Reengineering the catabolite activator protein system creates a glucose-inducible promoter in E. Coli

YASH PERSHAD, RISHABH KAPOOR, ALEX DUNCAN BRADFIELD, TEAGHAN COWLES
STANFORD UNIVERSITY

Type 1 diabetes mellitus (T1D) is an endocrine disorder that affects over 1.25 million Americans. Safe, effective T1D treatments should mimic natural homeostatic functions by continually monitoring blood glucose levels (BGLs) and secreting an amount of insulin appropriate to current BGL. We envision a glucose-sensitive insulin-administration device to replace the function of damaged beta cells in T1D patients. Here, we prototyped a DNA-based glucose biosensor to eventually connect with an insulin actuator. We re-engineered the E. coli natural regulatory sequence in the lac operon, which normally increases downstream gene expression under low glucose concentrations, to increase gene expression under high glucose concentrations. Then, we assayed the function of this sensor through GFP expression. Fluorescent assays demonstrated that our construct exhibits glucose inducibility and responds actively to changing glucose concentrations from 0.007 to 0.28 mM. While our results do not suggest that the biosensor operates under physiological conditions, we have created a proof-of-concept DNA-based glucose biosensor. This prototype system has potential to be optimized for physiological conditions, transferred to mammalian cells capable of producing insulin, and ultimately be used for therapeutic applications. Our research represents a new synthetic biology approach to circumvent the current limitations of T1D treatment.

Introduction
Type 1 diabetes mellitus (T1D) is an endocrine disorder that affects upwards of 1.25 million Americans and causes $14 billion dollars of healthcare expenditures annually. [1] T1D results from an autoimmune attack on beta pancreatic cells, which normally secrete insulin in response to elevated blood glucose levels (BGLs). Without insulin, a peptide hormone that induces systemic cellular uptake of glucose, sustained hyperglycemia and a concomitant lack of cellular energy can result in multi-system failure. [2]

Traditional treatment regimens for T1D (Table 1) rely on insulin-replacement therapies to normalize BGL. Since intestinal proteases readily hydrolyze insulin, oral administration is not possible, presenting serious complications for delivery. Furthermore, standard insulin therapy regimens are patient-directed and focus on the administration of insulin in anticipation of elevated BGLs (i.e., before a meal).

However, BGLs are subject to variability from diet, activity level, and psychological stress, so patient-directed insulin dosage often does not correspond to actual insulin need. This mismatch often leads to dangerous hypoglycemic episodes from excess insulin administration. Finally, current treatment regimens for T1D are prohibitively expensive. [2][3]

A safer, more effective T1D treatment would mimic the body’s natural homeostatic functions by continually monitoring BGLs and secreting insulin only when a patient has high BGLs. This feedback loop would be responsive in real-time and thereby avoid complications from preemptive insulin injections and dosing error. Different therapeutic approaches are at various stages of development, but no single method has been widely adopted as a safe, effective, and cheap alternative to the current standard (Table 1).

In this research project, we aim to leverage genetic engineering to design a glucose-sensitive insulin-administration device to replace the function of damaged beta cells in T1D patients. Although beta cell response to BGLs is normally mediated by induced exocytosis of insulin, such a complex system could not be readily replicated through genetic engineering. [7] Instead, we envision a DNA-based glucose biosensor that actuates production of insulin (or an insulin-like alternative). Such a device integrated into a patient’s somatic cells via viral-vector- based and/or CRISPR/Cas9-based gene therapy would allow modified cells to continually sense BGLs and release appropriate amounts of insulin, thus restoring normal homeostatic functions. CRISPR-mediated therapeutic “knock-ins,” where a functional genetic construct is introduced into an organism’s genome to compensate for genetic errors in metabolism, have shown promise in preclinical studies for conditions like arginase deficiency. By reprogramming the patient’s own cells, this treatment avoids the complications of infection and immune rejection associated with artificial pancreases and pancreas transplants.

