HORMONIC: An artificial close-loop system for hormonal homeostatic regulation

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***Abstract*- The project consists on developing a device that can regulate thyroid hormone imbalances. This device will be designed to sense triiodothyronine (T3) at skin level through our engineered *Escherichia coli (E. coli)*, which will contain an intein-mediated protein splicing linked to eGFP expression. A DIY turbidostat will be considered to maintain the cellular culture conditions constant. eGFP luminescence will be electronically sensed and , based on this measurement, a PDI control system will generate a feedback response so as to self-regulate and restore the normal levels of T3 hormone in the human body.**

***Index Terms*-** AHL, Close-loop, eGFP, Feedback, iGEM, Intein, Lactone, sfGFP, T3 & Thyroid.

I. INTRODUCTION

The endocrine system is responsible for controlling the balance of hormones in the bloodstream by the use of intrinsic feedback loops, which can be disrupted leading to disease. Among these disruptions, thyroid disease is one of the most common, affecting millions of individuals throughout Europe, 5% of the general population [1]. Over 99% of affected patients suffer from hypothyroidism and are mainly prescribed levothyroxine: a medication that has a narrow range of doses at which is effective without adverse effects [2]. As a consequence, one-third of the patients who receive this treatment still exhibit symptomes.

Therefore, several studies have concluded that there is a clear need for patient-specific dosage optimization [1]. However, current medications mainly offer 11 possible dosages that cover the range of 25 µg to 200 µg [3] and are administered once a day, which causes difficulties when adjusting the levels of thyroid hormones with respect to the real homeostatic conditions.

Consequently, the aim of this study is to restore the feedback system by focusing on the development of a medical device similar to the insulin pump. Since T3 is a thyroid hormone, more active than thyroxine, sensing it constantly will give us a lot of information on how the feedback is operating, something that cannot be done regularly by conventional blood tests . Therefore, our reporter bacteria will sense T3 by producing a recombinant protein that contains a mini-intein domain (ΔI-SM). Inteins allow for protein splicing, and when activated, they produce a ligation of C-terminal and N-terminal external proteins [4]. This allows that in presence of T3, the mini intein will fold to attach the two halves of an enhance Green Fluorescence Protein (eGFP), thus creating a functional eGFP which, when excited, will emit a fluorescence that can be captured by the use of a photodiode. The obtained value can be then compared to a reference value (that could be previously defined by an endocrinologist) and the system will inject levothyroxine until the optimal concentration is reached.

II. *RESEARCH ELABORATIONS*

2.1. Intein mediated T3 sensing

To quantify the amount of T3 in the body we need to express a marker with an intensity proportional to the quantity of T3 present at the skin level. It has been proven that the previously engineered ΔI-SM mini-intein, derived from the Mycobacterium tuberculosis RecA wild-type intein (Mtu recA), can be modified so that it is activated by the T3 hormone [4]. This is possible by replacing residues 110-383 of the Mtu recA sequence by the thyroid receptor β1 (TRβ1) sequence, that will be acting as a ligand binding domain [5].

Considering the wide usage of the green fluorescence protein (GFP) as a reporter and its capabilities to be linked to an optogenetic circuit makes GFP a great tool for our purposes. Given the splicing activity of the inteins to fuse the two subunits of a protein linked to its terminals, and the usage of enhanced green fluorescence protein (eGFP) for this purpose, eGFP was spliced at the 70 residue, so that the residue at the N terminal linked to the modified intein is a cysteine, as it seems to give better results [6].

Having such a large protein complex when eGFP is fused to the intein can result in the formation of inclusion bodies and, therefore, eGFP could not be visible [7]. The novel Fh8 system used as a solubility tag can help to avoid the formation of these inclusion bodies when added to the terminals of the protein complex [8] (figure 1).

As the intein activity happens at the post-translational level, the constitutive promoter BBa_K880005 was added to the previous construct (splitted eGFP + modified ΔI-SM mini-intein + Fh8 tag), so that the expression of eGFP is only dependent on the activation of the intein by the fusion of the T3 hormone to the TRβ1 ligand binding domain.

