-based assays[56, 130-132]. In this system, the biosensor fusion protein is expressed using a CFPS system in the presence or absence of an unknown EDC sample. An engineered reporter enzyme domain on the biosensor protein exhibits ligand-dependent activity, resulting in a simple,

المنارة للاستشارات
www.manaraa.com

colorimetric readout. Unique CFPS characteristics, including its chemically accessible reaction environment, robustness, scalability, and control[88, 132, 133], make this technology a powerful biosensing platform for both simple and complex detection applications. In addition, the ability to lyophilize the CFPS components enables this type of biosensor to be stockpiled for emergencies and biothreat situations. Further, the robustness of the sensor design and simplicity of its visual readout could facilitate field-deployment, where assays of environmental samples could be carried out by minimally trained personnel in the absence of any conventional laboratory equipment. By leveraging the advantageous traits of CFPS, we have generated a highly practical and effective CFPS biosensor for uses in detecting toxic EDCs, as well as potentially valuable therapeutics against this important drug target class.

4.2 Experimental Section

4.2.1 Materials

The ligands used for this paper, 3,3',5-tiiodothyroacetic acid (TRIAC, 95%), 17-β-estradiol (E2), and 3,3′,5-triiodo-l-thyronine sodium salt hydrate (T_3, 95%), were purchased from Sigma-Aldrich.

4.2.2 Biosensor Design and Construction

The pET-based plasmid encoding the biosensor protein (MBP-I_N-hTR$_β$-I_C-βlac as illustrated in Figure 4-1,A) is based on our previously reported biosensor design for thyroid receptor (TR) ligands[127]. The biosensor fusion protein was inserted into the DHFR control plasmid supplied with the PureExpress® *In Vitro* Protein Synthesis Kit (New England Biolabs), which includes a T7 promoter to regulate expression of the target protein. Construction of the

www.manaraa.com

biosensor gene was accomplished by stepwise insertion of DNA segments encoding the maltose-binding domain (MBP), the intein-human TR fusion module (I_N-hTRβ-I_C), and the β-lactamase reporter protein (β-lac), where the resulting biosensor fusion gene replaces the DFHR expression control gene. In this case, the MBP was taken from the commercially available pMal-c2 expression vector (New England Biolabs), the I_N-hTRβ-I_C segment was taken from our previously reported TR biosensor plasmid[127], and the β-lac reporter protein was taken from a previously reported intein fusion expression plasmid[134].

4.2.3 Cell Extract Preparation

Cell extract preparation was performed as previously described[135]. Briefly, 5 ml of LB media was inoculated using *E. coli* BL21.DE3* strain in a cell culture tube. The culture was incubated overnight at 37 °C while shaking at 280 rpm. The culture was transferred to 100 ml LB media and upon reaching OD 2.0, it was transferred to 1 liter LB media in a Tunair flask. T7 RNA polymerase was overexpressed by inducing the culture with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD 0.6. The cells were harvested at the end of the exponential phase by centrifugation at 6000 RCF for 10 min at 4 °C. The cells were washed by suspending in pre-chilled Buffer A (10 mM Tris-acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium glutamate, and 1 mM dithiothreitol (DTT)), and subsequently centrifuged at 6000 RCF for 10 min 4 °C. The cells were resuspended in 1 ml Buffer A per gram cells and homogenized using EmulsiFlex French Press homogenizer at 20000 psi. The lysate was clarified by centrifugation at 12000 RCF for 30 min at 4 °C. The supernatant was incubated at 37 °C for 30 min while shaking at 280 rpm, flash frozen in liquid nitrogen, and then stored at -80 °C for later use as cell extract for CFPS.

المنارة للاستشارات
www.manaraa.com

4.2.4 Lyophilizing the Biosensor System

For lyophilized biosensor systems, CFPS reagents were mixed and lyophilized as described previously[102, 136] with slight modifications including that all reagents necessary for CFPS were combined and lyophilized together. Briefly, CFPS components were added to a pre-chilled tube in the following order while the tube rested on the ice: deionized water, magnesium glutamate, PANOxSP, and lastly the plasmid. The reaction mixture was mixed gently and transferred to a 1.5 ml Eppendorf tubes in 250 µl aliquots. Tubes were quickly placed into a liquid nitrogen container to flash freeze the reaction. The samples were lyophilized using FreeZone 2.5 Liter Benchtop Freeze Dry System (LABCONCO, Kansas City, MO) with the operating conditions of -50°C and <120 mTorr for 8 hr.

4.2.5 Cell-free Protein Synthesis Reaction

The CFPS reactions were performed in 96 well plate using PANOxSP system for 20 to 180 min at 37 °C[82]. The reactions contained 25 volume percent cell extract, 1.20 nM plasmid and the following components all from Sigma-Aldrich (St. Louis, MO): 10 to 15 mM magnesium glutamate, which was optimized based on the extract, 1 mM 1,4-diaminobutane, 1.5 mM spermidine, 33.33 mM phosphoenolpyruvate (PEP), 10 mM ammonium glutamate, 175 mM potassium glutamate, 2.7 mM potassium oxalate, 0.33 mM nicotinamide adenine dinucleotide (NAD), 0.27 mM coenzyme A (CoA), 1.2 mM ATP, 0.86 mM CTP, 0.86 mM GTP, 0.86 mM UTP, 0.17 mM folinic acid, 2 mM of all the canonical amino acids except glutamic acid. For experiments requiring the measurement of protein production yield using a scintillation counter, 5 µM l-[U-^{14}C] Leucine (PerkinElmer, Waltham, MA) was added to the reaction, and protein yield was calculated based on total and washed counts as described previously[83].

المنارة للاستشارات
www.manaraa.com

4.2.6 Environmental and Human Samples Tested in Cell-Free Protein Synthesis

Tap water, storm water, and pond water were collected at various locations in Utah County, USA. Soil and snow samples were collected in Salt Lake County, Utah, where soil samples were extracted into ddH_2O at a one to one (weight to volume) ratio. All of the wastewater treatment samples were collected from the Provo city water reclamation facility. Raw sewage was from the influent of the plant. Post clarifier sample was after the primary sedimentation basins. Post biological sample was the effluent of the aeration basins with activated sludge. Post filter sample was the activated sludge process effluent (final clarifier effluent) passed through anthracite filters. The effluent sample was the final product of the plant after chlorination and dechlorination treatments. The single donor human whole blood-Na heparin sample was obtained from Innovative Research (Peary Ct, Novi, MI). Urine samples were obtained from volunteers.

4.2.7 Hormone Biosensor Assay

The Hormone biosensor assay was performed in 2 stages. Stage 1: CFPS of the biosensor protein in a 96 well plate for 20 min in the presence of 0 to 10 μM TRIAC, T3, or E2 dissolved in dimethyl sulfoxide (DMSO). For consistency all CFPS reactions were adjusted to have 5 volume percent DMSO. Stage 2: After 20 min, the reactions were diluted 104-fold into PBS buffer, of which 25 μl was transferred into each well of a UV-transparent Corning® 96 well plate. The 104-fold CFPS dilution was introduced for optimal signal clarity and to eliminate overflow readings of our assay instruments, and was used in all experiments. To each well, 175 μl of 228.6 μM nitrocefin in PBS was additionally added to the wells at the same time to achieve a final nitrocefin concentration of 200 μM. The plates were then directly quantified via plate

للاستشارات المنارة
www.manaraa.com

reader (BioTek Synergy Mx) for a nitrocefin-based beta-lactamase activity assay [137]. Specifically, the absorbance was read at 390 and 490 nm wavelengths for unreacted and reacted substrate nitrocefin, respectively. Measurements were repeated at 1 min intervals, with 10 sec shaking at each interval to mix, for 15 min. At the end of the assay, the absorbance was read at 760 nm to provide a relative background level for the assay. The rate of nitrocefin conversion was determined at each ligand concentration using the time course measurements, and the resulting rates were used to determine the half maximal effective ligand concentration (EC50).

4.2.8 Analysis of Hormone Biosensor Assay Results

The nitrocefin conversion value (NCV) was calculated using Equation 1. The A_{390} is λ_{max} of the yellow substrate nitrocefin, while A_{490} is the λ_{max} of the red nitrocefin conversion product, and A_{760} is background absorbance of each well. In order to maximize the signal-to-noise ratio, the time point with the maximum difference between the NCVs of the negative control (zero ligand) and maximum ligand concentration was selected to calculate the dose-response curves. The Four-Parameter Logistic Function (Equation 2) was fitted to this data to yield the half maximal effective concentration (EC_{50})[138]. Parameters "a" and "b" define lower and upper plateau value of the function, respectively, while "k" is the slope factor.

$$\text{Nitrocefin Conversion Value (NVC)} = \frac{A_{490} - A_{760(median\ of\ all\ reaction\ wells)}}{A_{390} - A_{760(median\ of\ all\ reaction\ wells)}} \quad (4\text{-}1)$$

$$\text{Predicted NVC} = a + \frac{b-a}{1+(\exp(k(\log(ligand\ concentration) - \log(EC_{50}))))} \quad (4\text{-}2)$$

To generate percentage dose-response graphs, values and predicted values from the fitted function were normalized based on the equation 3.