As the first step to creating this synthetic glucose sensor and insulin actuator system, we aim to develop a DNA-based glucose biosensor. Due to the technical difficulties of mammalian cell culture and gene editing, we elected to construct a prototype glucose sensor system in E. coli as a proof-of-concept. If the sensor proves effective, it can be transferred to mammalian cells and subsequently optimized for therapeutic application. We selected E. coli as our model system for this sensor for the following reasons: (1) E. coli’s short doubling time render it a facile, low-cost expression system; (2) stable integration of DNA constructs is readily accomplished through transformation; (3) gene expression in E. coli is naturally responsive to changing glucose availability, providing natural regulatory mechanisms that we can re-purpose for our sensor.

Although glucose concentrations naturally affect gene expression in E. coli, glucose normally serves as a corepressor...
rather than inducer of gene expression. Thus, in order to create a glucose biosensor, we aim to re-engineer E. coli’s natural systems for increasing gene expression under low glucose concentrations to increase gene expression under high glucose concentrations. Glucose-inducible promoters have previously been engineered in both E. coli and S. cerevisiae [12], but not with the intention of creating a system that could continually respond to dynamic glucose levels, as would be required for our envisioned in vivo system.

Methods

Plasmid Construction and Validation

Device Design

As E. coli’s preferred energy source, glucose represses expression of genes of metabolism for other sugars. [9] For example, the well-characterized lactose (lac) operon is subject to positive control by the catabolite activator protein (CAP), also known as the cAMP-receptor protein (CRP). CAP binds to a site upstream of the promoter to recruit RNA polymerase when glucose concentrations are low; thus, transcription of the lac operon is repressed when glucose concentrations are high. [10][11]

In order to create an effective glucose biosensor, we aimed to re-engineer the native E. coli CAP regulatory system derived from the lac operon. We hypothesize that refactoring E. coli’s CAP regulatory system by placing the CAP binding site downstream, as opposed to upstream, of the promoter will create a glucose-inducible sensor. We anticipate that increased CAP binding in low glucose concentrations will repress transcription of downstream genes by sterically blocking RNA polymerase progression. Conversely, decreased CAP binding in elevated glucose concentrations will result in increased downstream transcription. Thus, swapping the relative positions of the CAP binding site and promoter should effectively transform CAP from a transcriptional activator to a repressor, which has been demonstrated by previous investigations. [13]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mechanism</th>
<th>Disadvantage(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous insulin injection²</td>
<td>Patient-administered insulin of various durations of action</td>
<td>Pain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lack of patient compliance</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Disruption of daily routine/quality of life</td>
</tr>
<tr>
<td>Insulin pump²</td>
<td>Electric pump connected to an in-dwelling catheter for continuous administration of slow-acting insulin and fast-acting bolus prior to a meal.</td>
<td>Infection risk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypoglycemic episodes</td>
</tr>
<tr>
<td>Pancreas transplant⁴</td>
<td>Replace dysfunctional beta cells with pancreatic transplants from a healthy</td>
<td>Immune rejection</td>
</tr>
<tr>
<td>Artificial pancreas⁵</td>
<td>Insulin-pump controlled by a continuous glucose monitor</td>
<td>Operative complications</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High cost</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Some models have low portability—utility restrict-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>More portable models are not fully automated; require finger-prick calibration and food-intake</td>
</tr>
</tbody>
</table>

Table 1. Traditional and developing treatment methodologies for T1D. Established regimens are unshaded, while currently developing methods are shaded in gray.
CAP’s repressive activity will lead to greater glucose sensitivity.

We placed the constitutive-promoter-CAP-binding-site complex upstream from GFP in order to assay expression levels through fluorescent intensity. We used the reporter protein GFP as a proxy for insulin because fluorescence enables straightforward tracking of expression levels.

2. Constitutive Promoter Plasmid (Plasmid C)

In order to ensure that this constitutive promoter works and does not itself exhibit a confounding glucose-concentration dependence, we designed a simple positive-control plasmid (Plasmid C) consisting of a constitutive promoter upstream of GFP in the promoterless pColi backbone (Figure 2).

