IBEHS 2P03: Health Solutions Design Projects
Milestone #6

Auto C.A.D.

A Novel Design for the Treatment of Atherosclerosis

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ABSTRACT

Atherosclerosis is the buildup of arterial plaque, resulting in reduced flow of oxygenated blood to the organs, and causes Coronary Artery Disease if it occurs around the heart. A novel design is described herein, utilizing synthetic biology to enhance fibrinolysis in the body. *E. Coli* cells will be modified using PCR, digestion, DNA purification, ligation and transformation. Using Matlab Simbiology, the circuit was modelled for two sets of half-life values. Using GPR and Doc half-life values of 102 and 510 minutes, respectively, Doc and Hly-A-tPA steady-state concentrations were 625 and 10 nmol/L (thrombospondin present). Similarly, for GPR and Doc half-life values of 15 and 5 minutes, Doc and Hly-A-tPA concentrations were 6 and 9 nmol/L, respectively. Without thrombospondin, both runs showed only AmpR, GPR and CREB production; no Doc or Hly-A-tPA. Design validity has been proven; subsequent work will determine specific kinetic constants and explore alternative host options.

1. INTRODUCTION

Atherosclerosis is a serious medical condition caused by the buildup of arterial plaque, which over time may harden and result in narrowing of arterial openings [1]. Eventually, the plaque may rupture and form a mobile blood clot, which can lead to a heart attack or stroke [1]. Plaque formation may be caused by smoking, high blood pressure, high cholesterol, type 2 diabetes, physical inactivity, obesity, high stress levels and high-fat diets [2]. When atherosclerosis occurs near the heart, it can cause Coronary Artery Disease (CAD), which is the second leading cause of death in Canada [3]. Approximately 2.4 million Canadians above the age of 20, live with ischemic heart disease; which is caused by the restriction of blood flow [3].

Synthetic biology was used to design a biological circuit that will enhance the body's own natural process for breaking down blood clots, namely, fibrinolysis. The target output is tissue-type plasminogen activator (tPA), a naturally occurring serine protease that hydrolyzes peptide bonds in plasminogen, between Arg-561 and Val-562, converting it to its active form, plasmin [4]. Plasmin plays a key role in the dissolution of blood clots, breaking down fibrin threads and inactivating factors V and XII, prothrombin and fibrinogen [4].

This circuit, which consists of two DNA plasmids on pBAD backbones, will be inserted into ClearColi™ host cells, which are genetically modified *E.coli* cells that perform protein expression without producing the endotoxin lipopolysaccharide (LPS) [5]. The pBAD backbone contains an AmpR promoter region; this promoter region was replaced with the CREB promoter for plasmid 2 [6]. On plasmid 1, the AmpR promoter is turned on by AmpR protein, to facilitate the production of Glycoprotein Ib-IX-V receptor (GPR) and cAMP Response Element Binding (CREB) protein. The input for this circuit is either Thrombospondin or Von Willebrand Factor; either of these will allow GPR to activate cGMP, therefore, for simplicity, thrombospondin will be considered the only input from this point forward. Once active, cGMP binds to CREB, forming a complex. This complex will then bind to the promoter plasmid 2, triggering the production of the main action proteins; Hly-A-tPA complex and Doc. The target output, tPA is transcribed in series with Haemolysin-A (Hly-A), which is a natural secretion system to allow for extracellular production of tPA [7].

2. MATERIALS AND METHODS

Experimental synthesis and testing has not yet been conducted but will follow the procedures outlined in Appendix A. To test the validity of the proposed design, Matlab Simbiology was used to model reaction kinetics for the circuit; specifically by defining the rates of transcription, translation, and degradation, according to the equations provided in Appendix B.1; a schematic of the model is provided in Appendix B.2. Kinetic constants were found for the following biological parts: AmpR and CREB promoters, AmpR, GPR, CREB, Doc, and Hly-A-tPA proteins, as summarized in Appendix B.3. Specifically, protein degradation constants were calculated using half-life values; CREB, Hly-A-tPA and AmpR half-life values were taken from experimental data collected under comparable conditions, whereas generic half-life values were used for GPR and the Doc protein. These generic values have a large range, and therefore, the non-specific degradation constants calculated were not as representative of this particular model's kinetic behaviour. Since these half-life values differ substantially compared to the more accurate literature-obtained values, a second set of results was collected, using new values for GPR and Doc protein half-life; 15 min and 5 min, respectively. Thrombospondin acts as the input for this system since its presence dictates that action of GPR on cGMP to allow the formation of the cGMP-CREB complex required to turn on the second plasmid.

