Developing New Modalities for Biosensing using Synthetic Biology

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ABSTRACT

Biosensors are devices that use biological components to detect important analytes. Biosensing systems have various applications in areas such as medicine, environmental monitoring, and process control. Classical biosensors are often based on bacteria or purified enzymes that have limitations on efficiency or stability. I have developed several new biosensors to overcome these disadvantages. Two preliminary biosensors were first created based on the extremely strong and specific interaction between biotin and (strept)avidin. Both biosensors showed high sensitivity and reliability for measuring biotin with detection limits of 50-1000 pg/ml and 20-100 ng/ml, respectively. Following these, a new biosensor was developed by coupling a mobile, functionalized microsurface with cell-free expression approaches. This biosensor demonstrated a dynamic range of 1-100 ng/ml. In addition, I also explored the possibility of combining these biosensing systems with engineered living cells. By leveraging the tools of synthetic biology, a genetic circuit was designed, constructed, and inserted into bacteria for enhanced biotin biosynthesis *in vivo*. Upon induction, a 17-fold increase in biotin production was measured in the engineered cells in comparison to wild type cells using the biosensors created herein. These new biosensors, particularly the mobile biosensing modality, form a building block for advanced biosensing and drug delivery systems due to enhancements in mobility and specificity. In the future, these biosensing and cellular production systems could impact a range of fields ranging from biomedicine to environmental monitoring.

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List of Abbreviations

MW: Molecular weight

ATP: Adenosine triphosphate

°C: Degree Celsius

µg: Micrograms

ml: Milliliter

L: Liter

DNA: Deoxyribonucleic acid

E. coli*: Escherichia coli*

B. sphaericus*: Bacillus sphaericus*

GFP: Green Fluorescent Protein

h: Hours

bps: Base pairs

IPTG: Isopropylthio-β-galactoside

ng: Nanogram

µg: Microgram

OD: Optical density

PCR: Polymerase Chain Reaction

RBS: Ribosome Binding Site

pI: Isoelectric point

HABA: 4'-hydroxyazobenzene-2-carboxylic acid

SPDP: Succinimidyl 3-(2-pyridyldithio) propionate

SMCC: Succinimidyl trans-4-(maleimidylmethyl) cyclohexane-1-Carboxylate

MWCO: Molecular weight cut-off

NHS: N-Hydroxysuccinimide

TMB: 3,3',5,5'-tetramethylbenzidine

HRP: Horseradish peroxidase

PBS: Phosphate buffered saline

ELISA: Enzyme-linked immunosorbent assay

HPLC: High Performance Liquid Chromatography

BSA: Bovine serum albumin

DMSO: Dimethyl sulfoxide

DTT: Dithiothreitol

b: Biotin

DTB: Desthiobiotin

SA: Streptavidin

Bb: BSA-biotin

SH: Streptavidin-HRP

bSH: Biotin-Streptavidin-HRP

BbSH: BSA-biotin-Streptavidin-HRP

ka: Kinetic constant of association

kd: Kinetic constant of dissociation

M: Mole/L

mM: Millimole/L

ODmax: Maximum optical density

ACC: Acetyl coenzyme (acetyl-CoA) carboxylase

OE-PCR: Overlap-extension PCR

SOEing: Splicing by Overlap Extension

MCS: Multi cloning sites

rpm: Revolutions per minute

LB: Luria-Bertani media

WT: Wild type

CML: Carboxylate modified

EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

PDMS: Polydimethylsiloxane

ms: Millisecond

ROI: Region of interest

DI: Deionized water

EDTA: Ethylenediaminetetraacetic acid

RT: Room temperature

Chapter 1

Introduction

1.1 Background and motivation

The ability to sense is ubiquitous in nature, from human sensing of gravity and vibration[1,2] to birds that sense magnetic fields[3]. In addition to these physical aspects, living cells have the ability to sense environmental and internal signals via chemical or biochemical molecules. By understanding these mechanisms, various sensor types have been developed[4,5,6]. Among them, biosensors are relatively new but promising[7].

A biosensor is a device that uses specific biochemical reactions, mediated by isolated biological components, to detect physicochemical compounds usually by electrical, thermal or optical signals[8]. Biosensors have been applied in the fields of medicine, food process control, environmental monitoring, as well as in defense and security[7]. The biological components in biosensors are typically enzymes, immune system components, tissues, organelles or whole cells. Enzymes are the most popular biological elements due to their high specificity and sensitivity[9]. However, they are relatively expensive to purify and difficult to maintain activity over a long period of time[10]. Another major biosensor group is comprised of microbial-based biosensors. Microbial biosensors (i.e., whole-cell biosensors) are easy to produce and manipulate, but have limitations since the entrapment of the cells causes additional diffusion resistance that damages sensitivity[11]. In these systems, a single strain of microorganism can only typically only respond to a specific

signal. To overcome these limitations, synergetic microbial communities have been applied[12].

However, the sensitivity and reliability of whole-cell biosensors are often unable to meet sensing needs[11]. Thus, specific and sensitive detection mechanisms are required. The biotin-(strept)avidin interaction system, for example, could be used to create biosensors because of its strong, non-covalent binding. Synthetic biology, in addition, has been utilized for rationally redesigning of biological systems. It also implements various engineering principles and technologies, such as computational modeling and microfluidics, for analysis and fabrication of the biological systems. Based on the above, I am interested in creating new biosensors that could be applied in various fields such as medicine, environmental monitoring and industrial process control. I am also interested exploring the possibility of using synthetic biology to develop more complex and advantageous biosensors, in comparison to the current technologies, in order to have better robustness, specificity and tunability.

1.2 Research Objectives

In this work, multiple modalities for biosensing were explored, developed and implemented. The specific objectives were:

(1) Developing, modeling and evaluating preliminary biotin sensors.

(2) Engineering and measuring the biosynthesis of biotin in *E. coli*.

(3) Creating a new biosensor design based on cell-free gene expression and exploring its applications.

1.3 Thesis organization

The thesis content is divided into six chapters. Chapter 1 contains basic background, motivation and objectives of the research. Chapter 2 describes current concepts and technologies that will be covered in this work in detail. Chapter 3 contains the methods and results of developing two preliminary biotin biosensors. Chapter 4 shows the results of the genetic circuit design and bacteria engineering for the biosynthesis of biotin. Chapter 5 describes the process and results of innovating a new biosensor. Chapter 6 contains the conclusion of current achievements and discussions about future work.

Chapter 2

Literature Review

2.1 Biotin, avidin and their interaction

Biotin (Fig. 1) is a small molecule (MW: 244.31 g/mol) composed of an ureido ring and a tetrahydrothiophene ring with a carboxyl substituent[13]. Although the required biotin level for most living organisms is relatively low[14], it is an essential molecule for cell functions. At the individual level, biotin is often recommended as a dietary supplement for strengthening hair, nails and skin[15]. Mammals obtain biotin mostly from many types of food, but also partly from intestinal bacteria synthesis[16]. This molecule acts as a CO_2 carrier (cofactor) between bicarbonate (HCO^{3-}) and an acceptor substrate by non-covalently binding to its partner enzymes[17] . When an energy source is available, N-carboxyl biotin is formed with（HCO^{3-}) and then serves to carry activated CO_2 to the substrates[18]. This type of biochemical reaction is essential in metabolic processes, especially during fatty acid biosynthesis and gluconeogenesis, as well as during secondary metabolism. In addition to its effects on metabolism, biotin is well known for its extremely strong interaction with avidin proteins[19].

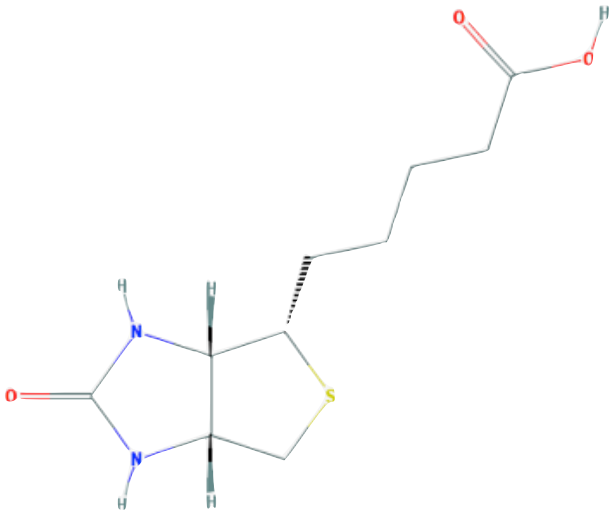

Figure 1: The structure of biotin. Biotin is composed of an ureido ring and a tetrahydrothiophene ring with a carboxyl substituent. The image above was obtained from pubchem[20]. National Center for Biotechnology Information. Pubchem Compound Database. http://pubchem.ncbi.nlm.nih.gov/compound/171548 (accessed May 15, 2015). Used under fair use, 2015.

Avidin is a 66,000 Dalton homo-tetrameric glycoprotein produced in the oviducts of birds, reptiles and amphibians, especially in egg-white[21,22]. Each of the subunits of avidin can bind to one biotin molecule with high affinity and specificity[19]. The biological functions of avidin are not completely understood, and possibly include antibiotic effects to prohibit bacterial growth, especially by its analog, streptavidin[23]. A well-established fact, however, is that the biotin-(strept)avidin interaction is one of the strongest non-covalent binding events found in nature[24] with an equilibrium dissociation constant around 10^{-15} M^{-1}. The tetrameric avidin is highly basic but extremely stable even under very harsh conditions. Moreover, avidin exhibits even higher stability when it is conjugated with biotin[25,26].

Streptavidin is also a biotin-binding protein and is produced by *Streptomyces avidinii*[27,28].

Streptavidin is almost identical to avidin structurally, merely lacking the sugar chain possessed by avidin. Thus, streptavidin potentially would be able to minimize the non-specificity issues since it does not bind to the carbohydrate receptors. In addition, the variations in its amino acid sequence from avidin result in a much lower pI (5 - 6)[29]. Therefore, streptavidin has better signal-to-noise ratios and would be better for conducting immunoassays. Fig. 2 shows the binding mechanism of the streptavidin-biotin interaction at one of streptavidin's active sites[30]. However, since biotin can be synthesized by a variety of organisms, such as plants, bacteria and some fungi[14], there are also analogs of biotin that can bind to avidin or streptavidin as well. One of these analogs, desthiobiotin, is the precursor of biotin synthesis in *E. coli*, and results in a dissociation constant only one to two orders of magnitudes lower than biotin's interaction with avidin[31]. Thus, specificity of biotin-(strept)avidin interaction-based biosensors could be potentially affected by the presence of desthiobiotin.

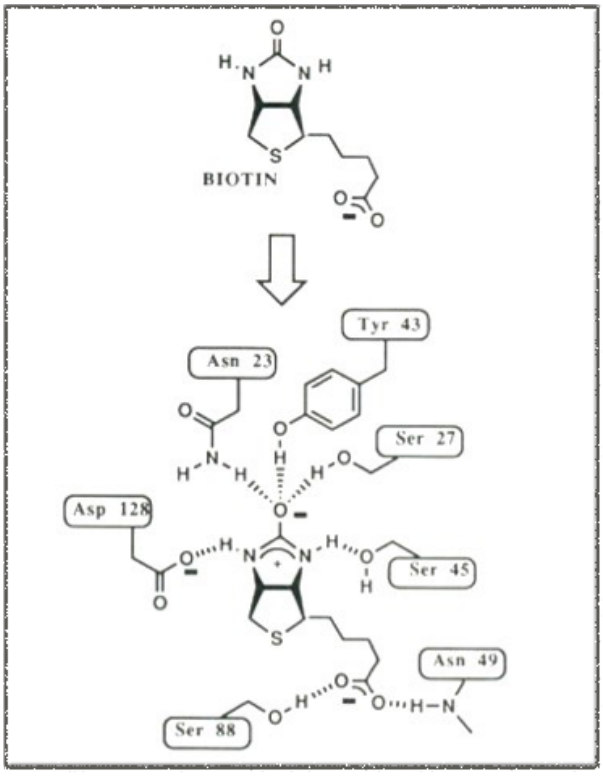

Figure 2: Binding mechanism of biotin and streptavidin. The figure shows the active site of one streptavidin subunit. Biotin is attached tightly into the active site via non-covalent binding. The image of the binding structure was obtained from the literature[23]. Hendrickson, W. A.; Pähler, A.; Smith, J. L.; Satow, Y.; Merritt, E. A.; Phizackerley, R. P. Crystal Structure of Core Streptavidin Determined from Multiwavelength Anomalous Diffraction of Synchrotron Radiation. *Proceedings of the National Academy of Sciences* **1989,** *86* (7), 2190–2194. Used under fair use, 2015.