المنارة للاستشارات
www.manaraa.com

Normalized Dose Response= $\frac{(NCV)-Min(NCV)}{Max(NCV)-Min(NCV)} * 100\%$ (4-3)

The overall quality of the assays was assessed using Z' factor, signal-to-noise ratio (S/N), and signal to background ratio (S/B) parameters. The parameters were calculated using a previously described method[128, 139]. Also, the limit of detection (LOD) was calculated based on IUPAC methodology by finding the corresponding concentration value for blank measurement added to its three times standard deviation[140]

4.3 Results and Discussion

4.3.1 RAPID Biosensor Design and Rationale

Here we report the RAPID (*R*apid *A*daptable *P*ortable *In-vitro D*etection) biosensor for NHR-binding ligands. The goal of this work was to create a near real-time biosensor platform by combining our previous cell-based allosterically activated, fusion protein approach[127] with the open flexibility of CFPS systems[23]. The fusion protein consists of four domains including: 1) maltose binding domain, which improves the solubility of the fusion protein[125]; 2) mini-intein domain, which acts as a stabilizing domain for the NHR domain[125]; 3) NHR ligand binding domain, which is the heart of the biosensor and acts as a switch to activate the reporter enzyme; and 4) the reporter enzyme. An interaction between a ligand/chemical and the NHR ligand binding domain causes a conformational change which results in improved reporter protein activity as previously described[141]. Hence, a signal results from the presence of a chemical/ligand that binds the NHR ligand binding domain during protein synthesis (Figure 4-1,A, Figure 4-2). Due to the cell-free nature of CFPS, there is no membrane transfer limitation for chemicals that might target NHRs[2], while the direct translation of the sensor protein

المنارة للاستشارات
www.manaraa.com

provides a fast, inexpensive, and convenient assay for the presence of EDC activity in unknown test chemicals.

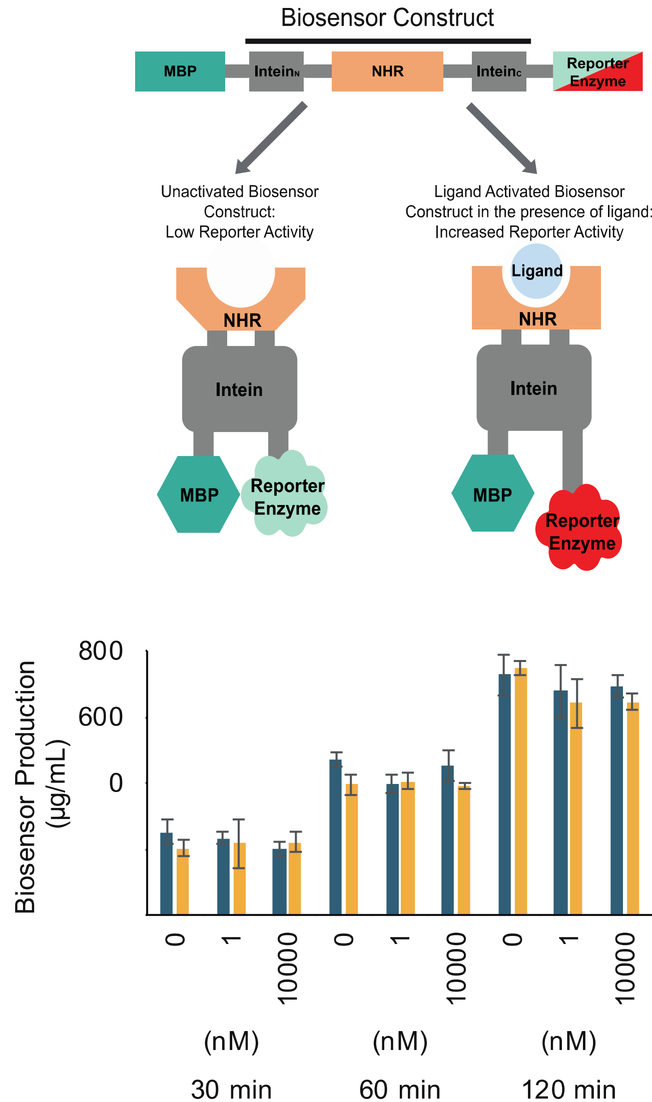


Figure 4-1(A) The protein construct for the RAPID biosensor. It includes the Maltose Binding Protein (MBP) at its N-terminus, a mini-intein splicing domain with an inserted NHR ligand binding domain (from hTRβ in this work), and a C-terminal reporter enzyme (β-lac in this work).The presence of ligand during expression of the protein changes the structure of the biosensor and improves accessibility of the reporter enzyme. (B) CFPS of the biosensor fusion protein with protein production yields reported for increasing reaction times and in the presence of three levels of the ligand T3 (total protein = dark bars, soluble protein = light bars, reaction volume was 20µl). The error bars represent one standard deviation and n=3.

www.manaraa.com

The initial step in creating the CFPS-based RAPID biosensor was to re-engineer the reporter protein domain for a rapid and straightforward colorimetric assay readout. Our previous bacterial biosensor platform employed the thymidylate synthase reporter enzyme to enable growth phenotype changes[125]. Unfortunately, *in vitro* assays for thymidylate synthase activity are cumbersome and require oxygen-sensitive reagents. For these reasons, the β-lactamase (β-lac) enzyme was selected to replace the thymidylate synthase enzyme due to its similarity in size and commercially available colorimetric activity assay.

To characterize our RAPID biosensor, the human thyroid receptor β (hTRβ) was chosen for the initial ligand binding domain due to its robust behavior in our bacterial biosensor[127]. It also has high sensitivity and selectivity to TRIAC, a potent agonist, with a half-maximal effective concentration value (EC_{50}) reported at 70 nM. Cloning work to incorporate the β-lac reporter and hTRβ ligand binding domain into the fusion protein is described in the methods section, with the final fusion protein sequence illustrated in Figure 4-1,A.

4.3.2 Cell-free Protein Synthesis of the Reporter Fusion Protein

The resulting fusion protein, containing the hTRβ ligand binding domain and β-lac, was expressed in an *E. coli*-based CFPS system as detailed in the methods section. To elucidate the mechanism of activation, total protein titer and protein solubility were measured by tracking the incorporation of C-14 radiolabeled leucine (Figure 4-1,B). The 92 kD MBP-I_N-hTRβ-I_C-β-lac fusion protein was expressed at yields up to 700 μg/mL in 3 hr and the expression level was unaffected by the presence of T3 ligand. Also, the protein solubility yields were consistently greater than 85%. The insensitivity of the expressed protein to this ligand, in addition to our previous work and subsequent experiments shown here, suggest that the expression system and

المنارة للاستشارات
www.manaraa.com

biosensor protein solubility are uncoupled from the ligand concentration. Thus, the scaffold is expressed in a soluble form regardless of ligand, further suggesting a signaling mechanism based on a structural change in the biosensor protein upon ligand binding, as opposed to a mechanism based on soluble biosensor expression.