3. RESULTS AND DISCUSSION

This biological circuit consists of two plasmid backbones, each with sequences illustrated in Figure 1 below. On plasmid 1, the AmpR promoter is regulated by AmpR protein, already present in the host cells. This triggers the transcription of AmpR, to ensure the promoter remains active, glycoprotein (GP) receptor protein complex, and cAMP Response Element Binding (CREB) protein. Transcription and subsequent translation of the GPR complex leads to the activation of cGMP through natural cellular processes. This occurs when the GPR complex comes into contact with thrombospondin, the system's input. cGMP binds to CREB, forming a cGMP-CREB complex. This complex induces transcription on the second backbone by binding to the CREB promoter; triggering transcription of Hly-A-tPA and Doc proteins. The Hly-A that is transcribed with the tPA allows tPA to be released from the host cells [7]. The tPA can then move to the site of a blood clot and initiate its dissolution. Doc protein enables the activation of ppGpp, which inhibits the antitoxin of a MazE/F killer mechanism (illustrated in Appendix C.1) in the host cells, allowing for destruction of the host bacteria cell once sufficient production of tPA has occurred [8][9].

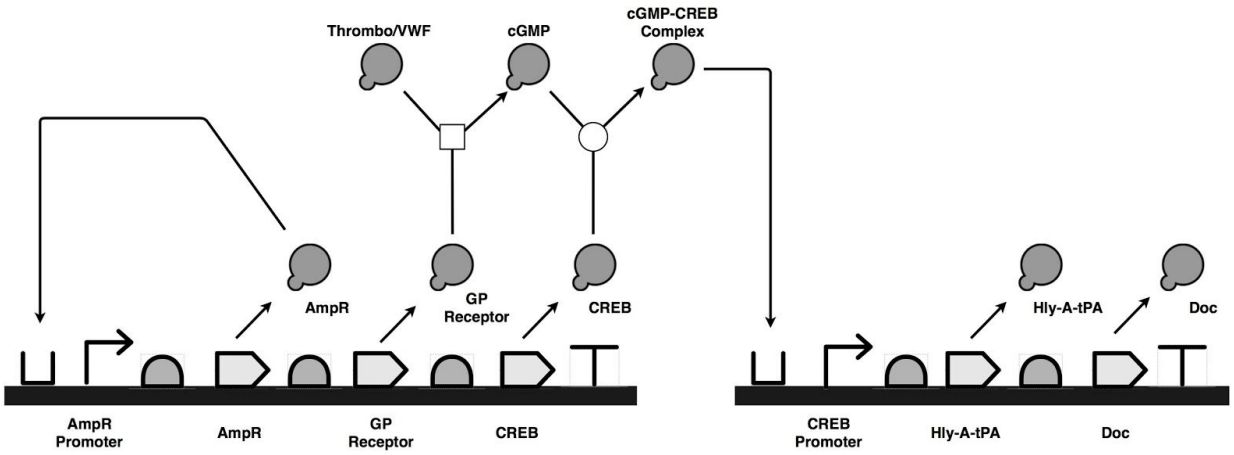

Figure 1 Synthetic Biology Open Language (SBOL) schematic that depicts two plasmid sequences that operate in series as a single biological circuit designed for the treatment of atherosclerosis; illustrates the action of protein products in the activation of promoter regions to facilitate downstream processes

Figure 2 shows the model's output for generic half-life values. As expected, in the presence of thrombospondin (Figure 2A), all components are produced. CREB and GPR reach steady state concentrations of 20 nmol/L and 140nmol/L, respectively. Concentration of AmpR remains constant at 10 nmol/L; this is required for regulation of the promoter on plasmid 1. The concentration of Doc protein quickly increased to 625 nmol/L, while Hly-A-tPA only reached 10nmol/L. In the absence of thrombospondin (Figure 2B), no Doc or Hly-A-tPA production is seen. Without thrombospondin, the GPR will not activate cGMP, preventing the formation of the cGMP-CREB complex required to initiate transcription on the second plasmid.