Due to the high affinity and stability, the biotin-(strept)avidin interaction has been widely applied in many available biotechnologies including non-isotopic immunoassay, acid hybridization, affinity chromatography, labeling techniques and DNA probes[29]. However, the disadvantage of avidin in those applications is the non-specificity that results from the existence of the sugar residues as well as the high isoelectric point (~10)[32,33]. Thus, streptavidin has become popular as an alternative substitute of avidin, despite the extra step of immobilization it requires in experimental processes[34]. One major application of the interaction system is the biotin immunoassay. Specifically, biotin immunoassays have

two types: immobilized or biotinylated. This is because both antibodies and antigens can be easily immobilized or biotinylated. These immunoassay designs potentially provide high sensitivity because up to 100-fold of amplification is introduced by the binding of 10-20 biotin molecules per antibody or 3 to 6 enzyme molecules per streptavidin, respectively[29]. The most popular enzymes for immunoassay conjugations are horseradish peroxide and alkaline phosphatase, because they are able to react with substrates to yield chromatic products. Other applications include nucleic acid hybridization assays. In addition to antibody biotinylation for Western blotting, biotinylated probes for Southern and Northern blotting have been developed to replace isotopic labeling since biotin can be incorporated easily with nucleic acids. Along this line, biotinylated primers have also been used for DNA sequencing[35]. Moreover, other applications take advantage of this interaction system to produce hybridoma cells by attaching biotin on the cell surface to mediate cell fusion, or to design drug targeting and oral delivery systems[36,37,38].

2.2 Biotin synthesis and metabolic regulations

As previously discussed, biotin is essential for living cells as it acts as a cofactor of enzymes in carboxylation reactions. Biotin deficiency could cause metabolic issues by affecting the biotin-dependent carboxylases[39]. Thus, industrial synthesis of biotin using chemical approaches has been developed to make it commercially available. The first total synthesis of biotin from L-cysteine occurred in 1942 by the Merck Research Laboratories[40]. Over the next 50 years, a variety of synthesis sequences have been developed from various substrates[41]. However, most of them take over 10 steps from substrate to product no matter which substrates are used. The chemical synthesis of biotin

dominated the early years of biotin production, until the rise of biosynthesis, when the advances in microbiological engineering started to take the lead in biotin manufacturing.

Biotin can be produced naturally by some species of bacteria and plants, as well as in a few fungi[14]. In this study, we performed genetic modifications on *E. coli* strains due to their fast reproduction rates and well-studied genomic background. In the wild type *E. coli*, the biotin production is mainly depended on the *bio* operon, which consists of two divergent parts the *bioA* operon and the *bioBFCD operon* that are negatively regulated through a common operator[42,43,44]. The complete metabolic pathway of biotin synthesis in *E. coli* is demonstrated in Fig. 3, which has been well studied[45]. In addition, it has been reported that the conversion from pimeloyl-CoA to 7-keto-8-pelargonic acid was the limiting step for biotin production[46].

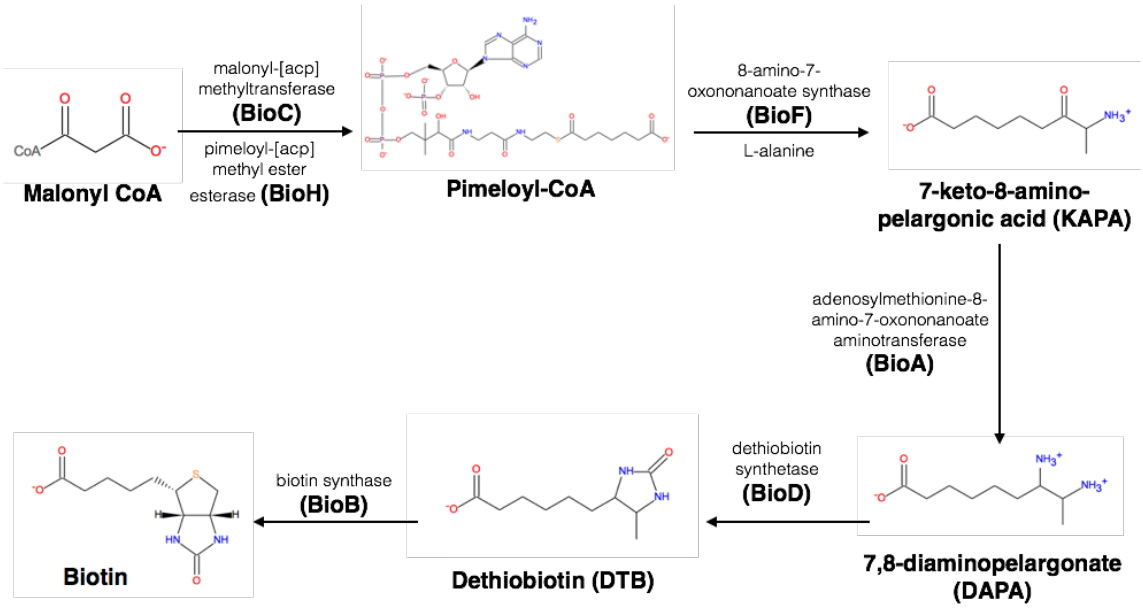

Figure 3: The metabolic pathway of biotin synthesis in *E. coli*. The biotin expression pathway in *E. coli* starts from malonyl-CoA and produces pimeloyl-CoA by use of the methyltransferase and esterase encoded by *bioC* and *bioH*, respectively. Pimeloyl-CoA can be converted to KAPA by the *bioF* gene encoded oxononanoate synthase. KAPA serves as the substrate for DAPA generation by the aminotransferase encoded by *bioA*. DAPA can be transformed into desthiobiotin (DTB) with *bioD*, a DTB synthase. Finally, DTB is the precursor of biotin and could be used for biotin synthesis. Images of the structures were obtained from ecocyc.org [47]. Keseler, I. M.; Mackie, A.; Peralta-Gil, M.; Santos-Zavaleta, A.; Gama-Castro, S.; Bonavides-Martinez, C.; Fulcher, C.; Huerta, A. M.; Kothari, A.; Krummenacker, M.; Latendresse, M.; Muniz-Rascado, L.; Ong, Q.; Paley, S.; Schroder, I.; Shearer, A. G.; Subhraveti, P.; Travers, M.; Weerasinghe, D.; Weiss, V.; Collado-Vides, J.; Gunsalus, R. P.; Paulsen, I.; Karp, P. D. Ecocyc: Fusing Model Organism Databases with Systems Biology. Nucleic Acids Res 2013, 41 (Database issue), D605-12. Used under fair use, 2015.

As a part of the metabolic network in living cells, the biotin synthesis pathway is regulated in multiple ways. As Fig. 4 shows, the biotin ligase BirA also recognizes biotin and covalently bonds itself to biotin to form a repressor complex that inactivates upstream biotin genes expression[48]. Additionally, the subunits of the acetyl coenzyme (acetyl-CoA) carboxylase (ACC) exhibit secondary repression over the BirA-biotin repressor[49]. All of those natural genetic regulations can potentially be engineered (weakened or strengthened) to achieve a higher biotin production *in-vivo*.

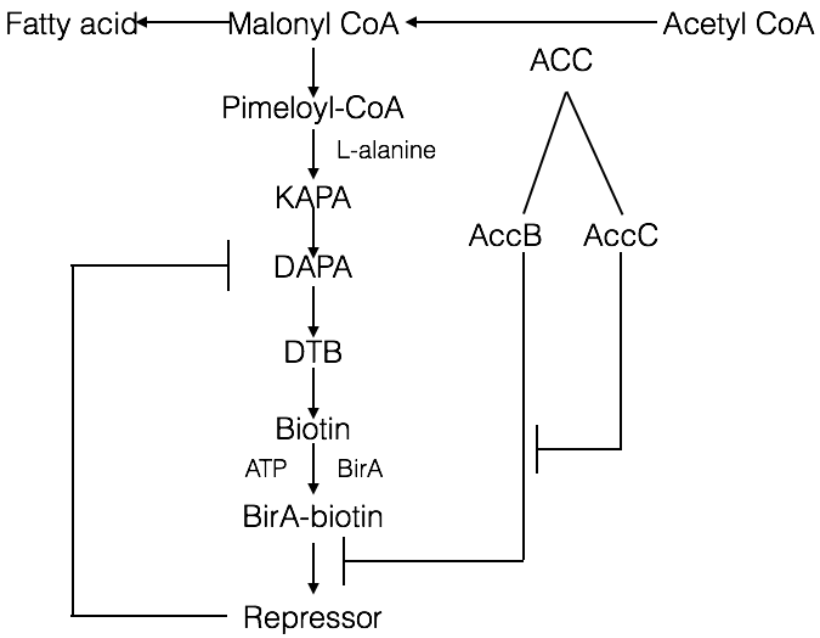

Figure 4: Biotin synthesis and regulations in *E. coli*. The precursor of biotin biosynthesis in *E. coli* is an intermediate of fatty acid synthesis, acetyl-CoA. Biotin expression is repressed by the complex biotin-BirA (biotin-biotin ligase). The subunits of the ACC enzyme also have sequential repressing functions on the BirA repressor.

2.3 Biotin assays

Due to the biotin uptake requirements, as well as the study of the biotin-(strept)avidin interaction system, several biotin measurement methods have been explored. Among these, the microbiological method is one of the first developed, is still widely used, and is relatively sensitive (10^{-9} g l^{-1})[50]. Many microorganisms have been used in this assay, such as *Lactobacillus plantarum*, *Lactobacillus casei*, *Saccharomyces cerevisiae*, *Rhizobium trifolii* and *Escherichia coli*. These experiments are based on the principle that the growth of many microorganisms depends on the presence of biotin supplementation in culture media[51]. However, the microbial assays are not always very specific since other nutrients, e.g., fatty acids, may also interfere with growth. In addition, these assays are potentially

time-consuming.

Another biotin detection method is the bioassay. One method of performing a bioassay is similarly based on cell growth. Additionally, biotin concentrations can be determined indirectly by measuring its biological function such as by assessing the activity of a biotin–dependent enzyme[51,52]. It is obvious that these approaches, if implemented to be dependent on animal growth, would take even longer than the microbial methods. They are not as sensitive as the others, and thus are limited to specific applications. Physicochemical methods have also been applied for biotin detection. However, due to their limited sensitivity, these methods can only measure biotin at high concentrations.

In contrast, biotin assays based on immunoassay binding have many advantages such as a fast operating time and high sensitivity. Specifically, the most used binding proteins are of the avidin family. In general, biotin assays are highly sensitive and specific although it is challenging to discriminate between biotin and biotin metabolites. This shortage could be compensated by the efficiency for high throughput assay compared to the more specific assays using HPLC methods[53]. Additionally, only some of the biotin analogs share the high affinity for avidin proteins that biotin has.

2.4 Synthetic biology

Synthetic biology is a relatively new field of research. The goals of synthetic biology are to understand and redesign biological systems, taking advantage of engineering and computational principles.

The early attempts at redesigning biological systems included construction of a genetic toggle switch[54] and an oscillator[55]. By applying the paradigm of electronic circuits, researchers have been working on building standardized parts that could be readily used and recombined to achieve different genetic functions. Among the first creations, BioBricks[56] were developed via a restriction enzyme-based cloning strategy. The Gibson assembly technique[57] has also been created and commercialized for efficient cloning applications. Moreover, a plug-and-play methodology has been designed that emphasizes post-assembly modification of genetic constructs to obtain high tunability[58].

To better understand living cells, it is critical to minimize the unknown components while still ensuring their function. Therefore, two conceptual approaches have been generated: "top down" and "bottom up." Minimal cells and the cell-free expression systems have been developed and are motivated by these concepts. The top-down approach refers to removing as many genes as possible from a living organism without compromising cell function in order to create a "minimal cell" and as many unrelated components as possible for lysate-based cell-free systems[59,60]. Alternatively, the bottom-up approach focuses on synthesizing new organisms and developing pure cell-free expression systems[61]. Cell-free expression is a rising area within synthetic biology since it allows high- throughput expression[62]. The expression is also well controlled and specific to designated, desirable genetic templates. Moreover, cell-free systems offer flexible reaction conditions to produce "difficult" proteins that are hard to express in living cells[63]. So far, synthetic biology has been successfully applied in various areas, from metabolic

engineering of biofuels to therapeutic treatments and medicine synthesis[64,65]. Thus, although there are still limitations associated with traditional biosensors, an advanced biosensor could be built by leveraging our knowledge of synthetic biology and molecular interactions.

In this work, the knowledge and techniques mentioned above have been applied to create new biosensor modalities. Focusing on the use of the biotin-(strept)avidin interaction, and with the aid of synthetic biology approaches and immunoassay concepts, multiple biosensors have been developed and evaluated.

Chapter 3

Construction of the Preliminary Biosensors

3.1 Background

The interaction between biotin and the avidin protein family is extremely specific and strong in nature[24]. It has been used in many analytical techniques and could be applicable to this study. Since one specific objective of my study focuses on engineering bacteria to synthesize biotin, it is critical to have a reliable assay that could measure differing biotin content in cell culture samples. Wild type *E. coli* strains produce around 1 ng/ml biotin naturally[66]. Although several biotin assays are already commercially available, their dynamic ranges are usually much higher than the desired level of biotin synthesis in *E. coli*. The Pierce™ Biotin Quantitation Kit, for instance, which is based on the competitive binding of biotin and its analog 4'-hydroxyazobenzene-2-carboxylic acid (HABA) to streptavidin (Fig. 5)[30], had detection limits of around 5 ug/ml as tested it in the laboratory. Thus, it was necessary to develop sensitive biotin biosensors to meet the requirements. More importantly, the construction of these biotin sensors would also be a preliminary step towards advanced biosensing designs.