Finally, the Flagx3 tag was added at the end the end of the construct to be able to inmuno detect its expression afterwards

and to see if the splicing is being done correctly depending on the molecular weight of the tagged protein.

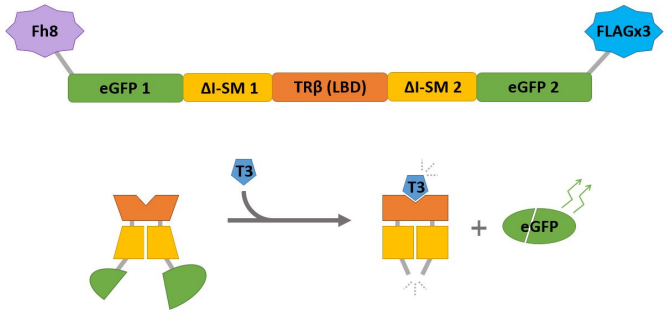

FIGURE 1: Construct of the T3 reporter cell containing the thyroid receptor (TRβ) in the middle, the 2 halves of the mini intein (ΔI-SM) and the 2 halves of the eGFP that will be spliced into a functional eGFP.

2.2. T3 sensing modelling

$$\frac{d[T3]}{dt} = m - \beta\,[T3][\,P\,] - \delta_{T3}[T3] \quad (1)$$

$$\frac{d[P]}{dt} = \alpha_p mRNA - \beta\,[T3][\,P\,] - \delta[P] \quad (2)$$

$$\frac{d[GFP]}{dt} = \alpha_{GFP}\;\beta\,[T3][\,P\,] - \delta_{GFP}[GFP] \quad (3)$$

In order to model the T3 reporter cell, a system of ordinary differential equations (ODE), that took into account the underlying dynamics of the T3 sensing construct was designed (equations 1-3). Because our construct (Figure 1) is produced at a constant rate the mRNA levels could be considered as in a steady state.

As T3 is a small hormone it can cross through the cell membrane via simple diffusion. This implies that the T3 concentration inside and outside is always the same, and thus the constant (m) represents the rate of entry of T3 into the system (1). In equation 2, T3 is consumed in contact with the construct protein (P) at a constant rate (β) and T3 is degraded at at a constant rate (δ_{T3}).

The construct protein (P) is produced at a stable rate (α_P) when in contact with mRNA (2) and is also consumed in contact with T3 at a constant rate (β). As with T3, the construct protein (P) is degraded at at a constant rate (δ).

In equation 4 the mini intein folds to form GFP at a stable rate (β) when T3 and the construct protein (P) are in contact. Due to the fact that the folding of the intein, to produce the splicing of the two halves of the GFP is not 100% reliable, a probability that the folding of the intein generates a functional GFP has been introduced (α_{GFP}).GFP is degraded at at a constant rate (δ_{GFP}).

2.3. Turbidostat

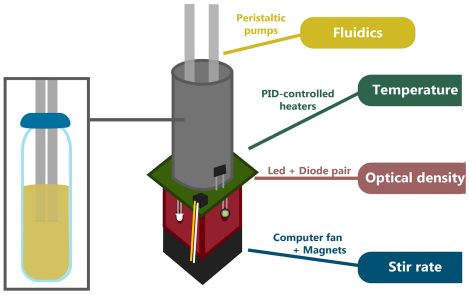

FIGURE 2: Turbidostat currently in development (circuit not included in the image).

To prove that our feedback system can work for extensive amounts of time, a turbidostat had to be built to ensure that cells are able to survive long enough. This device is based on the eVOLVER turbidostat designed by Wong B. et al. [9]. To do so, we counted with the help of the PhD Guillermo Nevot, who already built an imitation of the eVOLVER turbidostat achieving great results and could advise us during the whole process.

The structure of the turbidostat is compounded by five pieces: a 12V computer fan, a double methacrylate layer, a 3D printed integrated sleeve, an aluminium tube and a PCB (printed circuit board) or breadboard.

The 12V computer fan is used to rotate a stirring magnet placed into the vial where the cells are contained, thus we can keep the medium homogenous, which is a key aspect when measuring the OD (optical density) of the contained culture. To do so two small magnets are stuck in opposite blades of the fan. The methacrylate layer is used in order to separate the blades of the fan from the bottom part of the vial. The aluminium tube due to its thermal conductivity was used to keep the cells at an optimal temperature.