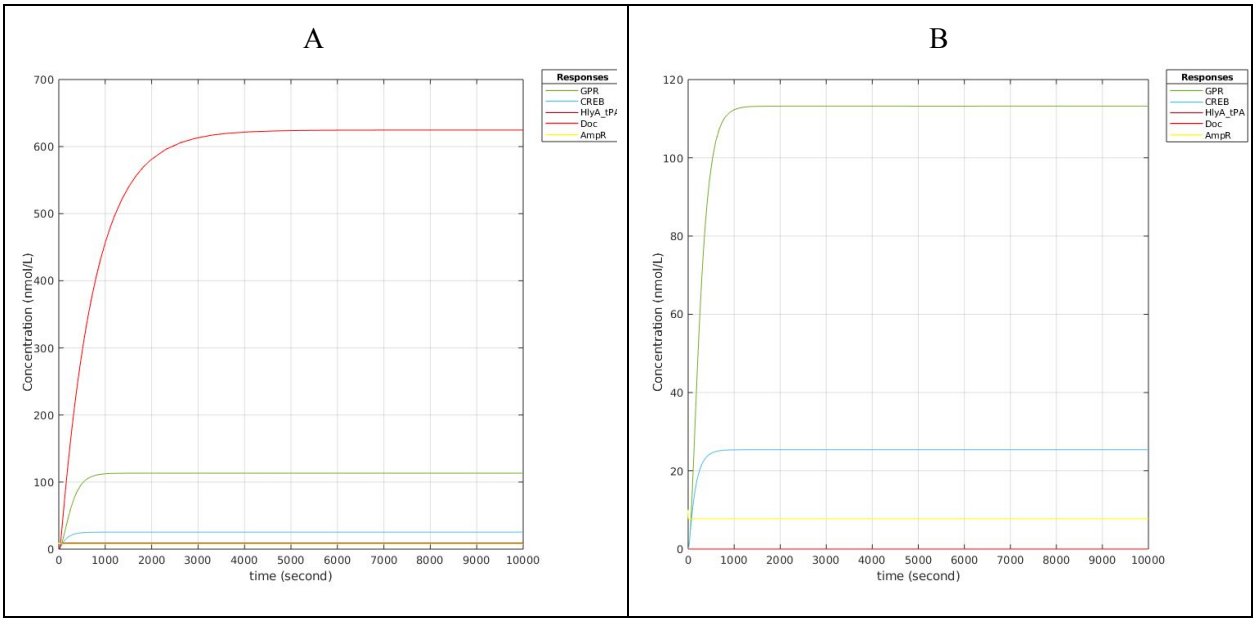

Figure 2 Displays the model's outputted concentration (nmol/L) of AmpR, GPR, CREB, Hly-A-tPA, and Doc proteins, as a function of time, with GPR and Doc half life values set to 102 min and 510 min, respectively; (A) in the presence of thrombospondin, and (B) in the absence of thrombospondin.

Figure 3 shows the model's output after adjusting the half-life values of GPR and Doc; new values were estimated based on the range of values from other proteins in the biological system, for which experimental data was found. As expected, this adjustment had no impact on which proteins were produced for a given input (i.e. thrombospondin present or absent); however, relative protein concentrations did change. In the presence of thrombospondin (Figure 3A), Doc reaches a stable concentration of only 6 nmol/L, compared to the 625 nmol/L it achieved with the generic half-life value (Figure 2A). Similarly, GPR concentration stabilized at 16 nmol/L, compared to 140 nmol/L originally reached. As expected, in the absence of thrombospondin, Figure 3B displays no production of Doc protein or the Hly-A-tPA complex.