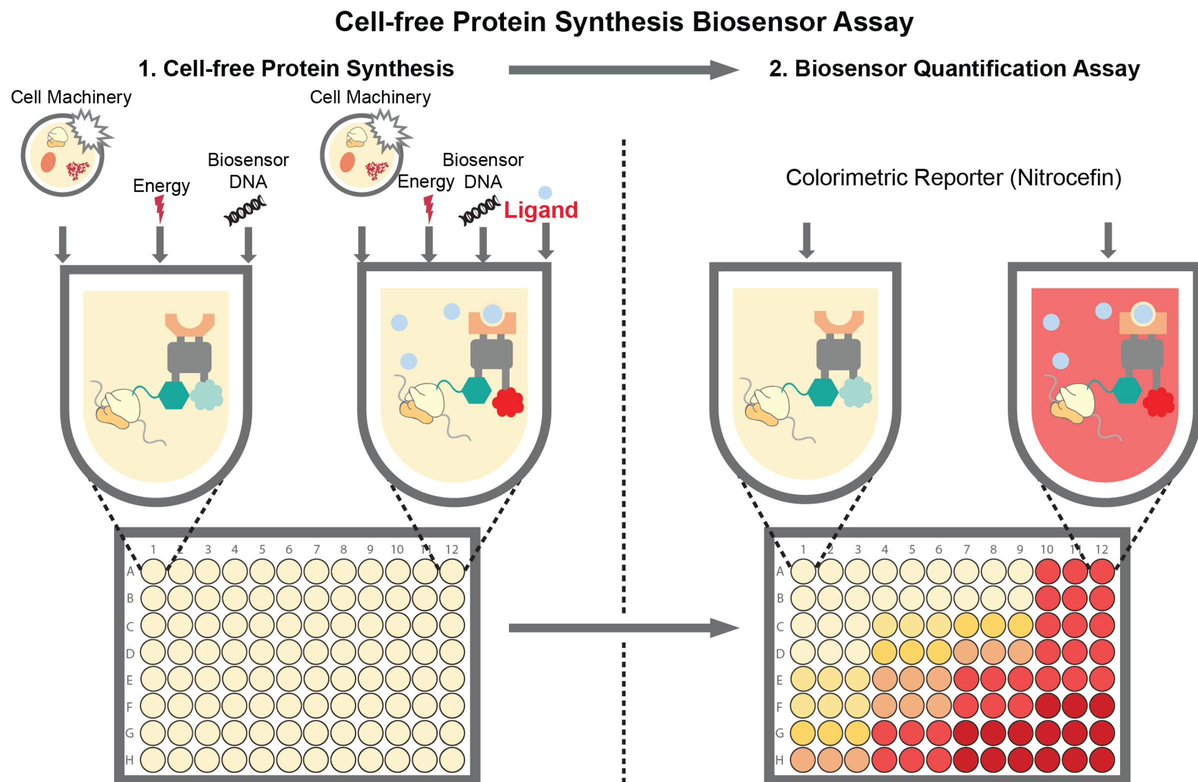


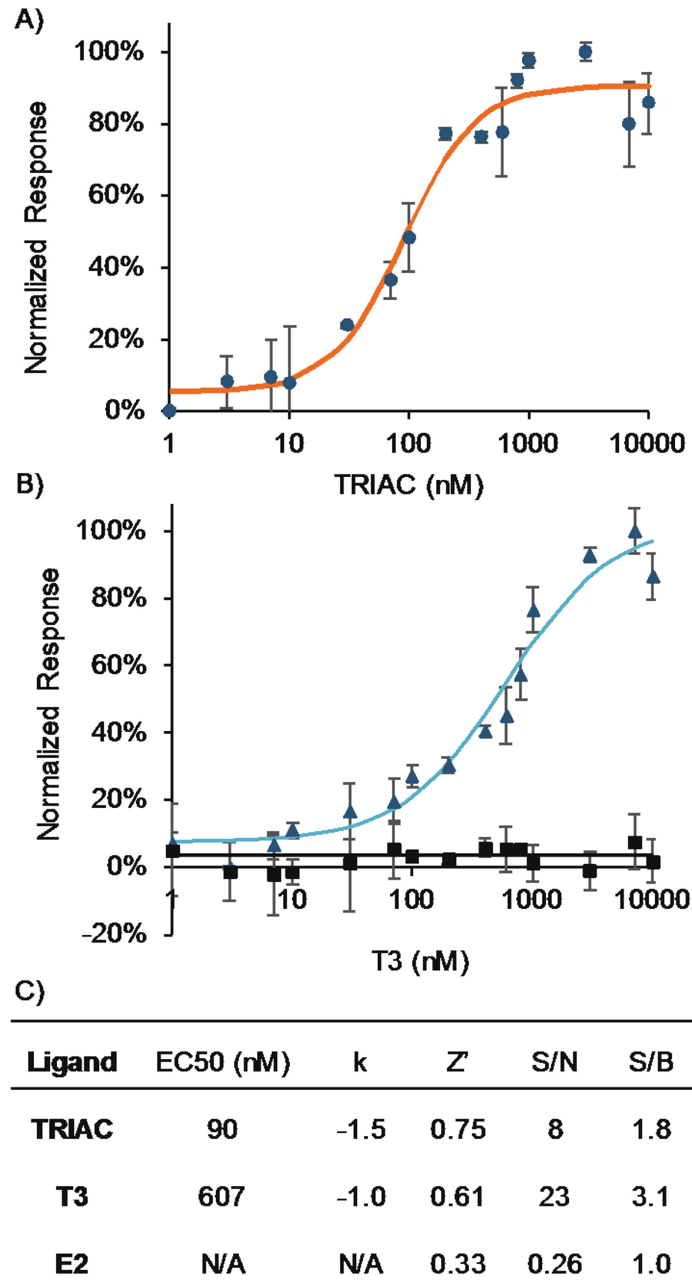
Figure 4-2 Scheme of the RAPID biosensor to detect chemicals that target NHRs. The biosensor assay includes two steps: 1- CFPS reaction to produce the biosensor, 2- colorimetric assay to quantify the biosensor protein activation. The presence of ligand during protein synthesis activates the biosensor by altering the conformation of biosensor enzyme and increasing the nitrocefin assay signal.

www.manaraa.com

4.3.3 Hormone Biosensor Assay

The hormone biosensor assay consists of two steps as illustrated in Figure 4-2. First, cell-free expression of the MBP-I_N-hTRβ-I_C-β-lac reporter fusion protein is performed in the presence of the sample to be tested. The resulting protein is then subjected to a colorimetric reporter enzyme activity assay, where NHR-ligand binding is reflected in the activity of the reporter enzyme domain (β-lac). The hormone sensing capability of this assay was assessed with 3 known endocrine disrupting chemicals; two chemicals that are known to target hTRβ (TRIAC and T3), and a negative control (estrogen) that targets the human estrogen receptor NHR but does not target hTRβ. The results are reported in Figure 4-3, where the EC_{50}, Z' factor, signal-to-noise ratio (S/N), and signal to background ratio (S/B) are calculated for each chemical. The Z' factor was between 0.5 to 1 for all assays, indicating "an excellent assay" for screening and sensing[128, 139]. The measured EC_{50} for TRIAC and T3 were 90 and 607 nM, respectively, which correspond well to the EC_{50} from our previous studies with the bacterial biosensor, 70 and 580 nM respectively (Figure 4-3)[127]. Also, the calculated LOD were 48 and 75 nM, respectively for TRIAC and T3. As expected, a statistically significant signal was not observed with the estrogen negative control (Figure 4-3,B, square markers, p-value of 0.84). TRIAC was 7-fold more potent than T3 against TRβ which is similar to our bacterial biosensor at 8-fold and other reported sensors at 6-fold[142]. Although some *in vitro* binding and transactivation assays can detect ligands with higher sensitivity, the simplicity, speed, and the lack of toxicity or cell-uptake complications make the RAPID system a strong candidate for screening of NHR-binding ligands[142-144].

المنارة للاستشارات
www.manaraa.com



Ligand	EC50 (nM)	k	Z'	S/N	S/B
TRIAC	90	-1.5	0.75	8	1.8
T3	607	-1.0	0.61	23	3.1
E2	N/A	N/A	0.33	0.26	1.0

Figure 4-3 A) Dose-response curve for the hTRβ biosensor in the presence of TRIAC. B) Dose-response curves of the hTRβ biosensor in the presence of T3 (triangles), and E2 (squares). C) The half-maximal effective concentration (EC50), slope factor (k), Z' factor, signal to noise ratio (S/N), and signal to background ratio (S/B) for the responses against TRIAC, T3, and E2. The solid lines represent fitted nitrocefin conversion values, the markers represent the average measured values, and the error bars represent one standard deviation for n=2.

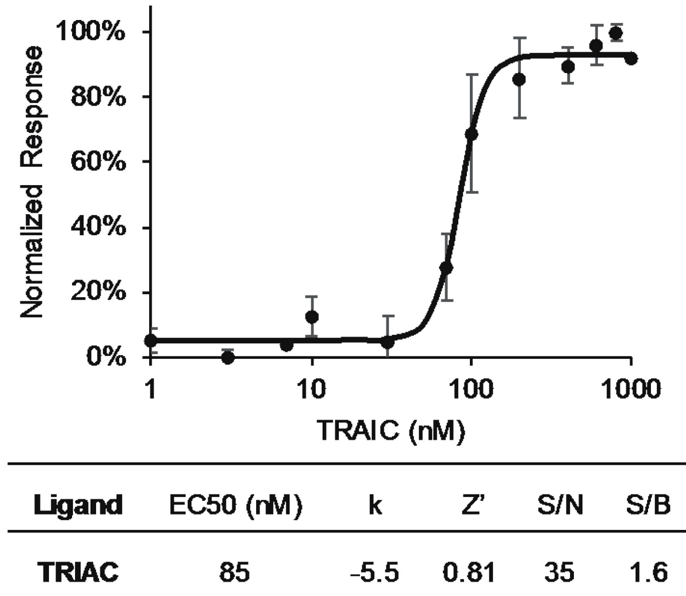
www.manaraa.com

One considerable strength of our RAPID biosensor is speed of the assay, with the total time needed to generate clear results being less than 30 min. Alternatively, mammalian-based assays may take days to weeks to complete and bacterial-based assays take 24-36 hours[127, 145]. Another strength of the cell-free system is the elimination of confounding issues associated with membrane transport of test chemicals, unknown or unintended side effects related to cell growth or toxicity, or cross activation of NHRs[23]. In contrast to other *in vitro* techniques, a further advantage of our system is that there is no need for any purification or complex enzyme stabilization steps[59]. Furthermore, the modular nature of the fusion protein opens the possibility of optimizing the system by rapidly incorporating new reporter enzymes, while also expanding the RAPID biosensor to include diverse nuclear hormone receptors for human and animal applications[146].