Figure 5: HABA biotin detection method. The Pierce™ Quantification Kit is based on the competitive binding of HABA and biotin to streptavidin. The detection limit remained at the microgram per ml level. The image was reproduced using information from Pierce[67]. Life Technologies. Pierce™ Biotin Quantitation Kit. https://http://www.lifetechnologies.com/order/catalog/product/28005 (accessed 15 May, 2015). Used under fair use, 2015.

In the preliminary sensors, the adsorption of protein on a polystyrene substrate was a fundamental first step towards biosensor development[68]. To functionalize the competitive binding schemes in this study, two conjugation mechanisms were required. Fig. 6 shows diagrams of the chemical reactions. As Fig. 6a indicates, crosslinkers activated by N-hydroxysuccinimide (NHS) esters, which are formed by the activation of carbodiimides, could react with primary amines groups of proteins in conditions of pH 7.2 to 9 to form stable amide bonds. Due to the high versatility, most of the conjugation reactions were conducted based on this chemical scheme, since both the crosslinkers and the biotinylation molecule possessed NHS ester tails (NHS-LC-LC-biotin, SPDP or SMCC), while proteins have primary amine groups on their surface. Those crosslinkers would attach to each other via the reactions of thiol containing molecules with maleimides to produce a thioether linkage, in ambient pH (6.5 ~ 7.5) conditions, as showed in Fig. 6b.

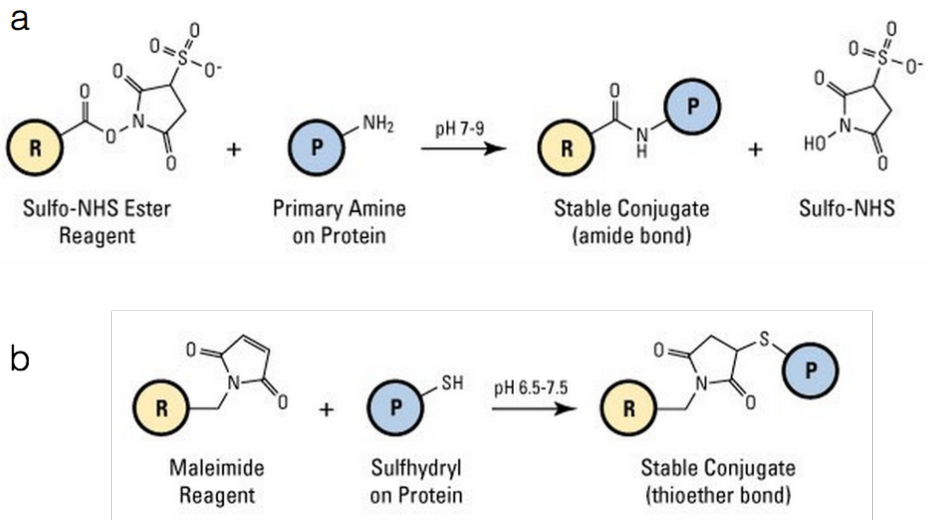

Figure 6: Conjugation mechanisms. (a) Reagents with NHS ester residues react with primary amines on proteins surface, (b) Maleimides react with sulfhydryl residues on the protein surface. Images were obtained from Pierce[69,70].
Life Technologies. Sulfhydryl-Reactive Crosslinker Chemistry. https://http://www.lifetechnologies.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/sulfhydryl-reactive-crosslinker-chemistry.html (accessed 15 May, 2015).
Life Technologies. Amine-Reactive Crosslinker Chemistry. https://http://www.lifetechnologies.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/amine-reactive-crosslinker-chemistry.html (accessed 15 May, 2015). Used under fair use, 2015.

3.2 Materials and methods

3.2.1 Materials

Streptavidin was used as the binding protein to develop the preliminary biosensors. The crosslinkers were succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and succinimidyl trans-4-(maleimidylmethyl) cyclohexane-1-Carboxylate (SMCC) and the biotinylation reagent was EZ-Link™ NHS-LC-LC-biotin. All the conjugation materials were purchased from Life Technologies. Concentrator columns with the molecular weight cut-off (MWCO) membrane of 5000 were purchased from Corning, Inc. and used for purification of the conjugates from unbound crosslinkers. Casein was used as a blocking

reagent. Sodium acetate was used to buffer a solution of 3,3',5,5'-tetramethylbenzidine (TMB), the substrate of horseradish peroxidase (HRP). Bovine serum albumin (BSA) was used as the adsorption protein. The pH condition of the sodium acetate solution was adjusted to 5.1 with NaOH and HCl before reaction. H_2SO_4 was the reaction termination reagent. Phosphate buffered saline (PBS) solution was supplemented with 0.01% Tween 80 to ensure efficient washing of the assays and to reduce non-specific binding. High coverage capacity well strips (Immulon™ 4 HBX, Thermo Fisher Scientific Inc.) were assembled on a 96 well plate frame. After the reactions, the results were read in a microplate reader (Synergy™ HT, Biotek, Inc.).

3.2.2 Complex preparation

The conjugation mechanisms were illustrated above. The NHS-LC-LC-biotin allowed convenient biotinylation of BSA or HRP simply by mixing the protein solutions with 20-fold more moles of NHS-LC-LC-biotin and incubating for 1.5 h at room temperature. Then, the conjugates were transferred into the concentrator tubes, centrifuged for 12 min at 9000 g, or when the volume reached 100 µl, then resuspended with buffer to 500 µl. The washing procedures were repeated 5 times.

The conjugation of streptavidin and HRP needed additional processes since neither of them had crosslinkers originally on the protein surface. Therefore, I applied crosslinkers SMCC and SPDP to each, respectively. Both crosslinkers were dissolved in DMSO to make a stock of 20 mg/ml. Proteins were prepared as 10 mg/ml stock solutions. For streptavidin, 1.4 µl of SPDP stock were added to 20 µl of streptavidin stock, incubated at

37 °C for 1 h while avoiding light. Then, 2.4 µl of 100 mM DTT solution was added and allowed to incubate again for 1 h. For HRP, 7.5 µl SMCC stock and 72 µl HRP stock (1 : 20 in molar ratio), was incubated at 37 °C for 1 h while avoiding light. Then, the solutions (SA-SPDP and HRP-SMCC) were transferred into concentrator tubes, concentrated, and washed 5 times to wash out the unconjugated crosslinkers. After the washing steps, 25 µl of SA-SPDP and HRP-SMCC were mixed together (molar ratio of SA : HRP = 5 : 1) to a total volume of 50 µl, reacted overnight to conjugate, and then aliquotted and stored at 4 °C.

3.2.3 Competitive binding procedures

The procedures to measure biotin with both preliminary biosensors were similar. The complete protocol is provided in Appendix A. A concentrated PBS-casein solution (0.25 g / 40 ml) was prepared first using a magnetic mixer with stirring. While the casein dissolved, the adsorption proteins (BSA-biotin or SA) were diluted with PBS. 100 µl of the dissolved solution was then added to the wells of the assay, and incubated at 37 °C for 1 h, covered with a plastic film. The wells were then washed 3 times with 200 ul PBS-Tween 80. After the immobilization, the wells were blocked with 200 µl PBS-casein at 37 °C for 1 h. After blocking, 20 µl of either samples containing known biotin or DTB content for use in creating calibration curves, or samples containing unknown biotin content, were added to the wells and then 80 µl of the conjugates (SA-HRP or biotin-HRP) were added and co-incubated at 37 °C for 1 h. For the chromogenic reaction, after washing the wells, sodium acetate was mixed with TMB and H_2O_2 (volume ratio of sodium acetate with TMB and H_2O_2 = 1000: 10: 1). 200 µl were added in each well

immediately after adding H_2O_2 to minimize the background signal. The solution was allowed to react for 15 min at room temperature. We used 50 µl of 2 M H_2SO_4 to stop the reaction and measured absorbance at 450 nm (OD_{450}) in the microplate reader.

The calibration curves were generated using a process that required several steps: converting the biotin concentrations to a $\log_{10}$ scale; deducting the background reading from each sample reading (OD_{450}); then subtracting each reading from the maximum readings (ODmax, with no biotin added as a control) and dividing by the maximum value; finally, the readings were converted to natural log scale and resulted in the final data (tOD). The conversion equation is shown below [Eq. 1]. After data processing, scatter graphs were plotted to determine the effective range of detection. Based on the signal distribution, the dynamic range or detection range of the biosensor was estimated. The reliability was evaluated by the statistical R-squared values. Regression equations for the calibration curves were obtained for biotin standards in each batch and used for estimation of unknown biotin samples.

$$\mathrm{tOD} = \ln\left(\frac{\mathrm{ODmax} - \mathrm{OD}}{\mathrm{ODmax}}\right) \qquad \text{[Equation 1]}$$

3.3 Biotin biosensor 1

3.3.1 Design of the biosensor

The biotinylated BSA was immobilized on the polystyrene surfaces of the 96 well plates by adsorption. External sources of biotin (i.e., biotin standards, unknown samples) were tested. The conjugated streptavidin-HRP was then added into the wells that had both

immobilized biotin and external biotin. It was assumed that both forms of biotin would interact with the conjugate. Therefore, a portion of the conjugates would attach (almost irreversibly, given streptavidin's strong affinity to biotin) to either free or immobilized biotin. Due to the linear relationship of HRP to the intensity of the catalyzed reaction, the ratio of the conjugates bound with immobilized biotin could be only affected by the concentration of external biotin. By establishing the calibration curves for each set of samples, an equation could be fit to the data and used to estimate the unknown biotin content in other samples. Fitness of the corresponding regression equations was evaluated by its R square values. Fig. 7 shows the diagram of the preliminary biosensor 1.

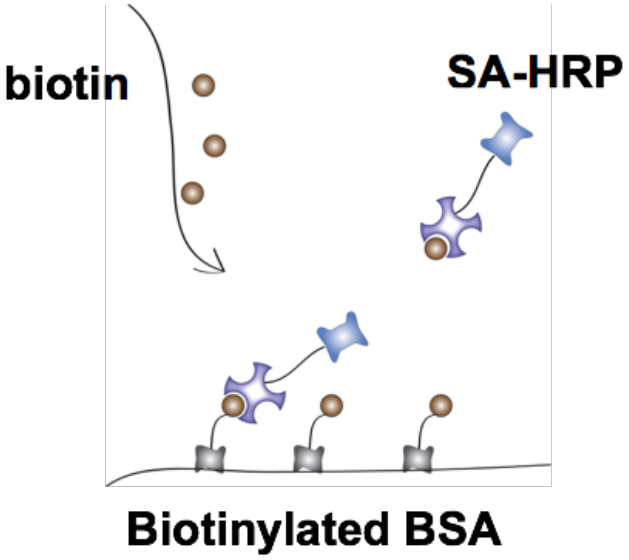

Figure 7: Diagram of biosensor 1. Biotin was immobilized via adsorption of biotinylated bovine serum albumin (BSA). Streptavidin (SA) and horseradish peroxidase (HRP) were conjugated via NHS ester reactions and carbodiimide reactions. The conjugate was co-incubated with external biotin or DTB to perform competitive binding.

3.3.2 Binding behavior of biosensor 1

For the assay to perform at its best, the data must contain the broadest dynamic range. The maximum absorbance, which is the chromogenic reaction with no external source of

biotin (i.e., control), should be as high as possible, but below the limit of the spectrometer's detection ability (ODmax = 4.0). Based on these requirements, the concentrations of the conjugates were optimized to be 0.04 ug/ml BSA-biotin and 0.2 μg/ml SA-HRP.

However, biotin has various analogs that can potentially interfere with the biosensor. DTB, especially, would affect the accuracy of the biosensor since it is the direct precursor of biotin in the biotin synthesis pathway of *E. coli*. To determine whether the presence of DTB in cell-synthesized samples was significant enough to effect assay behavior, a test was conducted to compare the binding affinity of biotin and DTB in the biosensor.

The same concentration gradient of biotin and DTB were evaluated. A significant difference was observed between their binding strength (Fig. 8). Consistent with the standard curve's behavior, the dynamic range of the biosensor was around 50–1000 pg/ml of biotin. However, the assay responded much less sensitively to DTB within the same range, indicating that DTB would not significantly interfere with the estimation of biotin concentration in samples of unknown biotin content. As the concentration increased by 100 ng/ml, DTB was able to occupy nearly as many binding sites of streptavidin as biotin did. These results matched the previous studies, which claimed the affinity of avidin for biotin is about 50-fold higher than its affinity for DTB[71].