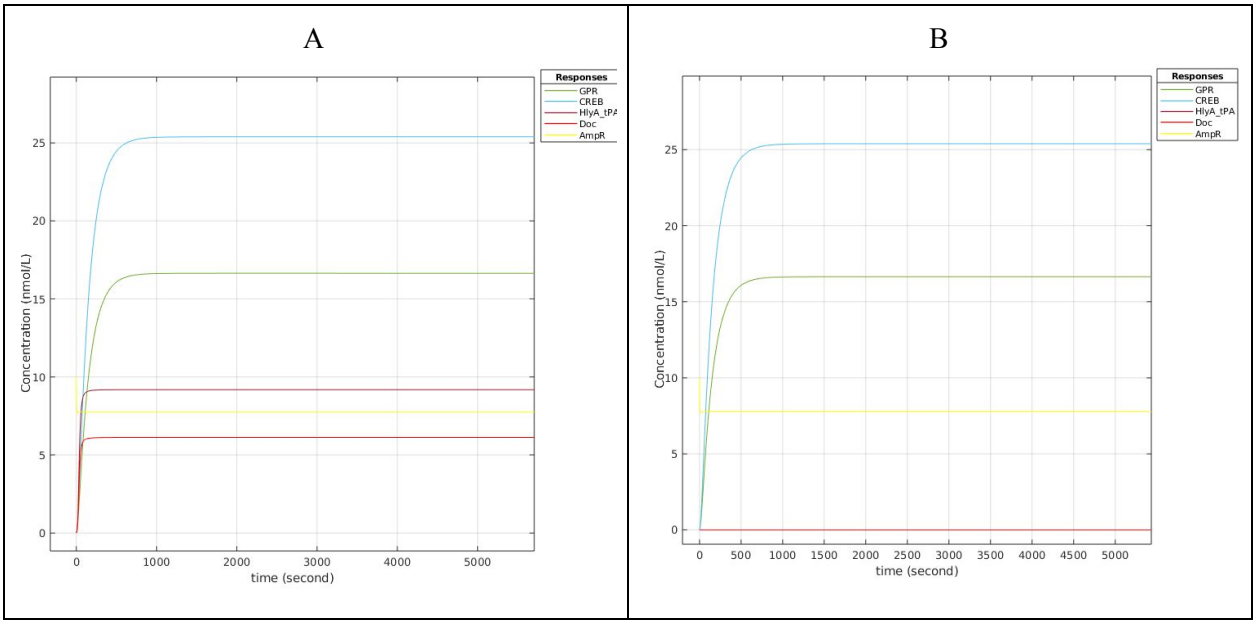

Figure 3: Displays the model's outputted concentration (nmol/L) of AmpR, GPR, CREB, Hly-A-tPA, and Doc proteins, as a function of time, with GPR and Doc half lifes set to 15 min and 5 min, respectively; (A) in the presence of thrombospondin and (B) in the absence of thrombospondin.

Degradation rate is inversely proportional to half-life according to Equation B.7 in Appendix B.1; therefore, a higher half-life value indicates that the protein will remain in the cell for longer before degradation occurs. Based on this observation, it is expected that protein products with higher half-life values will reach higher steady state concentrations; this result is demonstrated by contrasting Figures 2 and 3. In Figure 2A, the concentration of Doc protein is more than 60 times that of Hly-A-tPA. To determine the exact concentration of Doc needed to trigger the MazEF complex to perform apoptosis of the *E.Coli* cells, kinetic data would need to be collected experimentally. However, since Doc reaches its significantly larger concentration, relatively early in the cell's lifespan, it can be inferred that cell death would be triggered before sufficient production of Hly-A-tPA could occur. For a smaller half-life value (Figure 3), Doc production is inhibited, therefore allowing proper function of the circuit; tPA production

exceeds that of Doc. It is crucial that tPA production exceed Doc production to ensure that cell death will not occur prematurely, before sufficient production of tPA. A lower steady state concentration of Doc allows significantly more production time for Hly-A-tPA. This is expected to result in more effective treatment of atherosclerotic plaque; higher levels of Hly-A-tPA in the blood will lead to enhanced plaque dissolution.

Numerous limitations were encountered throughout the design process for the Simbiology model used. As stated previously, research resulted in generic inaccurate approximations for some half-life constants; more precise half-life values would yield better results from the model. Another unknown is the relative concentration of tPA needed within the bloodstream to effectively break down atherosclerotic plaque. Since this value likely varies depending on the patient's condition, it is difficult to predict. However the required tPA concentration will dictate whether or not the current proposed design would be effective; ensuring that the host cells are not broken down before sufficient tPA levels are achieved. Also, the exact mechanism by which Doc protein acts on *E.coli* cells is not well understood, and therefore requires further research. Finally, the exact concentration of AmpR required to turn on the promoter on Plasmid 1 is unknown; further studies must be conducted to determine minimize this uncertainty within the model's design.