4.3.4 Lyophilized Biosensor

To develop our RAPID biosensor platform for potential field use (*i.e.* outside of the laboratory), we assessed the possibility of lyophilizing the CFPS biosensor components. Previously, we reported lyophilized cell extracts remained active after 90 days of storage at room temperature, and demonstrated the potential for CFPS to be used in biotherapeutic protein production[147]. For this work, all essential elements, including cell extract, plasmid encoding the fusion protein, and necessary small molecule additives were combined and lyophilized at the same time, to create a "just-add-sample" CFPS assay. The results illustrate that lyophilized CFPS performed similarly to freshly prepared CFPS in detecting TRIAC (85 nM EC50, -5.5 k, 0.81 Z', 35 S/N, 1.6 S/B, 59 nM LOD) (Figure 4-4). Thus, the RAPID biosensor has the potential to be used as a field assay for *in situ* real-time detection of EDCs in essential infrastructure, such as watersheds.

المنارة للاستشارات
www.manaraa.com



Ligand	EC50 (nM)	k	Z'	S/N	S/B
TRIAC	85	-5.5	0.81	35	1.6

Figure 4-4 Dose-response graph and statistical analysis results for the RAPID biosensor with lyophilized CFPS components in the presence of TRIAC. The solid line represents fitted nitrocefin conversion values, while circle markers represent the measured values. Error bars repre-sent one standard deviation for n=2.

4.3.5 CFPS Performance in Different Environmental Samples

To understand the utility of this new NHR biosensor for evaluating environmental samples, we tested the performance of the CFPS system – a sensitive component of the RAPID biosensor – in various untreated water sources, raw sewage, and human bodily fluids (Figure 4-5). For all of the samples, CFPS produced a model protein GFP at sufficient protein production levels necessary for the biosensor assay. The water samples (tap, pond, snow, storm) and samples from various stages of a wastewater treatment plant did not significantly affect CFPS levels, with the exceptions being raw sewage wastewater and post clarifier wastewater. However, even after adding 47% by volume raw sewage or post clarifier wastewater to CFPS reactions, greater than 50% of protein production level was maintained. The robustness of CFPS across

المنارة للاستشارات
www.manaraa.com

diverse environmental samples indicates the potential for use in diverse environmental monitoring situations.

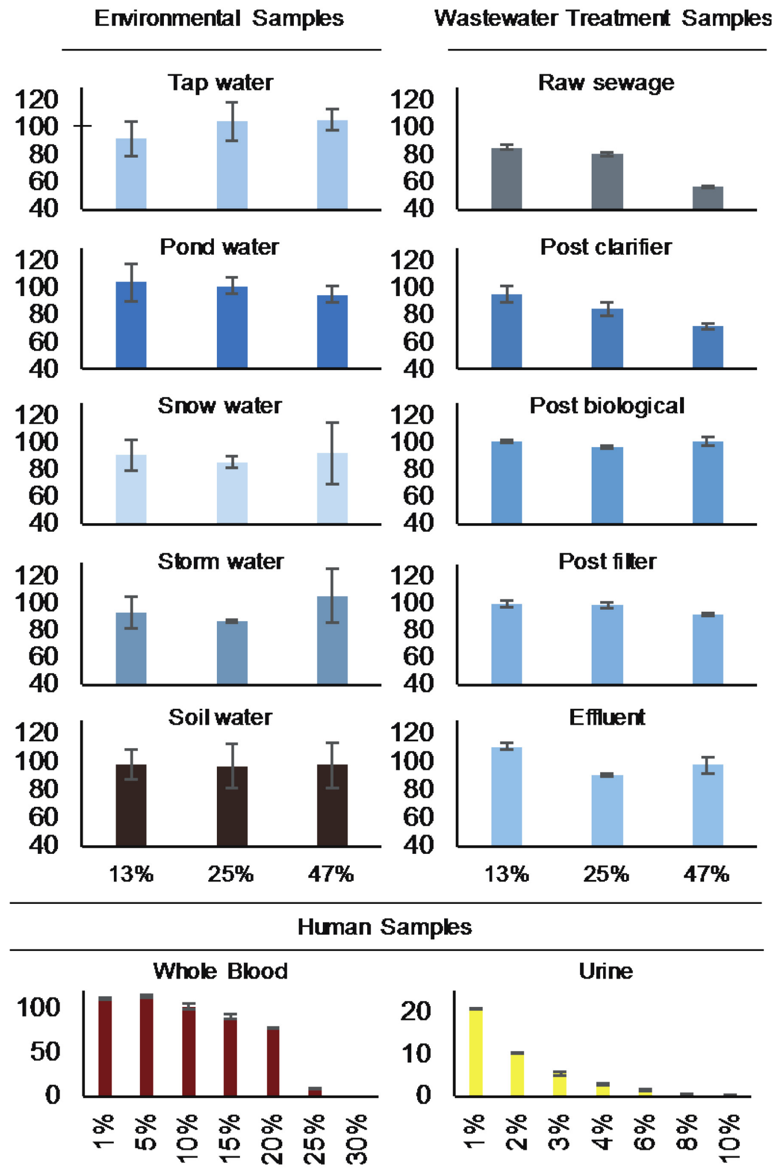


Figure 4-5 Protein production capability of CFPS in the presence of environmental and human samples. In all cases model protein GFP is expressed and the production level (y-axis) is normalized to GFP production in a standard CFPS with 100 corresponding to 100% of the GFP production level in standard CFPS. Each sample type is described in the methods section and the x-axis corresponds to the final concentration for the sample in the CFPS reaction by volume percent. The error bars represent one standard deviation for n=3.

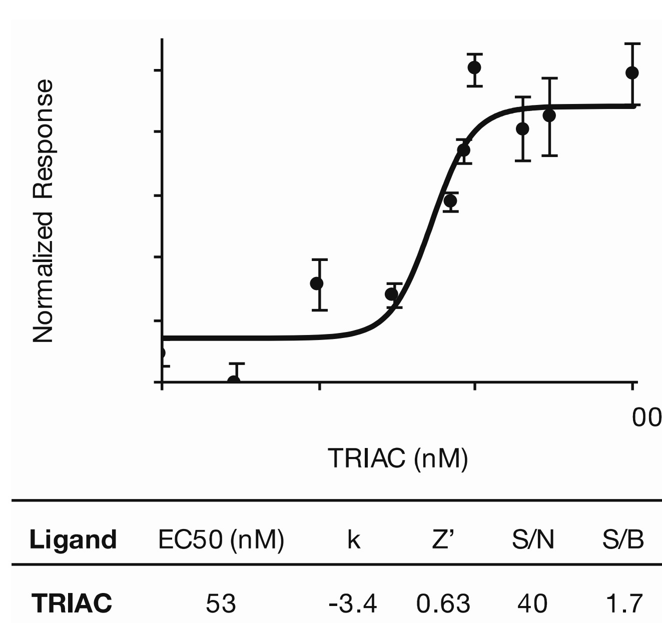
www.manaraa.com

Beyond environmental and wastewater samples, we examined CFPS tolerance to human medical samples, including blood and urine. Greater than 60% of the original CFPS activity was retained in reactions containing up to 20% by volume human blood. Additionally, we note that the blood we used in this work contained heparin as anticoagulant in lieu of EDTA, because EDTA at high concentrations can sequester magnesium and inactivate CFPS[82]. Expectedly, human urine, which contains a significant concentration (~280 mM) of the protein denaturant urea[148], had the greatest impact on CFPS activity. However, CFPS activity remained detectable at up to 8% by volume urine (1% original activity, with a standard deviation of 0.05 %). To account for significant yield changes caused by urine samples, a control CFPS reaction with a model protein such as GFP could be used in combination with the biosensor to ensure consistent dilution of the CFPS biosensing protein in the second colorimetric stage of the biosensor assay. Overall, the ability of CFPS to tolerate high levels of various contaminants, such as organic matter, bacteria, blood, urine and wastewater demonstrates its robustness as a biosensing platform.