Fluorescent Plate Reading

From the glycerol stock, we resuspended bacteria with each plasmid in 10 mL of LB broth or EZ-Rich Medium overnight before each experiment. Before each dynamic range experiment, we transferred bacteria from the culture media to solutions of varying glucose concentrations in a 96-well plate. Our experimental glucose concentrations ranged from 0.002-17.78 mM, with particular concentration ranges determined by the goals of each experiment. We selected this concentration range according to the lac operon’s natural glucose dynamic range from 0 to 5 mM and the relevant physiological BGLs from 7 to 15 mM. We chose the upper glucose concentration limit of 17.78 mM in order to simulate the post-meal hyperglycemic conditions in T1D patients.

To prepare fluorescent plates for analysis, we added 800 µL of EZ-Rich Medium, 16 µL of glucose solution of various concentrations, and 100 µL of suspended bacteria to one-mL wells in a 96-well plate. Then, we transferred 200 µL of the solution from each well to a transparent 96-well plate for measurement of fluorescence and absorbance.

To assay GFP expression, we measured fluorescence via 400-nm excitation and 515-nm emission. To quantify the number of bacteria present, we measured absorbance at 600 nm. From these values, we calculated an OD-600-normed fluorescent value to represent the fluorescence per cell in each condition. For each experiment, we measured these values every 15 minutes for the first three hours, and for experiments measuring response time of bacteria to changing conditions (i.e., glucose concentrations), we continued sampling each hour for nine hours after the initial three. The data were processed, analyzed, and plotted in Matlab.
Results

Device Validation
The gel electrophoresis confirmed correct length of the region between the 5’ regulatory sequence and the middle of the GFP coding region for all three plasmids (Figure 4).

Sequencing analysis corroborated that the constructs were accurately cloned. Therefore, we concluded that the cloning process to construct our plasmid was successful.

Experiment 1: Initial Dynamic Range Fluorescence Test for All Plasmids
We resuspended bacteria with each plasmid in 10 mL of LB broth overnight. For each plasmid, we measured fluorescence over glucose concentrations ranging from 17.78 mM to 0.03 mM using two-fold dilutions and 0 mM to characterize a dynamic range.

Plasmid E potentially demonstrates a dynamic range from 0 mM to 0.28 mM (Figure 5), albeit with overlapping error bars. This dynamic range does not extend to higher glucose concentrations, as the positive association between fluorescence and glucose concentration does not hold above a concentration of 0.28 mM.

Plasmid C exhibits a relatively constant fluorescence level across glucose concentrations, which is consistent with expected constitutive expression. This result suggests that Plasmid E’s concentration dependence arises from the presence of the CAP binding site downstream of its promoter rather than from the constitutive promoter.

Plasmid N exhibits a significant decline in fluorescence between 0.035 mM and 0.07 mM, which is consistent with the lac operon’s natural glucose-induced repression. However, this trend does not hold for subsequent increases in glucose concentration. Thus, it is unclear whether Plasmid N exhibits a dynamic range.

Experiment 2: Focused Dynamic Range Fluorescence Test for All Plasmids
Due to the clear lack of a trend in fluorescence beyond 0.56 mM for all plasmids and the high variability in results, we chose to repeat this experiment with a narrower range of seven glucose concentrations (1.11 mM to 0.035 mM with two-fold dilutions and 0 mM).

Within this smaller range of glucose values, the glucose dose-response curve demonstrates clear glucose-induced fluorescence for Plasmid E. For Plasmid C, we see no clear glucose-dependence. For Plasmid N, there is an indication of glucose-repressed fluorescence for lower glucose concentrations, but this negative correlation does not hold for higher values in this range. As in Experiment 1, the relative magnitudes of the normed fluorescence are similar for Plasmids E and C, both of which are higher than Plasmid N; this result is expected, since Plasmid N uses a promoter with less affinity for RNA polymerase.

The results of Experiments 1 and 2 corroborated previous findings and demonstrated evidence of a dynamic range from 0 mM to 1 mM of glucose for the experimental plasmid. Since Plasmids N and C were meant to troubleshoot Plasmid E and results have confirmed Plasmid E’s functionality, we chose to focus future experiments on Plasmid E.