4. CONCLUSION

Overall, the circuit behaved as expected within the model. In the presence of thrombospondin, both Doc and Hly-A-tPA were produced. When thrombospondin was absent, only the products for the first plasmid were produced; specifically, AmpR, GPR and CREB. These two cases were simulated for both inflated generic half-life constants as well as more reasonable adjusted values. Once the correct kinetic constants are determined experimentally, more accurate concentrations of each protein output can be modelled.

Several unknowns remain regarding the practical function of this proposed biological circuit. For example, details about the action of Doc are not fully understood; kinetics must be explored to ensure that Doc production rate fits within the desired tPA production time frame, to prevent damage within the body. Another challenge is ensuring that the body does not reject the modified host cells, because this could cause sepsis and lead to death. *E. Coli* was used as the model host to prove the validity of this design, however, mammalian cells would be more suitable for clinical purposes. Although more complex, this alternative host would promote better reception in the body. Finally, after laboratory procedures are completed to create the modified cells, the product must be tested in vitro to prove its effectiveness and later in vivo to obtain approval for medical use.

In practice, the modified cells would be delivered via intravenous injection by a health professional in a hospital, where the patient could be monitored. The frequency of treatments would vary depending on the severity of each patient's condition. This biological circuit takes advantage of the body's natural process for blood clot dissolution. The cells are designed to detect when they are in the bloodstream, since they require an input of thrombospondin to initiate, and the DNA sequence of each plasmid was specifically designed to build off each transcript to allow for the release of Tissue

Plasminogen Activator. This novel design for the treatment of atherosclerosis, which provides a unique method for breaking down blood clots, could be extremely valuable in preventing Coronary Artery Disease, and therefore should be pursued in further research.

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APPENDIX A

A.1 Experimental Materials & Methods - Polymerase Chain Reaction

Material List

- DNA templates for tPA, HlyA and Doc (Bacteriophage P1) genes
    - See 2.2.2 for Doc gene restriction digest.
- Stock solution of primers listed in Table 1
- Restriction Enzymes: HpaI (tPA, Doc), EcoRI (tPA, HlyA), SphI (HlyA), BcII (Doc)
- Nuclease free water
- Taq 2X Mastermix from New England Biolabs containing:
    - Taq DNA Polymerase
    - $MgCl_2$
    - dNTPs

Methods

DNA for the following genes were amplified using Polymerase Chain Reaction (PCR); Haemolysin (Hly) A, tissue-type plasminogen (tPA), and Doc. Denaturation was performed at 92-94℃ to separate the DNA. The forward and reverse primer sequences, shown in Table 1 below, were used during the annealing step, conducted at 37-72℃; primer sequences were ordered from Thermo Fisher Gene Art. Taq DNA polymerase was used for extension, at 72℃. Methods may be repeated for 10-20 cycles depending on the supply required. Once completed, gel electrophoresis will be used to verify PCR products.

Table 1 Summary of forward and reverse primers to be used for PCR

DNA Template	Forward Primer Sequence	Reverse Primer Sequence
Hly A	5'-ATGCCAAAACTCAATCGTTGC-3'	5'-TTAGTTCAAATCAAATTGAACCC-3'
tPA	5'-GTTAACATTTTATTGCACTGACT-3	5'-GAATTCCTGCCCTGGGCTT-3
Doc	5'-ATGAGGCATATATCACCGGAAGA-3'	5'-CTACTCCGCAGAACCATACA-3'

A.2 Experimental Materials & Methods - Restriction Digests

Material List

- Buffer R from Thermo Fisher
- PCR Products from the previous step

Methods

Restriction digest reactions were performed for both plasmid backbones at 37℃ for 45 minutes. Three reaction tubes were prepared for each backbone; tube 1 for the DNA backbone (pBAD LIC), tube 2 for our amplified genes or pre-amplified genes ordered from a supplier, and tube 3 as a control with the uncut backbone. All tubes also included nuclease free water, 10X RE buffer R; tube 1 and 2 included restriction

enzymes, but tube 3 did not. Following reaction completion, tubes were spun down in a centrifuge. A similar procedure will be used to extract the Doc gene from bacteriophage P1 for use in the genetic system.