4.3.6 NHR RAPID Biosensor Performance in an Environmental Sample

Raw sewage was chosen to investigate how the composite biosensor was affected by the presence of an actual environmental sample. CFPS reactions containing 40% final volume raw sewage and TRIAC at varying concentrations were reacted for 20 mins. Subsequently, the reactions were diluted and assayed using the described colorimetric assay, where the responses are independently normalized to the high and low EDC concentration readings of the assay. The resulting RAPID biosensor maintained its sensitivity for TRIAC (53 nM EC50, -3.4 k, 0.63 Z’, 40 S/N, 1.7 S/B, 28 nM LOD) (Figure 4-6).

المنارة للاستشارات
www.manaraa.com



Ligand	EC50 (nM)	k	Z'	S/N	S/B
TRIAC	53	-3.4	0.63	40	1.7

Figure 4-6 Dose-response graph and statistical analysis results for the RAPID biosensor in the presence of TRIAC and 40% by volume raw sewage. The solid line represents fitted nitrocefin conversion values, the circle markers represent the measured values, and the error bars represent one standard deviation for n=2.

4.4 Conclusion

Here we have developed a new RAPID biosensor platform for chemicals that target nuclear hormone receptors using a quick, versatile cell-free protein synthesis approach. The developed biosensor has some key advantages over existing biosensors, including near real-time readout, the potential for portable field use, and reduced labor and cost requirements. This biosensor is also a promising tool for studying various NHR-binding ligands in a high-throughput manner. Additionally, the ability of CFPS to perform protein synthesis in different human and environmental samples, showed strong potential of the biosensor for detecting NHR-targeting compounds directly, without requiring purification or modification of the sample. Overall the RAPID biosensor is an attractive alternative to currently available technology and

للاستشارات
www.manaraa.com

provides a fast, versatile platform for detecting potential NHR-binding ligands including EDCs and therapeutics.

المنارة للاستشارات
www.manaraa.com

5 CONCLUSIONS AND FUTURE WORK

In my presented research, I successfully worked with colleagues and collaborators to lead the engineering of cell-free protein synthesis technology for 1) sense codon emancipation for incorporation of multiple unnatural amino acids; 2) expressing a hard-to-express anticancer biotherapeutic and introducing a just-add-water system; and 3) biosensing ligand that interacts with nuclear hormone receptors. I have helped advance each field and am excited about the potential of cell-free systems to revolutionize approaches to these areas. As we move forward, I have the following thoughts on the direction of future work needed for the advancement of this research.

5.1 Future Work

5.1.1 Codon Emancipation

Our work successfully showed that we can decouple codon-amino acid links by degrading endogenous tRNA. To emancipate sense codons and eventually incorporating unnatural amino acids, we need to add back tRNA for a minimal set of tRNAs for the 20 canonical amino acids (one tRNA for each amino acid) and orthogonal tRNA for incorporating unnatural amino acids from other organisms such as *methanocaldococcus jannaschii* or *saccharomyces cerevisiae*. We used an *in vitro* transcription method successfully to synthesize tRNA for both incorporating natural and unnatural amino acids, and this method is expensive

www.manaraa.com

and labor intensive. For future work, we suggest that instead of making each individual tRNA for canonical amino acids, we use *E. coli* bulk-purified tRNAs and then target just the tRNA that codes for the specific codon selected for emancipation. To do so, we recommend using complementary single stranded DNA and making a DNA-tRNA hybrid for the selected tRNA, then using immobilized RNase H to degrade the tRNA. In this way, we can add back the rest of bulk tRNAs to the system. This method would have a few advantages including: lower cost, and being a less labor-intensive process, Also, in this approach tRNA comes with all the necessary post translational modifications. For orthogonal tRNAs, we recommend using the previous method, involving *in vitro* transcription.

5.1.2 Expressing a Hard-to-express Anticancer Biotherapeutics

For future work in this area, we suggest combining this technology with linear DNA template technology and making a large library of mutated genes for onconase. This combined method has the potential to screen onconcase in a high-throughput manner to find the most potent gene for producing onconase with the highest level of anticancer activity in a fraction of time needed for the *in vivo* approach.

5.1.3 Biosensing Ligand that Interact with Nuclear Hormone Receptors

For this project, we strongly suggest expanding applications of the biosensor by substituting the nuclear hormone receptor domain to target other known endocrine disrupting compounds and to screen environmentally released chemicals for potential endocrine disrupting activity. Also, we recommend using other reporter enzymes for decreasing the cost of enzyme substrate and improving sensitivity. The modularity of the presented biosensor makes it a versatile platform for several different biosensing applications.

المنارة للاستشارات
www.manaraa.com

REFERENCES

1. Hu, W.S., *Biochemical Engineering over Half a Century.* Chemical Engineering Progress, 2013. **109**(11): p. 35-39.
2. Swartz, J.R., *Transforming biochemical engineering with cell-free biology.* AIChE Journal, 2012. **58**(1): p. 5-13.
3. Carlson, E.D., et al., *Cell-free protein synthesis: Applications come of age.* Biotechnology Advances, 2012. **30**(5): p. 1185-1194.
4. Swartz, J., *Developing cell-free biology for industrial applications.* Journal of Industrial Microbiology & Biotechnology, 2006. **33**(7): p. 476-485.
5. Prat, L., et al., *Carbon source-dependent expansion of the genetic code in bacteria.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(51): p. 21070-21075.
6. Noren, C.J., et al., *A general method for site-specific incorporation of unnatural amino acids into proteins.* Science, 1989. **244**(4901): p. 182-188.
7. Mendel, D., V.W. Cornish, and P.G. Schultz, *Site-directed mutagenesis with an expanded genetic code.* Annual review of biophysics and biomolecular structure, 1995. **24**(1): p. 435-462.
8. Bain, J.D., et al., *RIBOSOME-MEDIATED INCORPORATION OF A NONSTANDARD AMINO-ACID INTO A PEPTIDE THROUGH EXPANSION OF THE GENETIC-CODE.* Nature, 1992. **356**(6369): p. 537-539.
9. Wang, L., et al., *Expanding the genetic code of Escherichia coli.* Science, 2001. **292**(5516): p. 498-500.
10. Wang, L. and P.G. Schultz, *Expanding the genetic code.* Chemical Communications, 2002(1): p. 1-11.
11. Chin, J.W., et al., *An expanded eukaryotic genetic code.* Science, 2003. **301**(5635): p. 964-967.
12. Liu, C.C. and P.G. Schultz, *Adding New Chemistries to the Genetic Code*, in *Annual Review of Biochemistry, Vol 79*, R.D. Kornberg, et al., Editors. 2010. p. 413-444.
13. Anderson, J.C., et al., *An expanded genetic code with a functional quadruplet codon.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(20): p. 7566-7571.
14. Albayrak, C. and J.R. Swartz, *Cell-free co-production of an orthogonal transfer RNA activates efficient site-specific non-natural amino acid incorporation.* Nucleic Acids Research, 2013. **41**(11): p. 5949-5963.
15. Bundy, B.C. and J.R. Swartz, *Site-Specific Incorporation of p-Propargyloxyphenylalanine in a Cell-Free Environment for Direct Protein−Protein Click Conjugation.* Bioconjugate Chemistry, 2010. **21**(2): p. 255-263.