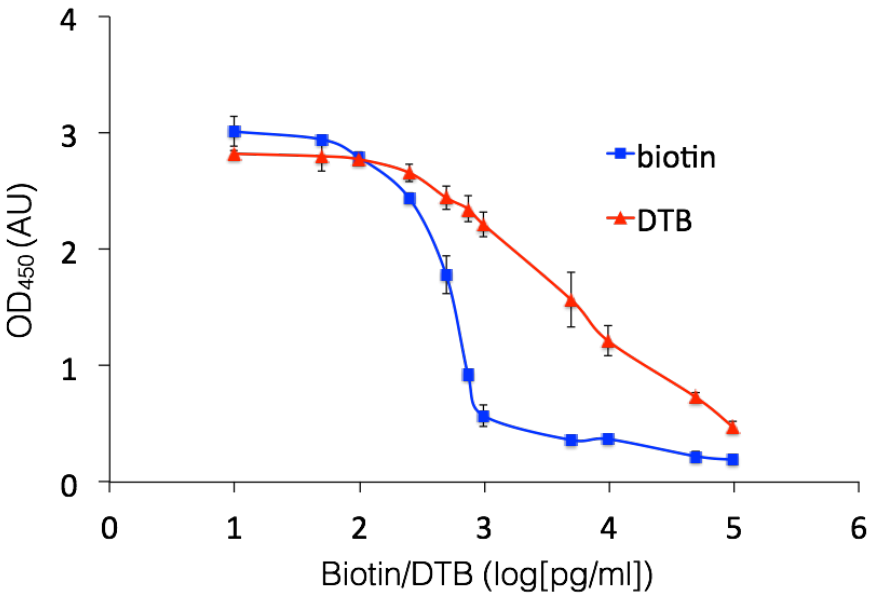

Figure 8: Different binding behaviors of biotin vs. DTB. The horizontal axis has units of log scale of concentrations of biotin or DTB (pg/ml). The vertical axis is the optical density with the arbitrary unit. The blue line shows binding behavior of biotin, while the red line shows binding behavior of DTB. Within the range of detection of the biosensor (100-1000 pg/ml of biotin), there was significant difference in binding affinity of biotin vs. DTB.

While the biosensing scheme described could be impacted by the presence of biotin analogs in uncharacterized samples, the chemical assembly steps that created the biosensor could also have impacted its function. Specifically, the order in which the conjugate (SA-HRP) was added to the samples and substrate (i.e., adding SA-HRP either prior to the addition of the biotin sample or after the addition of the biotin sample) could potentially have affected the behavior of the biosensor. The sensor was constructed with biotin first being immobilized on the substrate surface through adsorption. By adding the conjugate first, a portion of the immobilized biotin could be bound by the conjugate regardless of the amount of free biotin later added. This "biotin last" strategy could potentially have resulted in a higher background. Alternatively, if a "biotin first" approach was used, whereby samples of unknown biotin content were added to the

substrate prior to addition of the SA-HRP conjugate, the difference in the spatial distribution of immobilized and free biotin could affect the assay as SA-HRP would potentially bind free biotin before diffusing toward and binding the substrate.

The potential effect of procedure differences was tested and results are shown in Fig. 9. Overall, the difference between these two procedures is not significant, suggesting the diffusion rate is relatively high. When free biotin was added at higher concentrations, however, these approaches resulted in different signal levels, as expected.

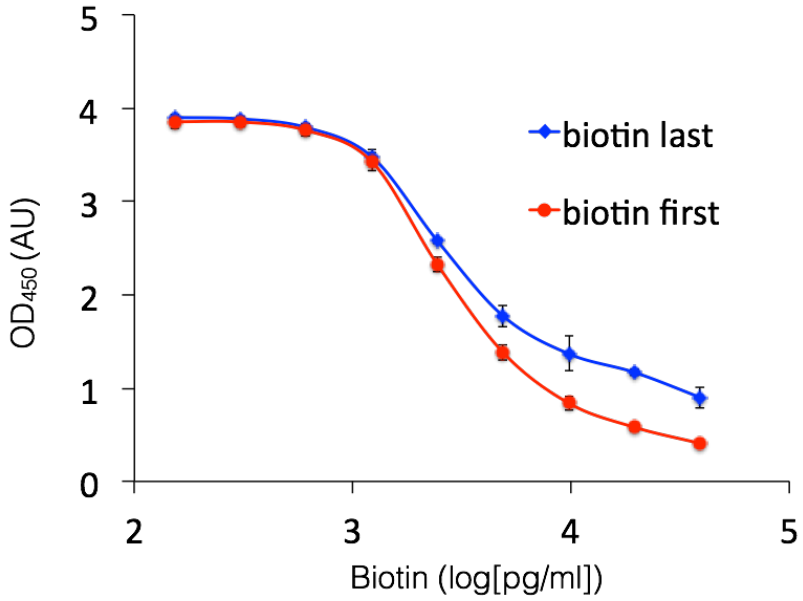

Figure 9: Effect of different strategies on the biosensor performance. The horizontal axis displays a log scale of biotin or DTB (pg/ml) concentrations. The vertical axis displays the optical density in arbitrary units (A.U.). Two strategies were used to measure the biosensing performance. 'Biotin first' refers to the biotin sample being added before the streptavidin conjugate; 'biotin last' refers to the opposite order of addition. The result shows little difference in binding affinity when applying these two methods. However, when approaching relatively high biotin concentrations, the biosensor performed better when biotin samples were added prior to addition of the conjugates.

3.3.4 Modeling of the competitive binding

The competitive binding behavior of the biosensor was modeled to study the parameters and provide an increased understanding of the kinetics involved. The binding events described above can be modeled with the following set of equations:

$$Bb_0 = Bb + BbSH \quad \text{[Equation 2a]}$$

$$b_0 = b + bSH \quad \text{[Equation 2b]}$$

$$SH_0 = SH + bSH + BbSH \quad \text{[Equation 2c]}$$

$$\frac{d(\mathrm{BbSH})}{dt} = ka_1 \times Bb \times SH - kd_1 \times BbSH \quad \text{[Equation 2d]}$$

$$\frac{d(\mathrm{bSH})}{dt} = ka_2 \times b \times SH - kd_2 \times bSH \quad \text{[Equation 2e]}$$

The association constants were defined as ka_1 and ka_2, while the dissociation constants were defined as kd_1 and kd_2. All of the bracketed symbols were defined as the concentrations of different chemical species (i.e., components) comprising the biosensor. Equation 2a describes the mass balance between the initial BSA-biotin adsorbed (Bb_0) and the unbound BSA-biotin (Bb) plus the bound form of BSA-biotin (i.e., the BSA-biotin-SA-HRP complex, labeled BbSH). Similarly, Equation 2b shows that initial free biotin in samples (b_0) would partly be captured and bound by SA-HRP forming a bound complex (bSH), with the remaining biotin remaining unbound (b). Equation 3c shows the mass balance between initial SA-HRP (SH_0) added to the system as a mixture of unbound conjugate (SH), conjugate bound to free biotin (bSH), and conjugate bound to BSA-

biotin (BbSH). Equation 2d and 2e describe the continuous competitive binding kinetics based on the dissociation and association of the bound SA-HRP to free biotin and BSA-biotin, respectively. As discussed in chapter 2, the Ka (equilibrium association constant) for the affinity of biotin with streptavidin has been measured as 1×10^{15} M^{-1}, and is one of the strongest non-covalent interactions found in nature[72]. Also, the constant rate of dissociation kd was measured to be 2.4×10^{-6} s^{-1} which makes it almost irreversible [73]. Based on these parameters, the association rate constant ka was estimated based on Equation 3, and could be used as the initial value for the parameters.

$$Ka = \frac{ka}{kd} \qquad \text{[Equation 3]}$$

The modeling presented here describes a system at equilibrium, assuming all the molecular interactions reached dynamic stability after a long period of time (1 h of incubation). This assumption results in a steady state where both the bSH and BbSH do not change over time:

$$\frac{d(\mathrm{BbSH})}{dt} = ka_1\times Bb\times SH - kd_1\times BbSH = 0 \qquad \text{[Equation 4a]}$$

$$\frac{d(\mathrm{bSH})}{dt} = ka_2\times b\times SH - kd_2\times bSH = 0 \qquad \text{[Equation 4b]}$$

Since the optical density from the colorimetric reactions was highly linearly correlated to the amount of HRP in the system, BbSH could be used to fit the OD_{450} data with a multiplication coefficient. With all the initial conditions known (as stated in Chapter

3.3.2), along with the kinetic constant values previously reported, the kinetic model should, in theory, describe the competitive binding accurately with the set of 5 linear equations (Eq. 2a, 2b, 2c, Eq. 4a and 4b) containing 5 free variables (b, SH, Bb, bSH and BbSH). However, it is difficult to determine the actual concentrations of the biomolecules immobilized or bound in solution. Further work would be required to determine the values of these variables and fit the parameters in the model.

3.3.5 Biosensor design evaluation

The biosensor's sensitivity and reliability were evaluated by establishing the calibration curve given below (Fig. 10). This figure shows that the biotin sensor has a stable detection range of spanning approximately 50 to 1000 pg/ml ($R^2 = 0.99$). In these tests, biotin samples were diluted 1 : 5 prior to measurement using the biosensor. This tight range of detection spans the biotin concentrations observed in wild type *E. coli* and, thus, could possibly be useful in measuring synthesis by engineered strains as well. Since it is a multi-step assay procedure, the range of detection would shift slightly between each batch. Thus, new standard curves were generated each time for measuring the unknown biotin samples.

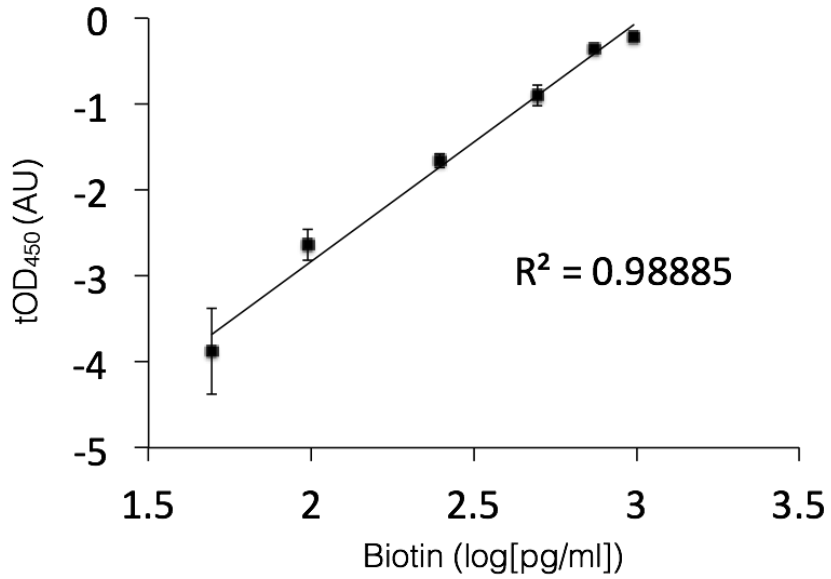

Figure 10: The Calibration curve of biosensor 1. The horizontal axis displays a log scale of concentrations of biotin or DTB (pg/ml) concentrations. The vertical axis displays optical density after conversion with Equation 1 in arbitrary units (A.U.). The calibration curve showed a high reliability for biosensor 1 when applied as a biotin assay method.

3.4 Biotin biosensor 2

3.4.1 Design of the biosensor

I created a second biosensor for measuring biotin using a reaction scheme similar to that described previous. This sensor's reaction scheme is shown below (Fig. 11). Unlike biosensor 1, described above, biosensor 2 used streptavidin adsorbed directly onto the polystyrene surface of the 96 well plates, in place of BSA-biotin. Then, biotinylated HRP was added as a conjugate, and competed with free biotin in samples for the adsorbed avidin. Biotinylated HRP that bound surface-adsorbed avidin was then able to generate chromogenic signals using the same HRP mechanism described above.

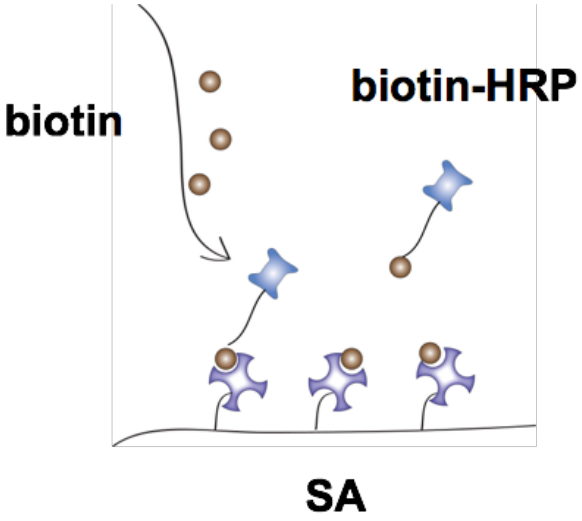

Figure 11: Diagram of biosensor 2. Streptavidin (SA) was immobilized via adsorption on polystyrene surfaces. Horseradish peroxidase (HRP) was biotinylated via NHS ester reactions. Biotinylated HRP was then co-incubated with biotin or DTB samples.

3.4.2 Binding behavior of biosensor 2

Similar to biosensor 1, the working molecular concentrations were optimized based on the principle of maximizing the absorbance readings within the detection range of the microplate reader. For biosensor 2, optimal initial concentrations of reagents were determined as 0.17 μg/ml of streptavidin and 0.2 μg/ml of HRP-biotin. The binding behavior of biosensor 2 was also studied. As Fig. 12 shows, the dynamic range of biosensor 2 was higher and broader than that of biosensor 1. Although each streptavidin molecule is theoretically able to bind 4 molecules of biotin, the conjugation of biotin or streptavidin with other reagents may affect their interaction ability. In biosensor 1, biotin was surface-immobilized through adsorption of the biotin-albumin conjugate to polystyrene. However, because biosensor 2 uses streptavidin directly adsorbed to the polystyrene surface, the some of the active sites of streptavidin are likely to be occluded by the polystyrene substrate, potentially resulting in loss of sensititivity.