A.3 Experimental Materials & Methods - DNA Purification

Material List

- PureLink PCR Purification Kit from Thermo Fisher containing:
    - Elution Buffer
    - Wash Buffer
    - Binding Buffer
    - Isopropanol
    - Ethanol
- PCR Products

Methods

DNA was purified using the PureLink PCR Purification Kit. Binding buffer with isopropanol was combined with each PCR product in PCR spin columns and centrifuged at room temperature; the flowthrough was discarded. Next, the DNA was washed using a wash buffer containing ethanol; the residual wash buffer was removed. Finally, an elution buffer was added to the DNA to permit extraction.

A.4 Experimental Materials & Methods - Ligation

Material List

- T4 DNA Ligase from Thermo Fisher
- T4 DNA Ligase Buffer from Thermo Fisher
- Promoter DNA from Thermo Fisher Gene Art
- Glycoprotein (GP) lb-IX-V Receptor DNA
- Human CREB Protein DNA
- Gibson Master Mix

Methods

Ligation reactions were completed at room temperature for 10 min - 2hr. Inserts were ligated in separate procedures to ensure proper order on the backbone. Reaction tubes contained backbone DNA, PCR gene products or supplier provided inserts (CREB promoter, CREB protein), ligase buffer, T4 DNA ligase, and water. Controls were prepared the same way, without the DNA inserts.

The Gibson Assembly method was used specifically for ligation of the Glycoprotein (GP) receptor DNA; this process was used to attach the individual segments of DNA that encode for the 4 subunits that make-up the GPR. It is useful for attaching DNA without leaving a scar (i.e. no gap between adjacent genes). PCR was used to create the overlap between adjacent segments, and then samples were left to incubate in the Gibson master mix. This process creates an overhang on each end, anneals them together and closes the gaps, resulting in a complete gene sequence that codes for the entire GPR.

A.5 Experimental Materials & Methods - Transformation

Material List

- ClearColi™
- DNA plasmids from previous step

Methods

Heat shock transformation is used to insert the modified plasmids into the *E. Coli* cells, by opening the pores of the cell membrane to allow DNA to enter. The plasmids will not be functional outside of the cells. Ligation products were combined with *E. Coli* cells, and tubes were incubated on ice for 10 minutes. Cells were heat-shocked at 42C for 20 seconds and then moved immediately onto ice for 2 minutes. Growth medium was added to the samples, followed by incubation at 37C for 30 minutes in the cell shaker. Finally, cells were plated on agar plates and spread; plates were incubated at 37C for 24 hours, re-plated in fresh media, and grown for another week.

A.6 Experimental Materials & Methods - Product Verification

Material List

- tPA Human ELISA Kit
- Final Bacterial Product
- Thrombospondin

Methods

Once the *E. Coli* cells have colonized and adequate time has passed to allow for the production of GPR and CREB protein, verification procedures were conducted. For the modification to be successful, the *E. Coli* cells must be able to recognize the presence of Thrombospondin (Thr), and trigger the production and release of tPA via the sequence of events outlined in section 1. The validity of our system was tested in vitro by introducing the bacteria to the signalling molecules (VW Factor and Thr) and measuring the amount of tPA produced, using an Enzyme-Linked Immunosorbent Assay (ELISA).

APPENDIX B

B.1 Rate Equations for Simbiology Model

$$Rate\ Transcription\ =\ k0_{tr} + (k_{tr}\frac{P^n}{K^n})/(1 + \frac{P^n}{K^n}) \quad \text{(B.1)}$$