المنارة للاستشارات
www.manaraa.com

16. Albayrak, C. and J.R. Swartz, *Using E. coli-based cell-free protein synthesis to evaluate the kinetic performance of an orthogonal tRNA and aminoacyl-tRNA synthetase pair.* Biochemical and Biophysical Research Communications, 2013. **431**(2): p. 291-295.
17. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2016.* CA: A cancer journal for clinicians, 2015.
18. Sommerfeld, S. and J. Strube, *Challenges in biotechnology production—generic processes and process optimization for monoclonal antibodies.* Chemical Engineering and Processing: Process Intensification, 2005. **44**(10): p. 1123-1137.
19. Sanchez-Garcia, L., et al., *Recombinant pharmaceuticals from microbial cells: a 2015 update.* Microbial cell factories, 2016. **15**(1): p. 1.
20. Boohaker, R., et al., *The use of therapeutic peptides to target and to kill cancer cells.* Current medicinal chemistry, 2012. **19**(22): p. 3794.
21. Tyagi, A., et al., *CancerPPD: a database of anticancer peptides and proteins.* Nucleic acids research, 2014: p. gku892.
22. Group, U.C.S.W., *United States cancer statistics: 1999–2010 incidence and mortality web-based report.* Atlanta: US Department of Health and Human Services, Centers for Disease Control and Prevention and National Cancer Institute, 2013.
23. Smith, M.T., et al., *The emerging age of cell-free synthetic biology.* FEBS Letters, 2014. **588**(17): p. 2755-2761.
24. Smith, M.T., et al., *The incorporation of the A2 protein to produce novel Qβ virus-like particles using cell-free protein synthesis.* Biotechnology Progress, 2012. **28**(2): p. 549-555.
25. Salehi, A.S.M., et al., *Cell-free protein synthesis of a cytotoxic cancer therapeutic: Onconase production and a just-add-water cell-free system.* Biotechnology Journal, 2016. **11**(2): p. 274-281.
26. Zimmerman, E.S., et al., *Production of Site-Specific Antibody–Drug Conjugates Using Optimized Non-Natural Amino Acids in a Cell-Free Expression System.* Bioconjugate Chemistry, 2014. **25**(2): p. 351-361.
27. Kanter, G., et al., *Cell-free production of scFv fusion proteins: an efficient approach for personalized lymphoma vaccines.* Blood, 2007. **109**(8): p. 3393-3399.
28. Yan, X. and Z. Xu, *Ribosome-display technology: applications for directed evolution of functional proteins.* Drug discovery today, 2006. **11**(19): p. 911-916.
29. Casteleijn, M.G., A. Urtti, and S. Sarkhel, *Expression without boundaries: Cell-free protein synthesis in pharmaceutical research.* International Journal of Pharmaceutics, 2013. **440**(1): p. 39-47.
30. Panthu, B., et al., *In vitro translation in a hybrid cell free lysate with exogenous cellular ribosomes.* Biochemical Journal, 2015. **467**(3): p. 387-398.
31. Zárate, X., et al., *Development of high-yield autofluorescent protein microarrays using hybrid cell-free expression with combined Escherichia coli S30 and wheat germ extracts.* Proteome Science, 2010. **8**(1): p. 1-15.
32. Yang, J., et al., *Rapid expression of vaccine proteins for B‐cell lymphoma in a cell‐free system.* Biotechnology and bioengineering, 2005. **89**(5): p. 503-511.
33. Buchanan, A., et al., *Improved drug-like properties of therapeutic proteins by directed evolution.* Protein Engineering Design and Selection, 2012: p. gzs054.
34. Barras, D. and C. Widmann, *Promises of Apoptosis-Inducing Peptides in Cancer Therapeutics.* Current Pharmaceutical Biotechnology, 2011. **12**: p. 1153-1165.

المنارة للاستشارات
www.manaraa.com

35. Li, Z.J. and C.H. Cho, *Peptides as targeting probes against tumor vasculature for diagnosis and drug delivery.* J Transl Med, 2012. **10**(Suppl 1): p. S1.
36. Thundimadathil, J., *Cancer treatment using peptides: current therapies and future prospects.* Journal of amino acids, 2012. **2012**.
37. Lee, K.-H., et al., *Ribosomal synthesis and in situ isolation of peptide molecules in a cell-free translation system.* Protein expression and purification, 2010. **71**(1): p. 16-20.
38. Nettles, K.W. and G.L. Greene, *Ligand control of coregulator recruitment to nuclear receptors.* Annu. Rev. Physiol., 2005. **67**: p. 309-333.
39. Bourguet, W., P. Germain, and H. Gronemeyer, *Nuclear receptor ligand-binding domains: three-dimensional structures, molecular interactions and pharmacological implications.* Trends in pharmacological sciences, 2000. **21**(10): p. 381-388.
40. Hopkins, A.L. and C.R. Groom, *The druggable genome.* Nature reviews Drug discovery, 2002. **1**(9): p. 727-730.
41. Collazo, A.-M.G., et al., *Thyroid receptor ligands. Part 5: Novel bicyclic agonist ligands selective for the thyroid hormone receptor β.* Bioorganic & medicinal chemistry letters, 2006. **16**(5): p. 1240-1244.
42. Baxter, J.D. and P. Webb, *Thyroid hormone mimetics: potential applications in atherosclerosis, obesity and type 2 diabetes.* Nature reviews Drug discovery, 2009. **8**(4): p. 308-320.
43. Newbold, R.R., E. Padilla-Banks, and W.N. Jefferson, *Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations.* Endocrinology, 2006. **147**(6): p. s11-s17.
44. Bay, K., et al., *Testicular dysgenesis syndrome: possible role of endocrine disrupters.* Best Practice & Research Clinical Endocrinology & Metabolism, 2006. **20**(1): p. 77-90.
45. Schapaugh, A.W., et al., *Analysis of EPA's endocrine screening battery and recommendations for further review.* Regulatory Toxicology and Pharmacology, 2015. **72**(3): p. 552-561.
46. Fisher, J.S., *Are all EDC effects mediated via steroid hormone receptors?* Toxicology, 2004. **205**(1): p. 33-41.
47. Ohno, K.-i., et al., *Estrogen Receptor Binding Assay Method for Endocrine Disruptors Using Fluorescence Polarization.* Analytical Chemistry, 2002. **74**(17): p. 4391-4396.
48. Zacharewski, T., *Identification and assessment of endocrine disruptors: limitations of in vivo and in vitro assays.* Environmental Health Perspectives, 1998. **106**(Suppl 2): p. 577.
49. Mita, D.G., et al., *Enzymatic determination of BPA by means of tyrosinase immobilized on different carbon carriers.* Biosensors and Bioelectronics, 2007. **23**(1): p. 60-65.
50. Hu, L.-s., et al., *Label-free detection of endocrine disrupting chemicals by integrating a competitive binding assay with a piezoelectric ceramic resonator.* Biosensors and Bioelectronics, 2014. **53**: p. 406-413.
51. Xia, W., et al., *Electrochemical biosensor for estrogenic substance using lipid bilayers modified by Au nanoparticles.* Biosensors and Bioelectronics, 2010. **25**(10): p. 2253-2258.
52. Dempsey, E., D. Diamond, and A. Collier, *Development of a biosensor for endocrine disrupting compounds based on tyrosinase entrapped within a poly(thionine) film.* Biosensors and Bioelectronics, 2004. **20**(2): p. 367-377.
53. Soto, A.M., et al., *The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants.* Environmental health perspectives, 1995. **103**(Suppl 7): p. 113.

المنارة للاستشارات
www.manaraa.com

54. Colborn, T., *Commentary: Setting aside tradition when dealing with endocrine disruptors.* ILAR journal, 2004. **45**(4): p. 394-400.
55. CHOI, K.-C. and E.-B. JEUNG, *The biomarker and endocrine disruptors in mammals.* Journal of Reproduction and Development, 2003. **49**(5): p. 337-345.
56. DeFur, P., *Use and role of invertebrate models in endocrine disruptor research and testing.* ILAR journal/National Research Council, Institute of Laboratory Animal Resources, 2003. **45**(4): p. 484-493.
57. Stokes, W.S., *Selecting appropriate animal models and experimental designs for endocrine disruptor research and testing studies.* ILAR journal, 2004. **45**(4): p. 387-393.
58. Suzuki, S., et al., *Study on Interactions of Endocrine Disruptors with Estrogen Receptor-. BETA. Using Fluorescence Polarization.* Analytical sciences, 2003. **19**(8): p. 1103-1108.
59. Zacharewski, T., *In vitro bioassays for assessing estrogenic substances.* Environmental science & technology, 1997. **31**(3): p. 613-623.
60. Kim, S.H., et al., *A proteomic microarray approach for exploring ligand-initiated nuclear hormone receptor pharmacology, receptor selectivity, and heterodimer functionality.* Molecular & Cellular Proteomics, 2005. **4**(3): p. 267-277.
61. Breinholt, V. and J.C. Larsen, *Detection of weak estrogenic flavonoids using a recombinant yeast strain and a modified MCF7 cell proliferation assay.* Chemical research in toxicology, 1998. **11**(6): p. 622-629.
62. Joyeux, A., et al., *Engineered cell lines as a tool for monitoring biological activity of hormone analogs.* Analytical biochemistry, 1997. **249**(2): p. 119-130.
63. Cho, H., et al., *Optimized clinical performance of growth hormone with an expanded genetic code.* Proceedings of the National Academy of Sciences, 2011. **108**(22): p. 9060-9065.
64. Smith, M.T., A.K. Hawes, and B.C. Bundy, *Reengineering viruses and virus-like particles through chemical functionalization strategies.* Current Opinion in Biotechnology, 2013. **24**(4): p. 620-626.
65. Wu, J.C.Y., et al., *Enhanced enzyme stability through site-directed covalent immobilization.* Journal of biotechnology, 2015. **193**: p. 83-90.
66. Lang, K. and J.W. Chin, *Cellular Incorporation of Unnatural Amino Acids and Bioorthogonal Labeling of Proteins.* Chemical Reviews, 2014. **114**(9): p. 4764-4806.
67. Lajoie, M.J., et al., *Genomically Recoded Organisms Expand Biological Functions.* Science, 2013. **342**(6156): p. 357-360.
68. Crick, F.H.C., et al., *General Nature of the Genetic Code for Proteins.* Nature, 1961. **192**(4809): p. 1227-1232.
69. Wang, L. and P.G. Schultz, *Expanding the genetic code.* Angewandte Chemie International Edition, 2005. **44**(1): p. 34-66.
70. Budisa, N., *Prolegomena to future experimental efforts on genetic code engineering by expanding its amino acid repertoire.* Angewandte Chemie International Edition, 2004. **43**(47): p. 6426-6463.
71. Neumann, H., A.L. Slusarczyk, and J.W. Chin, *De Novo Generation of Mutually Orthogonal Aminoacyl-tRNA Synthetase/tRNA Pairs.* Journal of the American Chemical Society, 2010. **132**(7): p. 2142-2144.
72. Quast, R.B., et al., *Cotranslational incorporation of non-standard amino acids using cell-free protein synthesis.* FEBS Letters, 2015. **589**(15): p. 1703-1712.