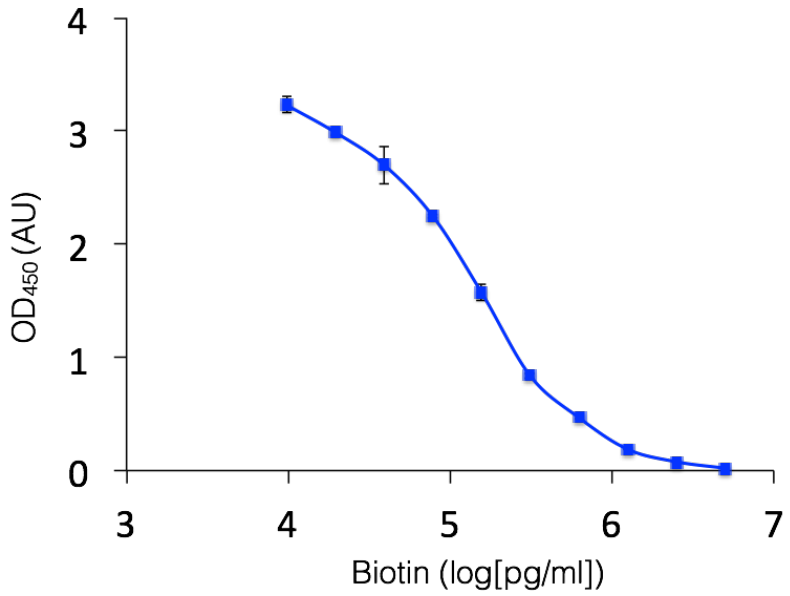

Figure 12: Binding behavior of biotin in the biosensor 2. The horizontal axis displays a log scale of biotin (pg/ml) concentration. The vertical axis displays optical density in arbitrary units (A.U.). The blue line shows the dynamic range of the biosensor 2 spanned from 10 to 1000 ng/ml of biotin, approximately.

3.4.3 Evaluation

A calibration curve of the biosensor 2 is shown below (Fig. 13). Similar optimization processes were conducted as for biosensor 1. Specifically, 0.17 μg/ml of SA was immobilized on the surface of 96 well plates, and 0.20 μg/ml of biotin-HRP and various biotin standard samples were added to the wells afterwards. As the calibration curve shows, the biotin assay has a stable detection range of biotin spanning 20 to 100 ng/ml ($R^2 = 0.97$) and was used to measure 1 : 5 dilutions of actual samples.

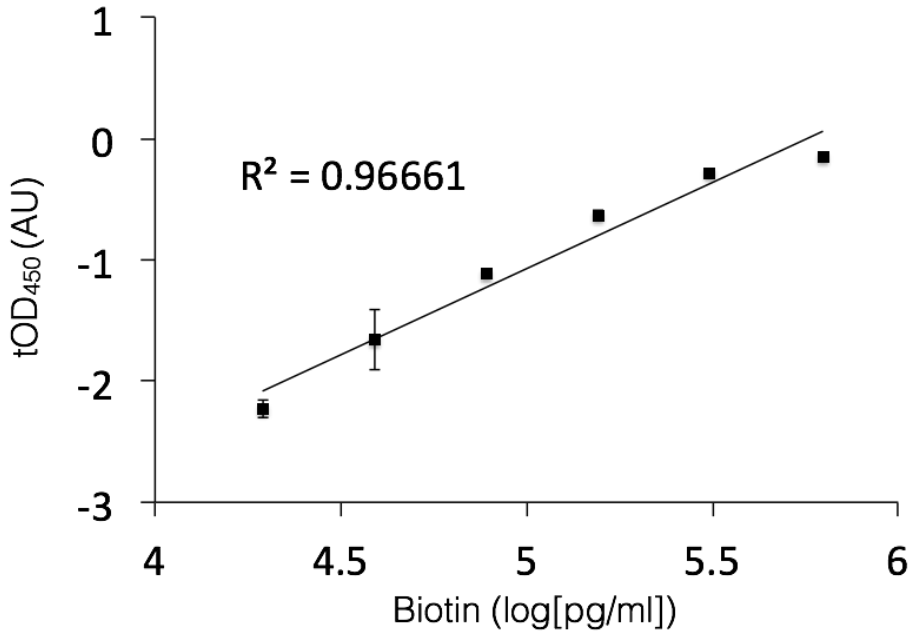

Figure 13: The Calibration curve of biosensor 2.

The horizontal axis displays a log scale of biotin (pg/ml) concentrations. The vertical axis shows the optical density after conversion with Equation 1 in arbitrary units (A.U.). The calibration curve shows high reliability of the biosensor 2 when applied as a biotin assay method.

3.5 Comparison of the biosensors

Although the concentrations of HRP were of the same magnitude for both sensors, significantly different dynamic ranges were observed. This is likely due to the effect of differing immobilized molecule types, as well as the number of biotinylated amines for the conjugates as the number of primary amine groups varies among proteins. In addition, as the reaction scheme of each biosensor type differed, the availability of both molecules of the binding pair would change as a result. For instance, with streptavidin directly absorbed onto surfaces in assay 2, it would be more difficult for biotin or biotinylated conjugates to reach all of its active sites. On the other hand, in biosensor 1, biotin was conjugated to BSA that occupied the surface of the wells. This characteristic does not

exist in the second assay and could potentially increase the chance of the biotin-streptavidin interaction. Considering all of the above, I expected to see the lower biotin dynamic range observed in biosensor 1. Both sensors have been evaluated as reliable assay methods for biotin measurement with differing detection ranges. The characteristics of these biosensors could be further investigated by simulating the kinetic model encapsulated within the equations provided. Moreover, the comparison of these two biosensors served as the preliminary step toward a more advanced biosensor based on surface functionalization as shown in Chapter 5.

Chapter 4

Engineered Biosynthesis of Biotin

4.1 Background

Biotin is an essential trace molecule for living organisms. Biotin deficiency (i.e. hypobiotinosis) leads to multiple diseases since biotin regulates fatty acid biosynthesis[74]. In addition, biotin has been reported to be beneficial for nail and hair growth[15]. Although biotin exists in many types of foods including milk and vegetables, it is not naturally produced in significant quantities by any known species[74]. Engineers have explored chemical as well as biological ways to synthesize biotin. The chemical synthesis methods that are popular in industry usually require complicated reaction steps[75]. On the other hand, biological engineers have also developed biosynthesis methods that utilize microorganisms. *E. coli* has been used as the main engineered microbial platform. Some researchers have overexpressed the entire biotin operon from *E. coli*[46]. Some introduced the operon from *B. sphaericus* into *E. coli* and grew the resulting engineered cells in a bioreactor to achieve a higher yield[76]. Alternatively, some researchers only used the *bioB* gene from *B. sphaericus*, which encodes a biotin synthase that converts DTB to biotin, and then supplemented cultures with DTB[76]. In this work, my aim was not to optimize biotin biosynthesis, but rather to generate a new threshold of biotin signal that could be recognized by the engineered biosensors I developed. Thus, a simple and robust gene circuit driving biotin synthesis was designed and tested.

4.2 Materials and methods

4.2.1 Plasmids and strains

The *bioB* gene was isolated from the genome of *E. coli* MG1655 wild type strain purchased from ATCC. The plasmids pKDL071 and pKE1-MCS were obtained from the laboratory of James Collins at Boston University (now at MIT). After engineering the synthetic cassettes containing the *bioB* gene, they were inserted into the pKE1-MCS expression vector to build a complete circuit (Fig. 14). The NEB Turbo strain (New England Biolabs) was used for cloning, while the final constructed plasmid was transformed into the *E. coli* MG1655 strain for biotin biosynthesis measurements.

4.2.2 Design of the genetic circuit

I designed the genetic circuit to have two parts: a repressor and the biotin expression gene. The repressor cassette contained the *lacI* gene driven by the $P_{LtetO-1}$ promoter[77] to constitutively express the repressor LacI. For maximizing biotin production, the *bioB* gene encoding biotin synthase was placed downstream of a strong synthetic Ribosome Binding Site (RBS) generated based on the RBS Calculator (from the Salis Lab, v1.1) and concatenated with the P_{trc-2} promoter[78]. LacI represses the P_{trc-2} promoter and its repression can be lifted by induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG). Finally, both the cassettes for the LacI repressor and BioB were inserted into the pKE1-MCS vector in divergent directions. Since LacI is a specific repressor for the P_{trc-2} promoter, the *bioB* gene would only activate when the inducer prevented the repressor from blocking the transcription sites. The diagram of the circuit design is shown in Fig. 14.

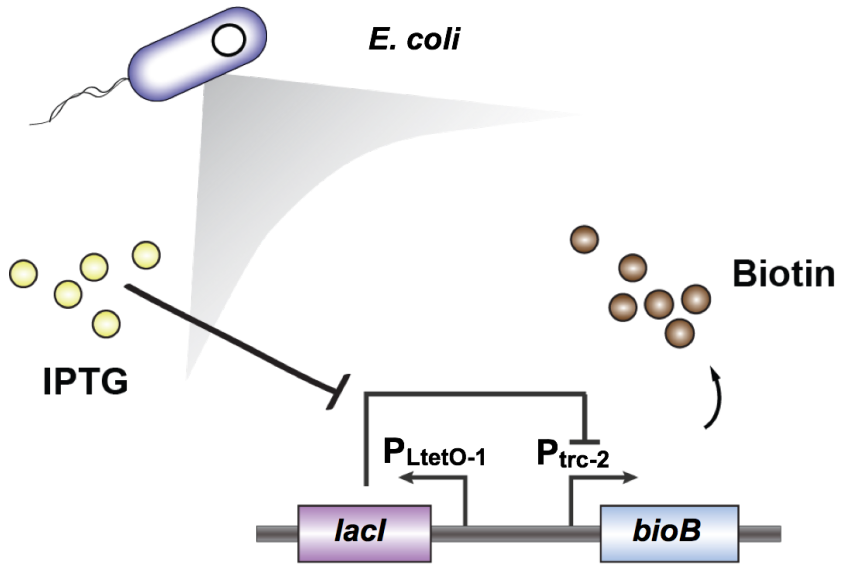

Figure 14: Design of the genetic circuit for biotin biosynthesis. This genetic circuit allows inducible expression of the *bioB* gene. BioB is biotin synthase, responsible for DTB conversion to biotin. The *bioB* gene was driven by the P_{trc-2} promoter, which is repressed by LacI, and can be de-repressed (activated) by IPTG.

4.2.3 Molecular cloning

General molecular cloning technologies have long been used for gene engineering. However, for rapid design and realization of gene circuits, we used recently developed standardized gene-level modules that could be easily tuned and recombined[58]. The P_{trc-2} promoter and the synthetic *lacI* cassette were extracted from the pKDL071 plasmid. The *bioB* gene was extracted from the *E. coli* MG1655 genome. The synthetic RBS segments were assembled into the cassette by primers. Overlap-extension PCR, also known as SOEing (Splicing by Overlap Extension), is a powerful method for assembling gene circuits[79]. By designing primers with overlapping regions of sequences to be assembled, genetic parts can be combined in a single PCR reaction. The PCR products were identified by agarose gel electrophoresis and purified using Gel Extraction kits. The

intact plasmids were collected using miniprep kits. All the cloning kits were purchased from Epoch Life Science, Inc. Double digestions were conducted on both cassettes as well as the vector, providing the same sticky ends for ligation. Digestion enzymes and the DNA ligase were purchased from New England Biolabs.

Both the *lacI* and *bioB* cassettes were inserted into the pKE1-MCS vector. The engineered plasmids were transformed into the NEB turbo competent cells and picked from LB-antibiotic- 1.5 % agar plates. After construct confirmation by digestion and gel electrophoresis, target plasmids were transferred into the *E. coli* MG1655 strain. The engineered strains were incubated for 24 h at 37 °C, and agitated at 400 rpm. The biotin concentration of the cell culture media was measured afterwards using the biosensors previously discussed.

The use of single-stranded DNA primers was essential to generate nucleotide sequences of interest from templates and to create desired genetic constructs. To extract the *bioB* gene from the *E. coli* genome, specific primers were designed and used in whole cell PCR. Primers were also designed to obtain the promoters, terminators and the *lacI* cassette from pKDL071. In addition, primers for SOEing were used for construction of the *bioB* cassette. All the primers used were synthesized by Life Technologies™ and are shown in Table 1 below.

Table 1: Primers used in this work.