$$k_{tr} = tps_{active} - tps_{repr} = tps_{active} \quad \text{(B.2)}$$

$$k0_{tr} = tps_{repr} \quad \text{(B.3)}$$

$$Rate\ Translation\ = k_{tl}[mRNA] \quad \text{(B.4)}$$

$$k_{tl} = \frac{translation\ efficiency}{mRNA\ half\ life} \quad \text{(B.5)}$$

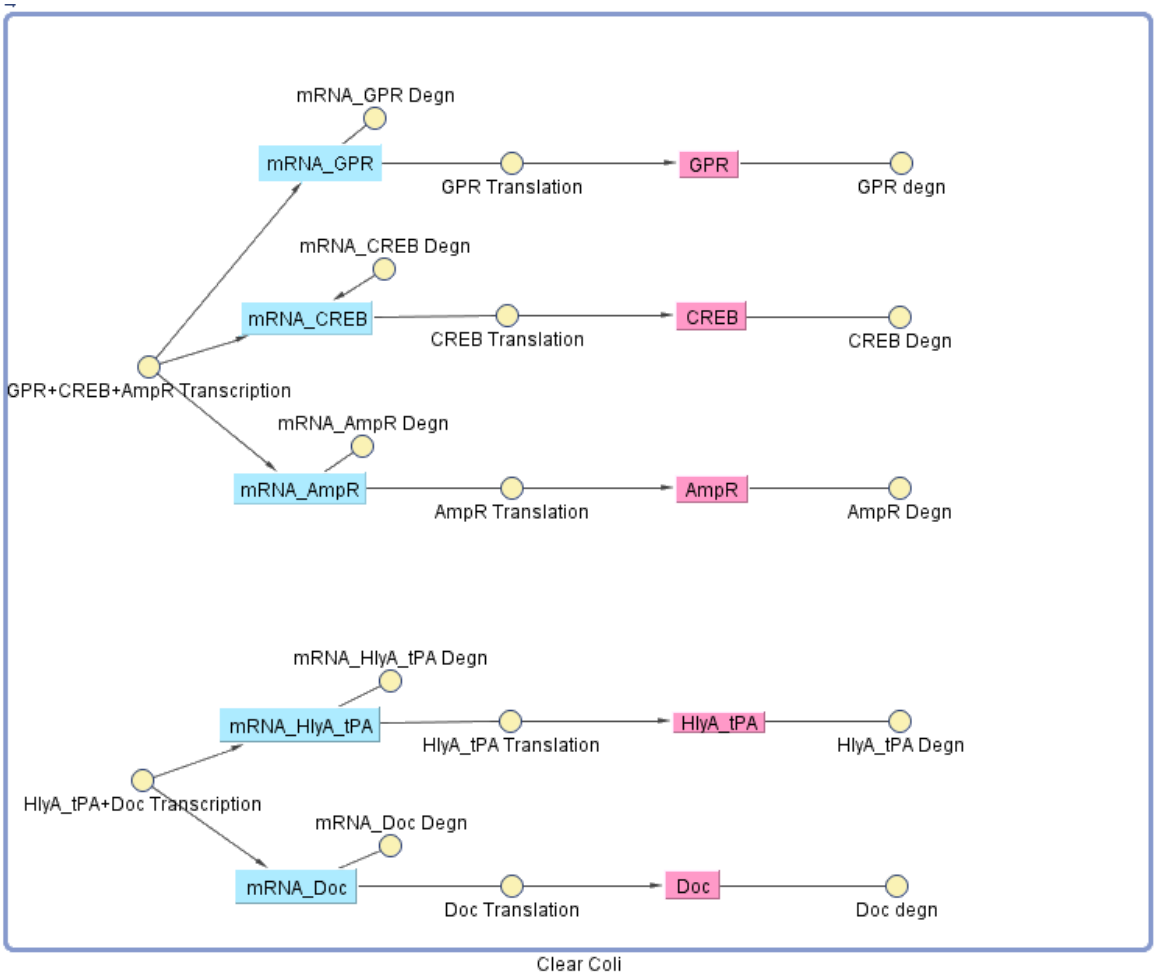
$$Rate\ Protein\ Degradation\ =\ kd_{prot}[Protein] \quad \text{(B.6)}$$