المنارة للاستشارات
www.manaraa.com

73. Mukai, T., et al., *Reassignment of a rare sense codon to a non-canonical amino acid in Escherichia coli.* Nucleic Acids Research, 2015.
74. Brocker, M.J., et al., *Recoding the Genetic Code with Selenocysteine.* Angewandte Chemie-International Edition, 2014. **53**(1): p. 319-323.
75. Hong, S.H., et al., *Cell-free Protein Synthesis from a Release Factor 1 Deficient Escherichia coli Activates Efficient and Multiple Site-specific Nonstandard Amino Acid Incorporation.* ACS Synthetic Biology, 2013. **3**(6): p. 398-409.
76. Des Soye, B.J., et al., *Repurposing the translation apparatus for synthetic biology.* Current Opinion in Chemical Biology, 2015. **28**: p. 83-90.
77. Cui, Z., et al., *Semisynthetic tRNA Complement Mediates in Vitro Protein Synthesis.* Journal of the American Chemical Society, 2015. **137**(13): p. 4404-4413.
78. Iwane, Y., et al., *Expanding the amino acid repertoire of ribosomal polypeptide synthesis via the artificial division of codon boxes.* Nature Chemistry, 2016.
79. Goto, Y., T. Katoh, and H. Suga, *Flexizymes for genetic code reprogramming.* Nature protocols, 2011. **6**(6): p. 779-790.
80. Fechter, P., et al., *Ribozyme processed tRNA transcripts with unfriendly internal promoter for T7 RNA polymerase: production and activity.* FEBS letters, 1998. **436**(1): p. 99-103.
81. Smith, M.T., et al., *Alternative fermentation conditions for improved Escherichia coli-based cell-free protein synthesis for proteins requiring supplemental components for proper synthesis.* Process Biochemistry, 2014. **49**(2): p. 217-222.
82. Jewett, M.C. and J.R. Swartz, *Mimicking the Escherichia coli cytoplasmic environment activates long-lived and efficient cell-free protein synthesis.* Biotechnology and Bioengineering, 2004. **86**(1): p. 19-26.
83. Wuu, J.J. and J.R. Swartz, *High yield cell-free production of integral membrane proteins without refolding or detergents.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2008. **1778**(5): p. 1237-1250.
84. Kunda, T., et al., *An Easy Cell-Free Protein Synthesis System Dependent on the Addition of Crude Escherichia coli tRNA.* Journal of Biochemistry, 2000. **127**(1): p. 37-41.
85. Suhasini, A.N. and R. Sirdeshmukh, *Transfer RNA cleavages by onconase reveal unusual cleavage sites.* Journal of Biological Chemistry, 2006. **281**(18): p. 12201-12209.
86. Shrestha, P., M.T. Smith, and B.C. Bundy, *Cell-free unnatural amino acid incorporation with alternative energy systems and linear expression templates.* New Biotechnology, 2014. **31**(1): p. 28-34.
87. Ranji, A., et al., *Chapter 15 - Transforming Synthetic Biology with Cell-Free Systems*, in *Synthetic Biology*, H. Zhao, Editor. 2013, Academic Press: Boston. p. 277-301.
88. Salehi, A.S., et al., *Escherichia coli-based Cell-free Extract Development for Protein-based Cancer Therapeutic Production.* International Journal of Developmental Biology, 2016.
89. Walsh, G., *Biopharmaceutical benchmarks 2014.* Nat Biotech, 2014. **32**(10): p. 992-1000.
90. Lindsley, C.W., *2013 Statistics for Global Prescription Medications: CNS Therapeutics Maintain a Leading Position among Small Molecule Therapeutics.* ACS Chemical Neuroscience, 2014. **5**(4): p. 250-251.
91. Kantardjieff, A. and W. Zhou, *Mammalian Cell Cultures for Biologics Manufacturing*, in *Mammalian Cell Cultures for Biologics Manufacturing*, W. Zhou and A. Kantardjieff, Editors. 2014, Springer Berlin Heidelberg. p. 1-9.

المنارة للاستشارات
www.manaraa.com

92. Fisher, J.A., M.D. Cottingham, and C.A. Kalbaugh, *Peering into the pharmaceutical "pipeline": Investigational drugs, clinical trials, and industry priorities.* Social Science & Medicine, 2015. **131**(0): p. 322-330.
93. Ardelt, W., K. Shogen, and Z. Darzynkiewicz, *Onconase and amphinase, the antitumor ribonucleases from Rana pipiens oocytes.* Current Pharmaceutical Biotechnology, 2008. **9**(3): p. 215-225.
94. Fiorini, C., et al., *Onconase induces autophagy sensitizing pancreatic cancer cells to gemcitabine and activates Akt/mTOR pathway in a ROS-dependent manner.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2015. **1853**(3): p. 549-560.
95. Smolewski, P., Witkowska, M., Zwolinska, M., Cebula-Obrzut, B., Majchrzak, A., Jeske, A., Darzynkiewicz, Z., Ardelt, W., Ardelt, B., Robak, T., *Cytotoxic activity of the amphibian ribonucleases onconase and r-amphinase on tumor cells from B cell lymphoproliferative disorders.* International Journal of Oncology, 2014. **45**(1): p. 419-425.
96. Ardelt, W., S.M. Mikulski, and K. Shogen, *Amino acid sequence of an anti-tumor protein from Rana pipiens oocytes and early embryos. Homology to pancreatic ribonucleases.* Journal of Biological Chemistry, 1991. **266**(1): p. 245-251.
97. Singh, U.P., et al., *Enzymatic and Structural Characterisation of Amphinase, a Novel Cytotoxic Ribonuclease from Rana pipiens Oocytes.* Journal of Molecular Biology, 2007. **371**(1): p. 93-111.
98. Notomista, E., et al., *Effective expression and purification of recombinant onconase, an antitumor protein.* FEBS Letters, 1999. **463**(3): p. 211-215.
99. Zhao, H.L., et al., *Secretory expression of glycosylated and aglycosylated mutein of onconase from Pichia pastoris using different secretion signals and their purification and characterization*. Vol. 9. 2009. 591-599.
100. Schoborg, J.A., et al., *Substrate replenishment and byproduct removal improve yeast cell-free protein synthesis.* Biotechnology Journal, 2014. **9**(5): p. 630-640.
101. Smith, M.T., et al., *The incorporation of the A2 protein to produce novel Q β virus ‐like particles using cell ‐free protein synthesis.* Biotechnology progress, 2012. **28**(2): p. 549-555.
102. Smith, M.T., et al., *Lyophilized Escherichia coli-based cell-free systems for robust, high-density, long-term storage.* BioTechniques, 2014. **56**(4): p. 186-193.
103. Carlson, E.D., et al., *Cell-free protein synthesis: Applications come of age.* Biotechnol Adv, 2012. **30**(5): p. 1185-1194.
104. Sachse, R., et al., *Synthesis of membrane proteins in eukaryotic cell-free systems.* Engineering in Life Sciences, 2013. **13**(1): p. 39-48.
105. Caschera, F. and V. Noireaux, *Synthesis of 2.3 mg/ml of protein with an all Escherichia coli cell-free transcription–translation system.* Biochimie, 2014. **99**(0): p. 162-168.
106. Boyer, M.E., et al., *Cell-free synthesis and maturation of [FeFe] hydrogenases.* Biotechnology and Bioengineering, 2008. **99**(1): p. 59-67.
107. Yang, J., et al., *Rapid expression of vaccine proteins for B-cell lymphoma in a cell-free system.* Biotechnol Bioeng, 2005. **89**(5): p. 503-511.
108. Park, C.G., et al., *Cell-free synthesis and multifold screening of Candida antarctica lipase B (CalB) variants after combinatorial mutagenesis of hot spots.* Biotechnol Prog, 2011. **27**(1): p. 47-53.