Time	Sequence (5'->3')	Usage
1F	CCGCCGGAATTCTCCCTATCAGTGATAGAGATT	LacI cassette extraction
1R	CCGACGTCTCACTGCCCGCTTTCCAGTC	LacI cassette extraction
ins1f	GGTCATGGATGTGTATGGGTGC	Biotin operon extraction
Ins7r	GTATTCTCTCGCTTGCCTCGTG	Biotin operon extraction
nbioB1F	CCCAAGCTTCTGAAATGAGCTGTTGACAATTAATCAT	Promoter of *bioB*
nbioB1R	GGGGGGTTCTTTTAATAAAGGTACCGTGTGAAATTGTT	Promoter of *bioB*
nbioB2F1	ACCCCCCTAAGGAGGTCATCATGGCTCACCGCCCACG	*bioB* extraction
nbioB2F2	ACGGTACCTTTATTAAAAGAACCCCCCTAAGGAGGTCATC	Add synthetic RBS
nbioB2R	TCCCCGCGGTCATAATGCTGCCGCGTTGTAATATTC	*bioB* extraction
p1f	GCAATAAACCAGCCAGCCGGAAGGGCCGAT	Cell-free template
b-p1f	Biotin - GCAATAAACCAGCCAGCCGGAAGGGCCGAT	Biotinylated forward primer
p1r	ATTCTCACCAATAAAAAACGCCCGGCGGCAACC	Cell-free template

4.2.4 Medium composition and cell culturing

For normal cell culture, LB media was used (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl). To exclude any external source of biotin when measuring biotin synthesized by the *E. coli* strains, a minimal media recipe was developed based on M9 salts[80]. Table 2 shows the composition of the minimal media. Glucose was chosen to be the carbon source as it may contribute to the restricted repression of the P_{trc-2} promoter[78]. Biotin-free, casamino acids (BD Difco™) were used to ensure no biotin existed in the media outside of that synthesized by cells.

For whole cell PCR, plasmid extraction or inoculation, *E. coli* strains were grown in LB media at 37 °C, 400 rpm overnight. Antibiotics were added if necessary for selection. To measure biotin synthesis, 5% (adjusting based on OD_{600} reads of each culture) of the above LB cultures were inoculated into the minimal media at 37 °C, 400 rpm for 24 h. Cell growth conditions were measure in the microplate reader at OD_{600}. The cultures were then centrifuged at 9000 rpm for 3 min, and the supernatants were measured by the biosensor 1 discussed in Chapter 3.

Table 2: Minimal media recipe (100 ml).

Name	Volume	Final Concentration
5X M9 salts	20 ml	1X
Deionized Water	76.8 ml	N/A
1 M $MgSO_4$	200 ul	2 mM
1M $CaCl_2$	10 ul	0.1 mM
20% Glucose	2 ml	0.4 %
2% biotin-free Casamino acid	1 ml	0.02 %

4.3 Results

4.3.1 Gene cloning

Touchdown-PCR[81] was applied for most PCR reactions in the work described here. Fig. 15 shows all the relevant gel electrophoresis results that confirm construction of the correct plasmid constructs. First, the *bioB* gene was extracted from wild type *E. coli* genome using whole cell PCR and purified from agarose gels. The length of the *bioB*

gene is 1041 bps. The 75 bps promoter P_{trc-2} and the 1193 bps *lacI* cassette were isolated from the pKDL071 plasmid separately. Then SOEing PCR was applied to concatenate the P_{trc-2} promoter and the *bioB* gene with the synthetic RBS (32 bps) in between, giving a total length of 1154 bps. After constructing the circuit as well as transforming the plasmid into the cells, confirmation tests were conducted by digesting the plasmid with the same restriction enzymes as used previously. Only the digestion test results of the final transformation are shown here. The gene circuit was successfully constructed and further expression tests have been performed to validate function.

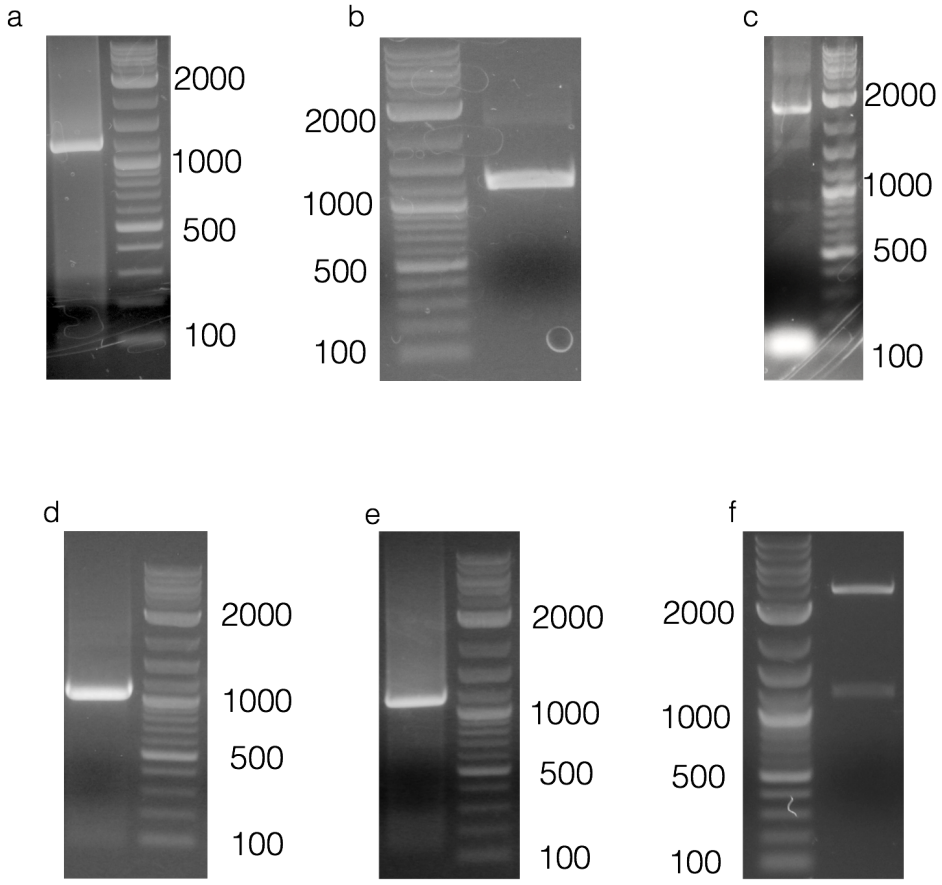

Figure 15: Cloning results. The agarose gels show the PCR results for both the *lacI* and *bioB* operon. (a) The *lacI* cassette was extracted from pKDL071 (1193 bps). (b) The *lacI* cassette was transformed into *E. coli* and test digested. The left band shows the empty pKE1-MCS vector (2376 bps) and right band shows digested band of the construct having separate vector and the *lacI* cassette. (c) The 75 bps promoter P_{trc-2} was also isolated from pDKL071 plasmid. The non-specific band around 2000 was due to multiple P_{trc-2} promoters existing in the plasmid. (d) The *bioB* gene (1041 bps) was isolated from *E. coli* MG1655 genome using whole-cell PCR. (e) SOEing PCR was applied to concatenate the P_{trc-2} promoter and the *bioB* gene with the 32 bps synthetic RBS in between with several restriction sites, giving a total length of 1154 bps. (f) Digestion test was conducted for the *bioB* gene cassette, showing the right size of insert (1154 bps) and the remaining vector containing the *lacI* cassette (3569 bps).

4.3.2 Gene expression

A parallel construct replacing the *bioB* gene with mCherry was also cloned to test the induction of IPTG. As expected, mCherry expression was well regulated by the IPTG

induction (Fig. 16). The induction of this designed genetic circuit was evaluated by applying different concentrations of IPTG (0.1 mM - 5 mM) for activation of the mCherry expression. All concentrations of the inducer show very similar intensity of the expression except for the control. Based on this data, it was confirmed that the *lacI* operon was correct and functioning. However, the full activation of mCherry expression upon low levels of inducer implied the relative low strength of the repression.

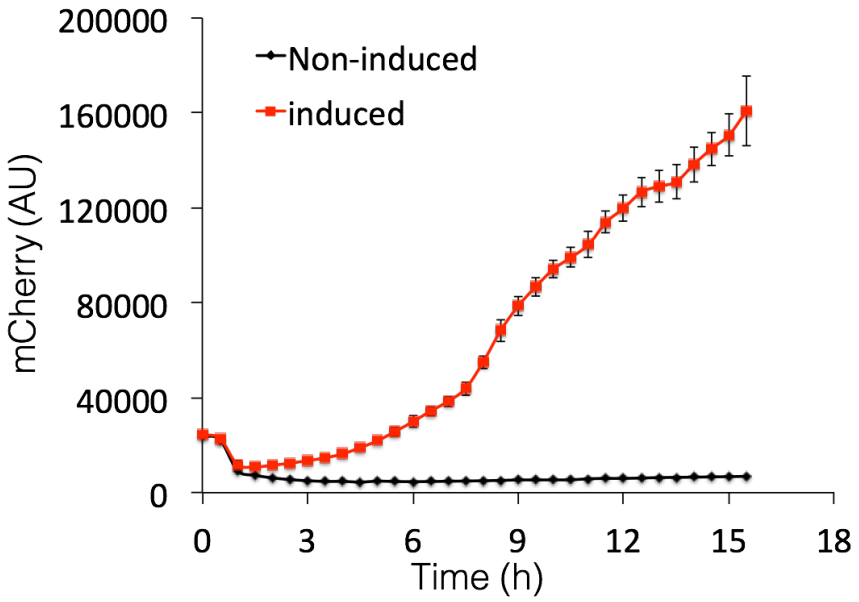

Figure 16: Expression of mCherry with induction. The horizontal axis displays time (h). The vertical axis displays arbitrary units (A.U.) of red fluorescent intensity. The mCherry expression was completely repressed without IPTG and activated by 1mM of IPTG. The black line shows baseline expression of mCherry when no inducer was added. The red line shows mCherry expression of various IPTG concentrations (0.1 mM - 5 mM). All concentrations of the inducer show very similar intensity of the expression except for the control.

4.3.3 Engineered biotin synthesis

To examine biotin synthesis in the engineered strain, the cells were inoculated 5% from overnight LB media into the minimal media described previously in Table 2. 30 ng/ml of

DTB was supplemented into the cell culture upon incubation at 37 °C for 24 h. Wild type *E. coli* were also cultured with or without DTB under the same conditions. As Fig. 17 shows, 30 ng/ml of DTB was hardly detectable by biosensor 1. Thus, DTB has been shown to be a suitable precursor of biotin signal generation for these biosensing systems. The recorded biotin production by the wild type cells without DTB addition matches previous studies and is around 1 ng/ml due to a 1 : 5 dilution when conducting the measurement. With DTB supplementation, the wild type cells produced 17-fold of biotin, indicating the abundance or the high efficiency of the biotin synthase naturally. For the engineered strain, a much higher biotin synthesis was observed for both induced and non-induced cells. The concentrations of biotin in culture were estimated to be around 17 ng/ml. The conversion rate of DTB to biotin was calculated accordingly to be 56.7 %.

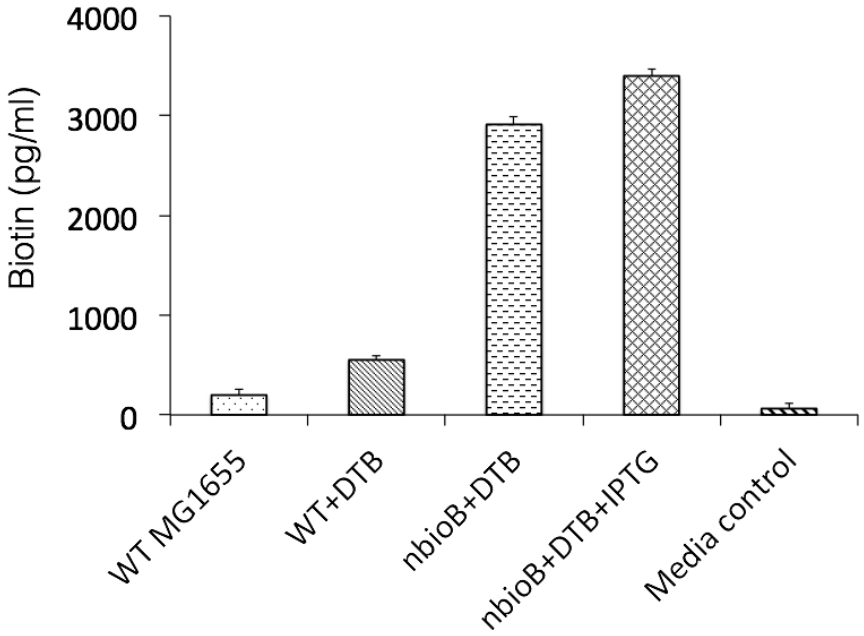

Figure 17: Biosynthesis of biotin in the wild type (WT) and recombinant strains. The horizontal axis displays different experimental conditions in each sample type. From left to right: wild type *E. coli*, wild type *E. coli* with 30 ng/ml of DTB, engineered *E. coli* with 30 ng/ml of DTB, engineered *E. coli* with 30 ng/ml of DTB and 1 mM of IPTG, pure media control with 30 ng/ml. The horizontal axis displays biotin concentration (pg/ml). These concentrations were measured in culture media samples that have been diluted 1 : 5.