Figure 5. Time-averaged dose-response curves for Plasmids E (black), C (blue), and N (red) from initial time point to three hours with sampling every 15 minutes. Glucose concentrations ranged from 0.035 mM to 17.78 mM with each concentration doubling the previous. Normed fluorescence levels at a glucose concentration of 0 mM (not shown on semi-log plot) fell below the error bars of the fluorescence level for 0.035 mM for Plasmid E. Mean ± standard error shown for each point.
Experiment 3: Dynamic Range Time Course for Plasmid E Cultured in Glucose-Free Media

Experiments 1 and 2 do not reveal the time course of the sensor’s response to changing glucose concentrations, as the LB culture media does not have a defined glucose concentration. Therefore, we performed another dynamic range experiment by culturing the bacteria overnight in EZ-Rich Medium without glucose and transferring them to higher glucose concentrations. This allows us to determine the time course of the sensor’s equilibration to an increase in glucose concentration. We tested a greater number of glucose concentrations by performing two-fold dilutions from 1.11 mM to 0.002 mM, along with 0 mM. By increasing the number of dilutions, we aimed to establish the lower limit of the sensor’s dynamic range.

Although the overlapping error bars complicate analysis, the dynamic range began between 0.002 mM to 1.11 mM and shifted to 0.007 to 0.28 by 150 minutes (Figure 7). This final dynamic range appears to remain relatively constant after 150 minutes.

Experiment 4: Focused Dynamic Range Fluorescence Test for Plasmid E Cultured in Glucose-Rich Media

We performed a complementary experiment to our last by incubating bacteria transformed with Plasmid E in a glucose-rich medium. Figure 7 shows the glucose dose-response plots for Plasmid E over time from 0 to 210 minutes, with sampling every 30 minutes. Glucose concentrations ranged from 0.002 mM to 1.11 mM with each concentration doubling the previous. Bacteria were cultured in EZ-Rich Medium without glucose. Mean ± standard error shown for each point.

Figure 6. Time-averaged dose-response curves for Plasmids E (black), C (blue), and N (red) from initial time point to three hours with sampling every 15 minutes. Glucose concentrations ranged from 0.035 mM to 1.11 mM with each concentration doubling the previous. Normed fluorescence levels at a glucose concentration of 0 mM (not shown on semi-log plot) fell below the error bars of the fluorescence levels for 0.035 mM for Plasmid E. Mean ± standard error shown for each point.
environment (0.28 mM) and transferring them to glucose concentrations ranging from 0.002 mM to 0.56 mM. This allows us to analyze the sensor’s equilibration to a decrease in glucose concentration. After transfer to glucose-rich media, the bacteria began to show glucose-dependent expression at 60 minutes and maintained this glucose-induced fluorescence relationship consistently for all subsequent measurements (Figure 8). This response occurred faster than for the bacteria that incubated in glucose-starved media and also appeared to be more precise, as indicated by the narrower error bars. The sensor appeared to exhibit a clear dynamic range over 0.014 to 0.28 mM, which roughly corresponds to the range determined from Experiment 3.

Discussion

For this project, we sought to create a synthetic DNA-based glucose biosensor in E. coli to eventually be paired with insulin-actuator system to treat elevated BGLs for T1D. To do this, we re-engineered E. coli’s natural glucose-repressed lac operon to create a glucose-inducible promoter. We designed Plasmid E and hypothesized that placing the CAP binding site downstream from a constitutive promoter would create a glucose-inducible system that could be assayed by expressing GFP. Our experiments aimed to establish a dynamic range for Plasmid E that demonstrates clear glucose-induced fluorescence.

In Experiment 1, we observed glucose-dependent expression at sub-millimolar glucose concentrations, but this relationship was not present at higher, physiologically relevant levels of glucose. This dynamic range corroborates previous research with the CAP binding site. From this experiment, we concluded that the current construct is not glucose-inducible at physiological levels. Experiment 2 corroborated the existence of a dynamic range from 0.007 to 0.28 mM.