The 3D printed piece that covers part of the aluminum tube includes holes that are used to introduce the LED and the photodiode used to obtain the OD. This allows to avoid as much external visible light as possible, as it can interfere in the photodiode measures. These measures are crucial since they will determine what the turbidostat will do to reach a desired value of OD set by the user. Thus, the turbidostat can either wait for the cells to reproduce so that the OD increases or dilute the culture with LB (lysogeny broth) medium to decrease the OD. Fixing the OD of the culture at a certain level ensures that a long term *in vitro* experiment can be performed without being influenced by the cells concentration.

Finally, a PCB or breadboard is used to provide a structure where all the components that synchronize the activity of the turbidostat are included: which are resistors, capacitors, transistors, LEDs for the OD and the GFP excitement.

Nevertheless, although a detailed description of the circuit is out of the scope of this summary article, it has to be stated that all the control of the electronic components of the turbidostat is carried out with Arduino, an open-source electronic prototyping platform, and through a sophisticated software called Firmata Node-Red. This software was programmed to control the main parameters of the turbidostat by some proportional-integral-derivative (PID) controller. The basic idea behind a PID controller is to read a sensor, then compute the desired actuator output by calculating proportional, integral, and derivative responses and summing those three components to compute the output.

III. RESULTS AND FINDINGS

3.1 T3 Sensing

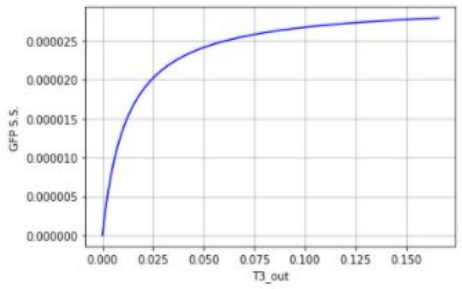

FIGURE 3: Final GFP steady states of with respect to T3_out

$$GFP = \frac{\alpha_{GFP}\,\beta\,T3\,\gamma_P}{\delta_{GFP}(\beta T3+\delta_p)} \qquad (5)$$

In order to evaluate the viability of the T3 reporter cell as a sensor the transfer function (5) of the ODE system was evaluated (figure 3).

3.2 Turbidostat

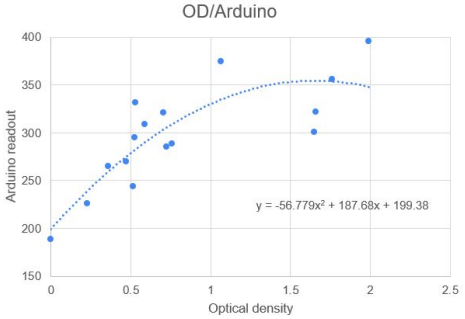

FIGURE 4: Transfer function between our arduino OD sensor and the optical density measured in a spectrophotometer

As our turbidostat needs to maintain a constant optical density (OD) in time a transfer function must be drawn between our arduino OD sensor and the optical density measured in a spectrophotometer (figure 4). This allows us to measure the optical density of the culture in the turbidostat using our arduino OD sensor, thus allowing an implementation of a fast PID controller in the Arduino to control the OD by pumping out old media with cells and pumping in new fresh media, from an erlenmeyer, at the turbidostat.

IV. DISCUSSION

This current project constitutes the first steps towards continuous in vivo hormone autoregulation; developing a small electronic device located on the skin that gives your body the regular levothyroxine it needs. The obtained first results from the plate reader showed that it is possible to correlate T3 hormone levels in an external medium to GFP fluorescence, however, reading this value with the electronic circuit incorporated in the proposed DIY turbidostat is still in progress. The implemented PID code on the turbidostat has experimentally shown to provide a robust, fast and accurate response over the control of the temperature and the management of the peristaltic pump responsible for delivering the right T3 hormone value. These findings enable progress towards developing a revolutionary therapy to hormonal imbalances using the demonstrated fully automatic feedback system.