Biotin production in the recombinant strain confirmed the functionality of the *bioB* gene in the engineered genetic circuit. Upon induction, the engineered *E. coli* was able to synthesize 17-fold more biotin in comparison to the wild-type strain when grown in the same conditions. The conversion rate of DTB to biotin was calculated to be 56.7 % in the engineered strain. These results indicate the possibility of engineering bacteria to generate appropriate signal to be recognized by the following components of the designed biosensor.

However, there was significant leak evident in the non-induced recombinant strain (85.7

%). Since the repression of LacI has been tested by the mCherry construct, the high biotin concentration in the un-induced recombinant strain was possibly due to the strong RBS for the bioB gene that could not be fully repressed by LacI. Future work for enhancing control of *bioB* would include strengthening *lacI* expression and its subsequent activation of *bioB*. Alternatively, the RBS driving *bioB* could be replaced with a weaker RBS.

Chapter 5

Development of a New Biosensing Modality

5.1 Background

After developing two robust and accurate preliminary biosensors, attempts to expand this technology to create a platform for solving a broader ranging of sensing applications were made. Frequently, sensors need to be portable (e.g., for deployment within the environment or at the clinical bedside). The previous biosensors we built were constrained, to some extent, by their required use of a stationary adsorption surface (i.e., the polystyrene surfaces of 96-well plates). In order to create a more mobile sensing platform, I used the tools of miniaturization (i.e., microparticles) to anchor biosensing components[82], and created a new transducing mechanism based on cell-free synthetic biology. The use of cell-free systems has become a valuable technique in synthetic biology due to their rapid and specific expression *in vitro*[83]. By leveraging knowledge of molecular interactions with the engineering principles in synthetic biology, I was able to create a unique biosensor that could have multiple applications in a range of fields.

5.2 Materials and methods

5.2.1 Bead functionalization

Carboxylate modified (CML) latex beads were purchased from Invitrogen™. I bonded streptavidin to carboxylated microbeads via a carbodiimide reaction[84], mediated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in ambient conditions. Next, I designed and synthesized biotinylated DNA encoding green fluorescent protein (GFP)

driven by the T_7 phage promoter (P_{T7}). I then co-incubated this DNA with the functionalized beads to test a range of different biotin samples, at 37 °C for 1 h. As Fig. 18 shows, the functionalized beads specifically respond to biotin and do not bind biotinylated DNA when SA's active sites have been occupied by free biotin. The bound DNA, immobilized on the microparticle surface, and then becomes the template for an *in vitro* expression system. The biotinylated dsDNA was synthesized via PCR reaction using a biotinylated forward primer and a standard reverse primer that I designed. Primers were synthesized from Life Technologies™.

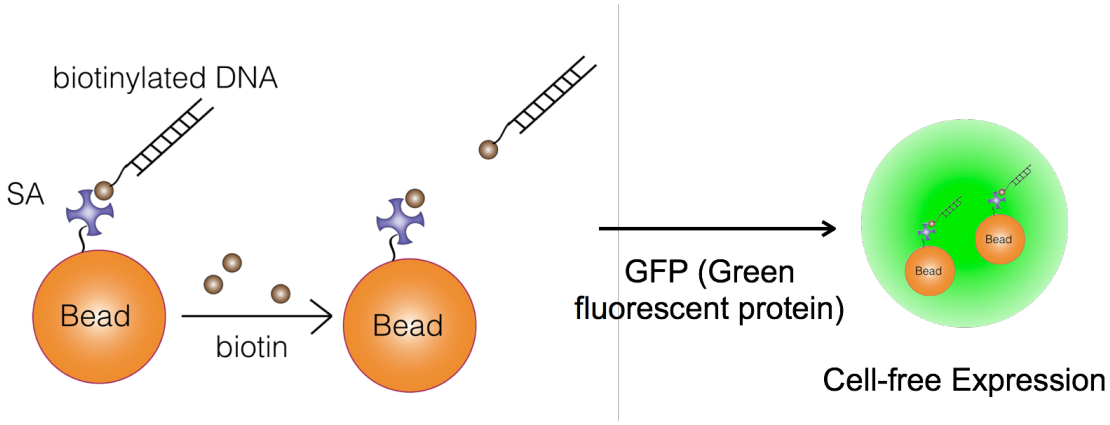

Figure 18: Diagram of the new biosensor. Latex microbeads functionalized with carboxyl groups were bonded to streptavidin (SA) and then bound to biotinylated DNA encoding the GFP gene driven by a T_7 promoter. Similar to previously described biosensors, the streptavidin-biotin interaction was mediated by the presence of external biotin. The functionalized microbeads were then applied in a cell-free system as the template for GFP expression.

5.2.2 Cell-free expression

After incubation, the mixture was washed and briefly centrifuged at 9000 rpm to pellet the beads. After discarding the supernatants, the numbers of beads were counted using a flow cytometer (Accuri™ C6, Becton, Dickinson and Company), to accurately measure

the number of microbeads. The number of beads for each batch was then standardized to be 10000 – 20000 microbeads per µl and remained consistent throughout each reaction batch. Typically, 3 µl of the concentrated beads were mixed with 7 µl of the cell-free expression solution (PURExpress®, New England Biolabs). An RNAse inhibitor solution was added to inhibit RNA degradation. The cell-free expression mixtures were incubated at 37 °C for 4 h with constant shaking at 400 rpm. After the reaction, the supernatant was separated by centrifugation at 6000 rpm.

5.2.3 Observation procedure

To observe the GFP expression, microfluidic chambers (height: 100 µm) were used with the reaction solution and enveloped in an oil shell. The oil was made of HFE 7500 (3M™ Novec™) with 1.8 % of the surfactant KRYTOK® 157 HFE (DuPont™). Upon mixing 5 µl of the reaction solution with 20 µl of oil and vortexing, oil-water droplets formed. Finally, the droplets were added into a microfluidic chamber to be observed under a fluorescent microscope (Eclipse Ti, Nikon).

The microfluidic chips containing the chambers were made from the (polydimethylsiloxane) PDMS (SYLGARD® 184, Dow Corning Corporation). The detailed protocol for making PDMS chips is listed in Appendix B. The chips were then moved onto a microscope stage holder to be observed. Fluorescent intensity was measured using a 40X oil immersion objective. Fluorescence images were captured using an ANDOR Zyla scientific CMOS (sCMOS) camera using an exposure time 50 ms for all samples. Since several droplets could be seen in the field of view, three measurements

were conducted for each sample by creating matched circular regions of interest (ROIs) for the droplets and recording the average GFP intensity within each ROI. All samples were background subtracted. Fig. 19 displays essential devices used in this study. The chambers were designed with one inlet and outlet each, with a barrier on one side to trap droplets with the chamber (Fig. 19a). The chamber dimensions were 40 mm in length and 20 mm in width. Fig. 19b shows two PDMS chips with several chambers in each. The size of the chip is shown compared to a 1.5 ml microcentrifuge tube for size reference. The complete microbeads functionalization and cell-free expression and observation procedure is listed in Appendix C.

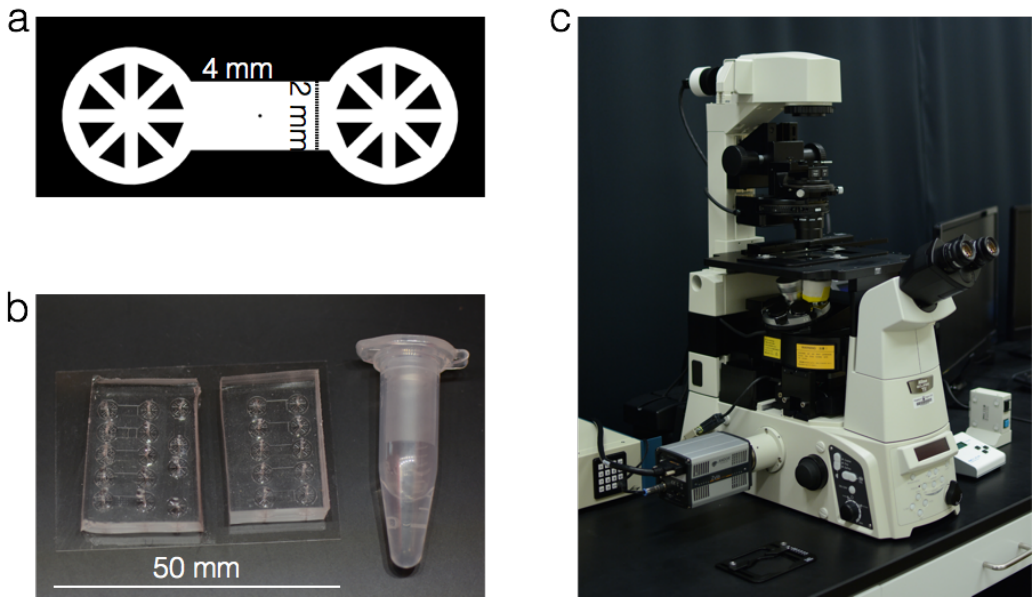

Figure 19: Devices used for developing the new biosensor. Key devices used in this work: (a) the design of the microfluidic chambers (working area dimensions: 4 mm × 2 mm). (b) Microfluidic chamber and a comparison to a 1.5 ml tube as a size reference. (c) The fluorescent microscope: an automated Nikon Ti Eclipse with ANDOR Zyla sCMOS camera.

5.3 Results

5.3.1 Cell-free expression

Cell-free expression of GFP was observed using fluorescent imaging (Fig. 20). Oil-encapsulated droplets ranged in size from 50-100 μm. The green spheres show the oil droplets generating a GFP signal. The small, dark spheres inside are the functionalized microbeads. To measure the GFP intensity of these droplets, these microbeads were removed before measurements were made. The effect of biotin on cell-free expression was examined using four different conditions. The details of these experiments are shown in Fig. 21. As shown, all templates showed similar fluorescence intensity after incubation. However, reactions with linear DNA and free supplemented biotin showed a significant decrease in expression, but not those reactions using biotinylated DNA. This may be due to the potential interaction of free biotin with different components in the expression solution. Thus, removing biotin from the cell-free system was critical for conducting the dynamic range study.

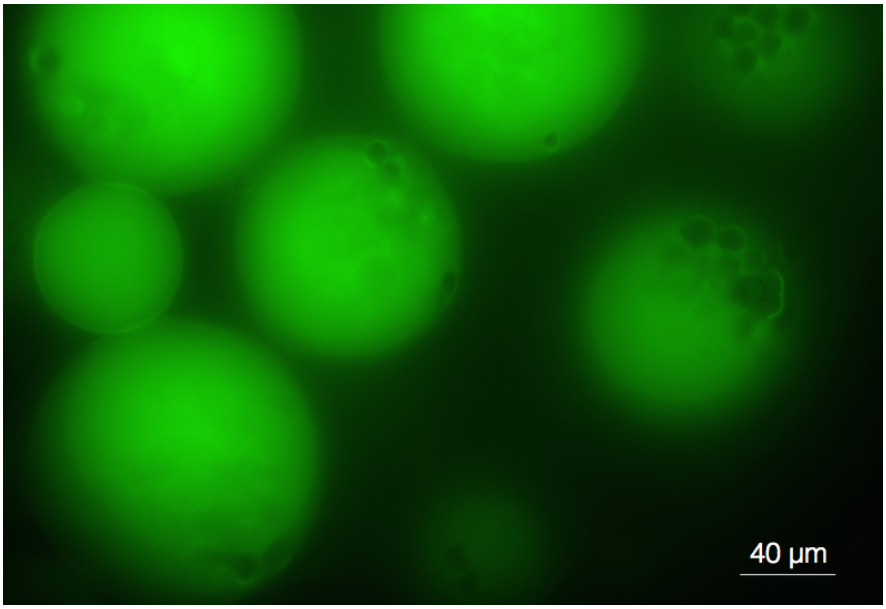

Figure 20: GFP droplet imaging. Droplets are shown consisting of hydrocarbon oil encapsulating an aqueous cell-free expression system with a microbead-DNA template. The small spheres within the droplets are the functionalized microbeads. When measuring GFP expression intensity, the microbeads were removed from the reaction solutions.

In addition, there were several sources of signal interference, including multiple layers of the droplets outside the focal plane causing high background signal. Additionally, the inclusion of beads resulted in measurement variance. The problem of multiple layers could be avoided by controlling the density of the droplets placed in the chambers, as well as by changing the height of the chamber to match the diameter of droplets, thus confining all droplets to a single focal plane. A homogeneous size of the droplets could be potentially achieved by utilizing a droplet forming microfluidic channels[85].