Experiments 3 and 4 included lower glucose concentrations, which demonstrated a dynamic range from 0.014 to 0.28 mM. Additionally, our sensor can respond to changing glucose concentrations, which would be necessary for physiological applications. Furthermore, the bacteria respond faster and more uniformly to a decrease in glucose concentrations than to an increase. This difference could alternatively be attributed to irregularities of gene expression and metabolism in the absence of glucose; a follow-up experiment might investigate the response when bacteria are cultured in low but non-zero glucose concentrations and then transferred to higher concentrations.

Our findings suggest that our construct works successfully as a glucose-inducible promoter at low glucose concentrations and responds actively to changing glucose concentrations. However, therapeutic application of our sensor will be limited by its unresponsiveness at physiological BGLs and its slow response time.

If this glucose sensor is to be used as part of a gene-therapy for T1D, these limitations in dynamic range and response time must be addressed. Experimenters might attempt to place multiple CAP binding sites downstream from the constitutive promoter.
promoter, which would theoretically amplify the glucose-inducible expression. Additionally, future researchers could vary the distance between the CAP binding site and the promoter; in our construct, we placed a single site immediately downstream. Changing these parameters could potentially shift the plasmid’s dynamic range to include physiologically relevant values and quicken response times. Alternatively, researchers might investigate glucose-linked regulatory mechanisms in mammals to create a more responsive and physiologically appropriate sensor.16,17

While existing glucose-inducible promoters have been previously engineered in both E. coli10 and S. cerevisiae11, they were not with the intention of creating a system that could continually respond to dynamic glucose levels, as would be required for our envisioned diabetic treatment. While our results do not prove that the plasmid would operate under physiological conditions and E. coli cannot perform post-translational modifications to produce active insulin, we have created a proof-of-concept DNA-based glucose biosensor. This prototype system can be optimized for physiological conditions, transferred to mammalian cells capable of producing insulin, and used therapeutically. Our research represents a new synthetic biology approach to address the current limitations of T1D treatment.

Safety and Security
Our device would be incorporated into a larger glucose-sensitive insulin-administration device for use in T1D patients to monitor and regulate BGLs. This device would then be inserted into the genome of a T1D patient’s somatic cells in order to functionally replace damaged beta cells. Our biosensor could not be used for human patients, as currently it would not perform under physiological conditions and cannot adjust glucose levels via an insulin actuator. Thus, an accidental release of the bacteria into a non-laboratory setting would not result in negative consequences. Our bacteria do possess ampicillin resistance and would present problems if they were to be introduced into the world; however, as a BSL-1 hazard, the strain of E. coli used is not pathogenic.

Our completely engineered device would, in theory, respond to BGLs by secreting an appropriate amount of insulin into the diabetic patients’ bloodstream. A maliciously inclined individual could repurpose our designed pathway to secrete a harmful substance. The aforementioned scenario is highly unlikely, as the effort needed to repurpose our design would be much greater than alternative means of harm. If further experimentation was performed to optimize our system for use in mammalian cells, extensive testing would be necessary before prokaryotic DNA was introduced into a human host. Moreover, the human genome does not naturally encode the CAP protein; so, in addition to transfecting patient cells with the glucose-sensitive insulin device, we would also have to insert a CAP actuator. This construct would also require rigorous safety testing in mammalian tissue cultures and animal models to ensure that the introduction of CAP does not have a deleterious effect on cellular metabolism.

The low risk of adverse effects and potential misuse lead us to conclude that it is ethical to continue to develop our device. However, the cost-effectiveness of investing in our design over other potential diabetic treatments is a necessary consideration. How efficient is allocating money to our device when treatments to manage diabetes already exist? Because current therapies for T1D are prohibitively expensive and not physiologically specific, the medical need for better treatments is extremely high. A synthetic biology approach via a DNA-based glucose biosensor and insulin actuator offers a potential avenue toward creating better T1D treatments.

Acknowledgements
We thankfully acknowledge Stanford University’s BIOE44 (Fundamentals for Engineering Biology Laboratory) instructors, Paul Vorster and Lei Stanley Qi, for their guidance and instruction throughout this research. Additionally, we would like to thank Michael Becich and Samuel Bray, along with the rest of the BIOE44 teaching assistants, for their advice and help.

References