المنارة للاستشارات
www.manaraa.com

109. Smith, M.T., et al., *Alternative fermentation conditions for improved Escherichia coli-based cell-free protein synthesis for proteins requiring supplemental components for proper synthesis.* Process Biochemistry, 2013.
110. Leland, P.A., et al., *Ribonuclease A variants with potent cytotoxic activity.* Proceedings of the National Academy of Sciences, 1998. **95**(18): p. 10407-10412.
111. Lou, P.-J., et al., *Reversal of doxorubicin resistance in breast cancer cells by photochemical internalization.* International Journal of Cancer, 2006. **119**(11): p. 2692-2698.
112. Baneyx, F., *Recombinant protein expression in Escherichia coli.* Current opinion in biotechnology, 1999. **10**(5): p. 411-421.
113. Saxena, S.K., et al., *Entry into Cells and Selective Degradation of tRNAs by a Cytotoxic Member of the RNase A Family.* Journal of Biological Chemistry, 2002. **277**(17): p. 15142-15146.
114. Turcotte, R.F. and R.T. Raines, *Interaction of onconase with the human ribonuclease inhibitor protein.* Biochemical and Biophysical Research Communications, 2008. **377**(2): p. 512-514.
115. Leland, P.A. and R.T. Raines, *Cancer chemotherapy – ribonucleases to the rescue.* Chemistry & Biology, 2001. **8**(5): p. 405-413.
116. Kyner, D., P. Zabos, and D.H. Levin, *Inhibition of protein chain initiation in eukaryotes by deacylated transfer RNA and its reversibility by spermine.* Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis, 1973. **324**(3): p. 386-396.
117. Zawada, J.F., et al., *Microscale to manufacturing scale-up of cell-free cytokine production—a new approach for shortening protein production development timelines.* Biotechnol Bioeng, 2011. **108**(7): p. 1570-1578.
118. Castro, J., et al., *A cytotoxic ribonuclease reduces the expression level of P-glycoprotein in multidrug-resistant cell lines.* Investigational new drugs, 2012. **30**(3): p. 880-888.
119. Salehi, A.S.M., et al., *Cell-Free Protein Synthesis Approach to Biosensing hTRβ-Specific Endocrine Disruptors.* Analytical Chemistry, 2017.
120. Chen, T., et al., *Coactivators in assay design for nuclear hormone receptor drug discovery.* Assay and drug development technologies, 2003. **1**(6): p. 835-842.
121. Vamvakaki, V., K. Tsagaraki, and N. Chaniotakis, *Carbon Nanofiber-Based Glucose Biosensor.* Analytical Chemistry, 2006. **78**(15): p. 5538-5542.
122. Bahadır, E.B. and M.K. Sezgintürk, *Applications of commercial biosensors in clinical, food, environmental, and biothreat/biowarfare analyses.* Analytical Biochemistry, 2015. **478**: p. 107-120.
123. Ziegler, C., *Cell-based biosensors.* Fresenius' journal of analytical chemistry, 2000. **366**(6-7): p. 552-559.
124. Liu, Q., et al., *Cell-based biosensors and their application in biomedicine.* Chemical reviews, 2014. **114**(12): p. 6423-6461.
125. Skretas, G. and D.W. Wood, *A Bacterial Biosensor of Endocrine Modulators.* Journal of Molecular Biology, 2005. **349**(3): p. 464-474.
126. Skretas, G., et al., *Engineered Chimeric Enzymes as Tools for Drug Discovery: Generating Reliable Bacterial Screens for the Detection, Discovery, and Assessment of Estrogen Receptor Modulators.* Journal of the American Chemical Society, 2007. **129**(27): p. 8443-8457.

المنارة للاستشارات
www.manaraa.com

127. Gierach, I., et al., *Bacterial biosensors for screening isoform-selective ligands for human thyroid receptors α-1 and β-1.* FEBS open bio, 2012. **2**: p. 247-253.
128. Gierach, I., et al., *Bacterial biosensors for evaluating potential impacts of estrogenic endocrine disrupting compounds in multiple species.* Environmental toxicology, 2013. **28**(4): p. 179-189.
129. Shi, C., Q. Meng, and D.W. Wood, *Analysis of the roles of mutations in thyroid hormone receptor-β by a bacterial biosensor system.* Journal of Molecular Endocrinology, 2014. **52**(1): p. 55-66.
130. Charles, G.D., *In vitro models in endocrine disruptor screening.* Ilar Journal, 2004. **45**(4): p. 494-501.
131. Gray, L., et al., *Use of the laboratory rat as a model in endocrine disruptor screening and testing.* ILAR journal, 2004. **45**(4): p. 425-437.
132. Pardee, K., et al., *Paper-Based Synthetic Gene Networks.* Cell, 2014. **159**(4): p. 940-954.
133. Schinn, S.-M., et al., *Protein synthesis directly from PCR: progress and applications of cell-free protein synthesis with linear DNA.* New Biotechnology, 2016. **33**(4): p. 480-487.
134. Banki, M.R., L. Feng, and D.W. Wood, *Simple bioseparations using self-cleaving elastin-like polypeptide tags.* Nat Methods, 2005. **2**(9): p. 659-61.
135. Shrestha, P., T.M. Holland, and B.C. Bundy, *Streamlined extract preparation for Escherichia coli-based cell-free protein synthesis by sonication or bead vortex mixing.* BioTechniques, 2012. **53**(3): p. 12.
136. Smith, M.T., et al., *Creating a completely "cell‐free" system for protein synthesis.* Biotechnology progress, 2015. **31**(6): p. 1716-1719.
137. Bourgeois, S., et al., *Evaluation of critical formulation parameters influencing the bioactivity of β-lactamases entrapped in pectin beads.* International journal of pharmaceutics, 2006. **324**(1): p. 2-9.
138. O'Connell, M.A., B.A. Belanger, and P.D. Haaland, *Calibration and assay development using the four-parameter logistic model.* Chemometrics and Intelligent Laboratory Systems, 1993. **20**(2): p. 97-114.
139. Zhang, J.-H., T.D. Chung, and K.R. Oldenburg, *A simple statistical parameter for use in evaluation and validation of high throughput screening assays.* Journal of biomolecular screening, 1999. **4**(2): p. 67-73.
140. Vareiro, M.M.L.M., et al., *Surface Plasmon Fluorescence Measurements of Human Chorionic Gonadotrophin: Role of Antibody Orientation in Obtaining Enhanced Sensitivity and Limit of Detection.* Analytical Chemistry, 2005. **77**(8): p. 2426-2431.
141. Skretas, G. and D.W. Wood, *Regulation of protein activity with small‐molecule‐controlled inteins.* Protein Science, 2005. **14**(2): p. 523-532.
142. Martínez, L., et al., *Gaining ligand selectivity in thyroid hormone receptors via entropy.* Proceedings of the National Academy of Sciences, 2009. **106**(49): p. 20717-20722.
143. Takeda, T., et al., *Triiodothyroacetic acid has unique potential for therapy of resistance to thyroid hormone.* The Journal of Clinical Endocrinology & Metabolism, 1995. **80**(7): p. 2033-2040.
144. Marchesini, G.R., et al., *Biosensor Recognition of Thyroid-Disrupting Chemicals Using Transport Proteins.* Analytical Chemistry, 2006. **78**(4): p. 1107-1114.
145. Borgert, C.J., et al., *Evaluation of EPA's Tier 1 Endocrine Screening Battery and recommendations for improving the interpretation of screening results.* Regulatory Toxicology and Pharmacology, 2011. **59**(3): p. 397-411.

المنارة للاستشارات
www.manaraa.com

146. Gierach, I. and D.W. Wood, *Engineered Nuclear Hormone Receptor-Biosensors for Environmental Monitoring and Early Drug Discovery*, in *Biosensors for Health, Environment and Biosecurity*, P.A. Serra, Editor. 2011, InTech.
147. Salehi, A.S.M., et al., *Cell-free protein synthesis of a cytotoxic cancer therapeutic: Onconase production and a just-add-water cell-free system.* Biotechnology Journal, 2015: p. n/a-n/a.
148. Ipe, D.S., E. Horton, and G.C. Ulett, *The basics of bacteriuria: Strategies of microbes for persistence in urine.* Frontiers in cellular and infection microbiology, 2016. **6**.

المنارة للاستشارات
www.manaraa.com