Even though initial results seem promising this study has encountered some difficulties and limitations. On the one hand, the specificity and sensibility of our biosensor need to prove to be high enough not to cause a big error in the T3 hormone administration pump system. In other words, this study must demonstrate that the GFP luminescence is capable of providing a reliable indirect measurement of the level of T3 hormone in the body. Since this project aims at finding a revolutionary therapy applied to the complex human body, it needs to be highly secure and accurate. On the other hand, since this study deals with engineered bacteria as biosensors for a future human therapy, a reliable and practical method for biocontainment needs to be contemplated.

Further work to be done in this area constitutes a deeper characterization of the inteins and the study of strategies for making them more specific. This project also considers the use of the inteins design pipeline for making it specific to not only to T3, but also to other hormones. Moreover, a deeper study is proposed on the implementation and optimization of the final design, that is, the miniaturization of the final medical device; as well as developing a user friendly GUI to allow clinicians to remotely control the settings of the device.

V. CONCLUSION

All in all, ‘Hormonic’ device would be able to sense the patient’s levels of T3 and regulate its concentration in order to reach normal levels of this hormone. This would mean a patient-specific treatment for the patient and thus, and an improvement in the quality of life. Nevertheless, this project constitutes the first steps into this kind of therapy for thyroid imbalances, and so, further advances in its development will be finished at the end of the iGEM 2020 project.

REFERENCES

[1]: Chiovato, L., Magri, F. & Carlé, A. Hypothyroidism in Context: Where We've Been and Where We're Going. Adv Ther 36, 47–58 (2019). https://doi.org/10.1007/s12325-019-01080-8

[2]: Fliers, E., Demeneix, B., Bhaseen, A., & Brix, T. H. (2018). European Thyroid Association (ETA) and Thyroid Federation International (TFI) Joint Position Statement on the Interchangeability of Levothyroxine Products in EU Countries. European thyroid journal, 7(5), 238–242.

[3]: Gottwald-Hostalek, U., Uhl, W., Wolna, P., & Kahaly, G. J. (2017). New levothyroxine formulation meeting 95-105% specification over the whole shelf-life: results from two pharmacokinetic trials. Current medical research and opinion, 33(2), 169–174. https://doi.org/10.1080/03007995.2016.1246434

[4]: Skretas, G., & Wood, D. W. (2005). Regulation of protein activity with small-molecule-controlled inteins. Protein science : a publication of the Protein Society, 14(2), 523–532. https://doi.org/10.1110/ps.04996905

[5]: Gierach, I., Li, J., Wu, W. Y., Grover, G. J., & Wood, D. W. (2012). Bacterial biosensors for screening isoform-selective ligands for human thyroid receptors α-1 and β-1. FEBS open bio, 2, 247–253. https://doi.org/10.1016/j.fob.2012.08.002

[6]: Tornabene, P., Trapani, I., Minopoli, R., Centrulo, M., Lupo, M., de Simone, S., Tiberi, P., Dell'Aquila, F., Marrocco, E., Iodice, C., Iuliano, A., Gesualdo, C., Rossi, S., Giaquinto, L., Albert, S., Hoyng, C. B., Polishchuk, E., Cremers, F., Surace, E. M., Simonelli, F., … Auricchio, A. (2019). Intein-mediated protein trans-splicing expands adeno-associated virus transfer capacity in the retina. Science translational medicine, 11(492), eaav4523. https://doi.org/10.1126/scitranslmed.aav4523

[7]: Gangopadhyay, J. P., Jiang, S. Q., & Paulus, H. (2003). An in vitro screening system for protein splicing inhibitors based on green fluorescent protein as an indicator. Analytical chemistry, 75(10), 2456–2462. https://doi.org/10.1021/ac020756b

[8]: Costa S, Almeida A, Castro A and Domingues L (2014) Fusion tags for protein solubility, purification, and immunogenicity in Escherichia coli: the novel Fh8 system. Front. Microbiol. 5:63. doi: 10.3389/fmicb.2014.00063

[9]: Wong, B., Mancuso, C., Kiriakov, S. et al (2018). Precise, automated control of conditions for high-throughput growth of yeast and bacteria with eVOLVER. Nat Biotechnol 36, 614–623. https://doi.org/10.1038/nbt.4151