$$kd_{prot} = \frac{log(2)}{protein\ half\ life} \quad \text{(B.7)}$$

$$Rate\ mRNA\ Degradation\ =\ kd_{mRNA}[mRNA] \quad \text{(B.8)}$$

$$kd_{mRNA} = \frac{log(2)}{mRNA\ half\ life} \quad \text{(B.9)}$$

B.2 Matlab Simbiology Model



B.3 Summary of Kinetic Constants used in Simbiology Model

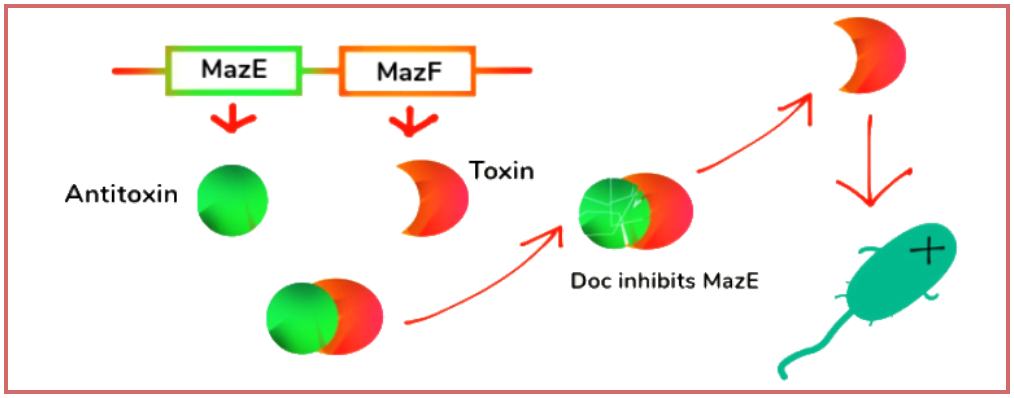
Component	Symbol	Value of Constant	Source	Rationale
Transcription Constant (KCTranscription)				
Plasmid 1 + 2	tps_active	1.8 nmol/L-min (0.03 nmol/L-hr)	[10]	• KCTranscription is calculated as tps_active - tps_repr • Assume tps_repr (rate of transcription during repression) is zero for this system • Value is for generic transcription in the same host cell (*E. Coli)* • See Equation 2
Translation Efficiency				

Plasmid 1 + 2	Translation efficiency	3.33 protein/mRNA/min (200 protein/mRNA/hour)	[11]	• This value is taken from the clearcoli manual
Activator/Repressor Coefficient (K)				
AmpR Promoter	K1	2.6 nmol/L	[10]	• Similar promoter-operator complex • Same host cell (*E. Coli*)
CREB Promoter	K2	1.7 nmol/L	[12]	• Experimental data for same protein-DNA binding complex
Hill Coefficient (n)				
AmpR Promoter	n	2	[13][14]	• Assume ideal cooperative binding Hill Coefficient
CREB Promoter	n	2	[13][14]	• Assume ideal cooperative binding Hill Coefficient
mRNA half-life				
GP Receptor mRNA	Degn_mRNA_GP	564 min	[15]	• Assume specific mRNA half-life for Glycoprotein (GP) Ib-IX-V is similar to that of another glycoprotein mRNA half-life • Both are transmembrane proteins from the same family with similar properties
CREB mRNA	Degn_mRNA_CREB	378 min	[16]	• Assume mRNA half-life for CREB mRNA is similar to that of ATF mRNA • Both are transcription factors with similar properties
Hly-A-tPA mRNA	Degn_mRNA_TPA	3.2 min	[17]	• Source states the degradation constant of tPA as 0.0036 when not in the presence of plasminogen • Half-life was calculated from Equation 7
Doc mRNA	Degn_mRNA_DOC	10.33 min	[10]	• Average bacterial mRNA half-life value assumed • Half-life ranges from 40 sec to 20 min; average value 10.33 min used
AmpR mRNA	Degn_mRNA_AMP	10.33 min	[10]	• Average bacterial mRNA half-life value assumed

				• Half-life ranges from 40 sec to 20 min; average value 10.33 min used
Protein half-life				
GP Receptor	Degn_GP	102 min	[10]	• Average mammalian protein half-life value assumed • Half-life ranges from 0.4-3 hrs; average value 1.7 hr used
CREB	Degn_CREB	15 min	[18]	• Source explicitly states half-life for CREB protein is 10-20 min • Average value 15 min used
Hly-A-tPA	Degn_TPA	7.5 min	[19]	• Hly-A-tPA half-life in *E. Coli* estimated to equal that in human bloodstream • System will operate in bloodstream; therefore, similar degradation kinetics can be assumed
Doc	Degn_DOC	510 min	[10]	• Average bacterial protein half-life value assumed
AmpR	Degn_AMP	7 min	[20] [21]	• Half-life of AmpR approximated as half-life of the enzyme beta-lactamase • This enzyme hydrolyses beta lactam ring in antibiotics with this structure

Appendix C

C.1 MazE/F Killer Mechanism



Reproduced from [22]