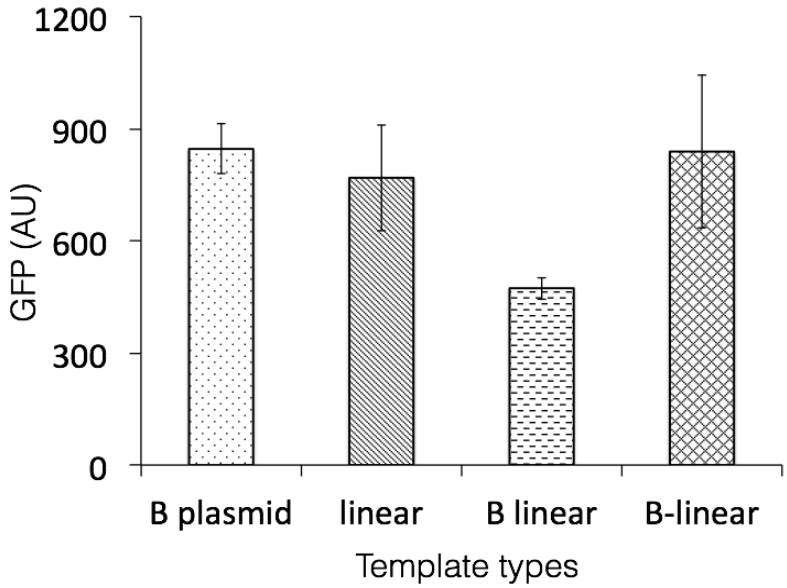

Figure 21: Effect of template types on cell-free expression. The horizontal axis shows the type of different templates for expression. The vertical axis is the arbitrary units of GFP intensity. From left to right: the plasmid containing GFP with free biotin (B plasmid), linearized GFP template (linear), linearized GFP template with biotin (B linear), and the biotinylated linear DNA encoding GFP (B-linear). Supplemented free biotin in "B plasmid" samples was identical to the actual biotin concentration in biotinylated DNA samples. A decrease in expression level was observed in the biotin-containing linearized template (B linear).

5.3.2 Dynamic range of the biosensing system

Similar to the preliminary biotin sensors, the dynamic range of the new system was investigated (Fig. 22). After streptavidin was immobilized on the beads surface, samples with a gradient of biotin concentrations were co-incubated with the biotinylated DNA. The biotin content affected the binding capacity of the modified DNA, with the dynamic range of biotin ranging from 1 to 100 ng/ml, which is similar to the dynamic range of biosensor 2 since they shared almost the same design for streptavidin immobilization.

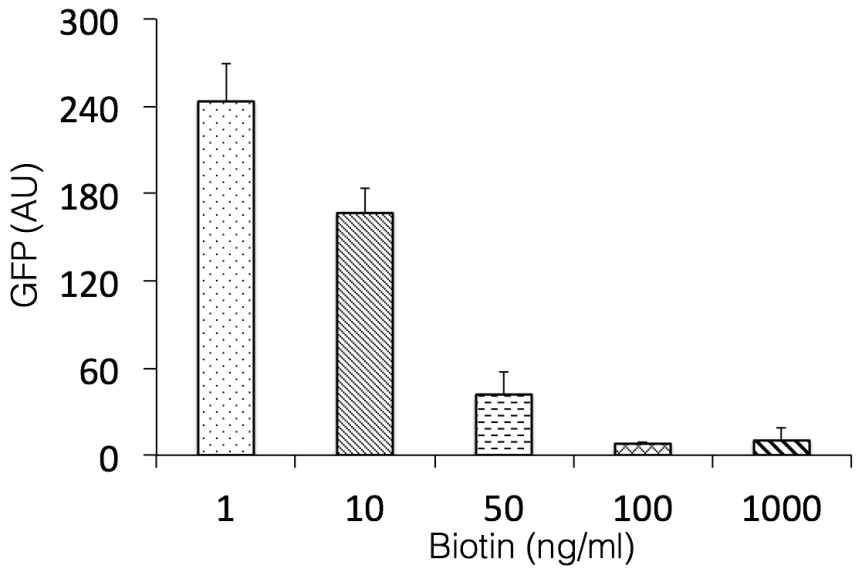

Figure 22: Dynamic range of the new biosensor. The horizontal axis is the biotin concentration gradient (ng/ml). The vertical axis is the GFP intensity in arbitrary units (A.U.). Within the range of 1 to 100 ng/ml of biotin, the biosensor shows a detectable dynamic range.

Our results revealed a dynamic range of GFP signals corresponding to biotin concentration. This mobile, functionalized surface would potentially serve as a building block for portable and automated biosensing devices and could have multiple applications.

Overall, the data on the system described here serves as a first step in linking a cellular signal to a non-living, cell-free expression system. We can envision a system where ultimately, the components and interactions within cell free systems could be encoded within the genetic information in engineered living cells.

Chapter 6

Conclusion and Discussion

Multiple biosensors have been developed in this study. The preliminary biosensors were designed based on the biotin-(strept)avidin interaction system and were evaluated and analyzed. Biosensor 1 was also modeled based on equilibrium kinetic conditions. These biosensors were relatively simple, yet robust and specific. They provided a way to understand the interaction system, a platform for designing more complicated biosensors based on them, and a tool for measuring biotin content at very low concentrations.

In addition, by exploring the possibility of the biosensors communicating with living cells, a recombinant *E. coli* strain was constructed to overexpress biotin controlled by induction. An increase of biotin production by around 17-fold was observed compared to wild type strains. This inducible biotin microbial cell factory builds a signaling bridge between response of living cells to environmental inputs and the biosensors based on the specific recognition of the very signal that the cells produce. By leveraging our knowledge of synthetic biology, the cells could be engineered to become receivers for various inputs in their environment, and thus create a whole new type of tunable, interactive biosensor.

The newly developed biosensing system was innovatively based on a functionalized mobile surface. Compared to the preliminary biosensors, it is more flexible without losing the sensitivity of a biotin-(strept)avidin interaction-based device. Compared to typical microbial-based biosensors, it would be superior in terms of specificity as well. This system has various applications, for instance, it could be a candidate for accurate drug delivery systems by replacing the reporter gene with pharmaceutical proteins. As we know, our intestinal bacteria produce biotin[15]. We could design an encapsulation system to release a protein-encoding DNA to be expressed in the intestines. Additionally, by engineering microfluidic devices, we could design portable and automated biosensors that meet various needs. Finally, by communicating with engineered cells via a biochemical interaction, this mobile, cell-free biosensor could have a huge impact as a building block of abiotic-biotic sensors that utilize the strengths of both living and non-living sensors.

Based on all the thoughts above, these are tentative steps to be explored for future work:

(1) For all the biosensors, a data-fitting algorithm could be developed for better simulation and prediction of the binding behavior.

(2) The induction leak of biotin synthesis could be addressed by optimizing the cell growth conditions and tuning the components of the synthetic circuit. Also, other biotin genes from the bio operon could be included to eliminate analog concerns, and to further improve biotin synthesis.

(3) Applications for the new biosensor could be explored by reengineering the genetic circuits for different purposes, and by designing advanced microfluidic devices.

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APPENDIX A

The protocol of biotin assay

0) Volume used for 1 plate (96 wells)

1) Prepare solutions:

20 mg/ml in DMSO for both SMCC and SPDP linkers (2mg into 100ul DMSO);

20 mg/ml LC-LC-biotin in DMSO;

10 mg/ml HRP in PBS buffer;

10 mg/ml SA in DI water (or PBS);

10 mg/ml BSA in PBS;

DTT diluted with DI water to make 100mM stock (15.4 mg into 1 ml DIW);

Add EDTA in PBS to make it 5mM (18.6 mg into 10 ml PBS);

0.5% casein in PBS;

20% Tween 80 stock (get rid of nonspecific binding);

50mM of sodium acetate, adjust pH to 5.1 using 3M HCl;

Dissolve TMB in DMSO to make it 1%;

H2O2 in DI water 3%;

2M H2SO4 used to stop reaction;

Washing buffer: PBS with 25ul/50ml 20% Tween 80 (150ml needed for an assay)

2) Add 1.4 µl SPDP to 20 µl SA (molar ration of 1:20), incubate at 37 °C for 1h avoiding light; Then add 2.4 µl of 100mM DTT 1h to 10mM final concentration in the mixture, incubate for another 1h;

3) Mix 200 µl BSA with 17 µl LC-LC-biotin (1:20) and incubate at RT 1.5h;

4) Add 7.5 µl SMCC to 72 µl HRP (1:20), incubate at 37 °C for 1h avoiding light;

5) Transfer the mixtures to Vivaspin tubes, bring volume up with desired buffer and ready for centrifugation at 10x 1000g speed;

6) For SA-SPDP use PBS-EDTA to wash; for HRP-SMCC and BSA-biotin simply use PBS;

7) Spin down SA-SPDP and HRP-SMCC 16 min or until the volume reaches 25 µl at 10x 1000g repeating 5 times, wash with buffer to 50 µl; spin down BSA-biotin 12min or when the volume reaches 100 µl at 10x 1000g repeating 5 times, wash with buffer to 500 µl;

8) Mix SA-HRP (molar ratio SA : HRP = 5:1, simply 25 + 25 ul) together to 50 µl, store in 4 °C and ligate overnight; Store BSA-biotin in 4 °C after bring the volume to 200 µl; dilute SA-HRP 1/4, freeze the rest and keep one tube for SA-HRP and BSA-biotin in fridge.

9) Second day, for 1/5 assay (20ul sample in 80ul conjugates), prepare concentrated PBS-casein of 0.25 g/40ml. (casein needs time to dissolve stirring with heat)

10) Dilute BSA-biotin (and BSA w/o biotin as control, 10ul into 490ul PBS) with PBS to 0.03 ug/ml, add 100 µl to each well (using a culture plate and a multipipette), incubate at 37 °C for 1h with wrap, wash 3 times (pipette 200ul PBS-Tween 80, wait for 2min, then decant the liquid);

11) Take out 24ml of concentrated PBS-casein, add 6ml of PBS to make it 0.05% then block the plate with 200 µl; incubate 1h at 37 °C, wash 3 times (pipette 200ul PBS-Tween 80, wait for 2min, then decant the liquid); Prepare samples while blocking;

12) Take out 12 ml of concentrated PBS-casein, add 7.5ul of Tween 80; (5 ul per 10 ml make it 0.01%)

13) Dilute the SA-HRP mixture to 1/10000 (0.10 ug/ml) with concentrated casein-PBS-Tween 80, add 80 ul to each well.

14) Add 20ul with biotin sample or DIW adjusted biotin/DTB standards (make final content of 0.5% casein and 0.01% Tween 80, could use left PBS-casein from step 11 20ul into standard biotin wells), incubate 1h at 37 °C, wash 3 times (pipette 200ul PBS-Tween 80, wait for 2min, then decant the liquid);

15) Mix sodium acetate/TMB/H_2O_2 = [1000/10/1] to a total of around 20 ml, 200 µl each well immediately after adding H_2O_2 (control background signal), react for 15 min in RT;

16) Use 50 µl 2 M H_2SO_4 to stop reaction and measure absorbance in OD_{450}.

APPENDIX B

PDMS fabrication procedure

(1) Mix 50 g of silicone and 5 g of crosslinkers from the PDMS kit; gently mix then put it into vacuum to get rid of bubbles for 1 h.

(2) Put the microfluidic chip mask on a piece of foil, trim to the mask's size, add mixture of silicone and crosslinkers and vacuum for another 1-2 h.

(3) Take out the mask and PDMS gel, put into the oven at 95 °C for 30 min.

(4) Carefully take off the foil and then separate the make with PDMS.

(5) Use microscope to check whether the chambers and channels are intact.

(6) Punch holes for the inlets and outlets using syringe needles.

(7) Use a penknife to cut the PDMS for desired channels.

(8) Clean the surface of PDMS and a piece of cover glass using DI then ethanol.

(9) Put both of them into the Plasma clean, adjust the valve until the pink-purple light is observed. Then wait for 1 min.

(10) Quickly take out the PDMS and the glass but make sure do not touch their surface. Then quickly attach them together.

APPENDIX C

Beads functionalization GFP protocol

(1) For 10 reactions, take 100 ul beads from stock, wash 3 times with PBS-Tween 80 (950 ul taken each time), keep 80 in volume finally.

(2) Add 10 ul of 1 mg/ml SA and 10 ul of 1M EDC, R.T. react for 1-2 h, using low speed of vortex.

(3) Wash 3 times, take as much supernatant as possible the last time, add 100 PBS to make SA-bead stock.

(4) For each reaction, take 10 ul of SA-beads from stock then add 36.5 ul PBS, add 1ug biotin-DNA, and 2 ul of biotin sample (droplet on the wall, mix DNA and sample then transfer into beads), mix well.

(5) 37 °C reaction for 1h in shaker 400 rpm.

(6) Wash 6 times, take as much supernatant as possible the last time (with 6-10 ul left).

(7) Take 1ul add into 99 ul DIW to count the number of beads in Flow Cytometer (run medium speed of 10 ul, threshold 5000).

(8) Take 2-3 ul (around 100000 beads per reaction) into 7 ul of cell-free expression system (for each aliquotted cell free stock of 17.5 ul, add 4 ul of DIW into solution A, B, then aliquot again to 3 tubes with 7 ul each).

(9) React cell-free expression 4 h in 37 °C with 400 rpm shaking, stands the 500 ul PCR tube of sample in a 1.5 ml EP tube which stands in a 15 ml cell culture tube, 0.5 reaction no shaking, let the beads go down.

(10) Take 5 ul of reaction supernatant into 20 ul of oil, shake in vortex, add 2 ul of

emulsion to a microfluidic chamber (make sure there is only one layer of droplets in chamber).

(11) Observe in microscope, set the exposure time the same (usually 50-100 ms).

(12) To measure GFP intensity, use circular ROI in 3 areas in each sample, extract the background signal besides the ROI of interest.