Engineering a 'Bioballoon' for Extraterrestrial Mid-Atmospheric Sensing

Synthetic Biological Applications of Latex and Chromogenic Proteins

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Abstract:

Ongoing research in extraterrestrial atmospheric sensing continues to give insight into the origin and history of planetary bodies. The comparison of different atmospheres to Earth's broadens our understanding of various atmospheric processes that have vast effects on potential biological life at the surface. The most significant limiting factors on the collection of atmospheric data on extraterrestrial bodies arise from the difficulty of engineering lightweight transportation vessels for sensing technologies capable of traveling the extensive vertical and horizontal distances required for useful measurement. The work herein details an application of synthetic biology to space exploration that provides an inexpensive and lightweight approach to extraterrestrial atmospheric flight through the biosynthesis of inflatable balloon membranes. Latex is the most widely used material for on-Earth high-altitude balloon membranes, making it an ideal candidate for an extraterrestrial flight membrane. We metabolically engineered *Escherichia coli* to produce a latex precursor by overexpressing isopentenyl diphosphate (IPP) through the endogenous methylerythritol phosphate pathway and transfecting cells with genetic constructs for key isoprene chain polymerization enzymes derived from *Hevea brasiliensis*. Thus, we present a novel mechanism for bacterial polyisoprene production. To sense temperature differentiation by altitude, we characterized the reversible color changes in a set of 12 chromogenic proteins at temperatures between 40°C and 90°C. These chromoproteins were flown at altitudes up to 100,000 ft on Earth via a high altitude latex balloon, confirming that the increased radiation exposure and low atmospheric pressures did not permanently disrupt their color changing properties.

Introduction

High-altitude weather balloons are the most commonly used tool for atmospheric research in tracking weather patterns, wind patterns, and monitoring atmospheric composition. Understanding our atmosphere and extraterrestrial atmospheres is critical for understanding climate change dynamics, atmospheric chemistry evolution, and the weather. However, large scale sampling of atmospheric properties is difficult owing to scale, economic feasibility, and the limited payload capacity of contemporary weather balloons. To address this issue, our team developed a biomaterial based balloon by engineering synthetic organisms for sensor and balloon membrane production. To make the balloon itself, we metabolically engineered *E. coli* to produce elastic polymers for incorporation into the membrane structure. Additional functionality was achieved by engineering biological temperature and small molecule biosensors, which could be imbedded in the balloon membrane via biotinylation. When combined, these components can be constructed into a balloon made entirely out of biological products. We achieved this goal through two important aims—development of a bacteria capable of producing a latex like polymer as an alternative to natural and synthetic rubber and characterization of a collection of chromogenic proteins sensitive to specific temperatures.

For the construction of the membrane, we focused primarily on latex synthesis owing to its high demand of use and distinct material properties. Used extensively as both a major structural component and dampening component in the construction of space exploration vehicles, latex is a critical building block for furthering space exploration.[1] Because of its large stretch ratio and high resilience to repeated stress, latex is an appealing material for construction of flexible structures that can adjust to variable mechanical stresses. However, the use of rubber materials in space can be limited by the cost of bulk material transport—thereby illuminating the need for a sustainable, scalable, and transportable method of latex production.

Currently, the only source of commercially usable natural rubber that can be processed into latex is available from the rubber tree *H. brasiliensis*.[2] While other plants are capable of producing rubber particles, they form materials that are weaker after processing, and require less extension to break compared to the former.[3, 4] However, rubber farming in recent years has been threatened by production shortfalls owing to diseases such as South American Leaf blight. *H. brasiliensis'* narrow genetic base also leads to most large acreage farms planting genetically identical trees, making them prone to large crop failure.[5] This problem is further exacerbated by deforestation and the growing acreage needed for agriculture, both of which limit rubber production by decreasing the amount of land available for rubber tree plantations.[6, 7]

While chemical synthesis of synthetic latex is an appealing alternative to natural latex, the chemical and physical properties of the two differ in subtle but important ways. Although the properties of synthetic latex can be fine tuned to a particular use by varying proportions of isoprene monomers in its composition, it lacks the mechanical and low temperature performance of its natural counterpart--attributes which are critical for atmospheric exploration.[8,9] Additionally, the increasing price of petroleum and butadiene has elevated prices in the synthetic rubber industry and consequently exacerbated the current market shortfall of natural rubbers.[10, 11] However, natural latex has associated weaknesses as well; it is difficult to handle due to natural allergens and exhibits diminished durability, resilience, and elasticity without vulcanization.[8, 12] Natural rubbers also suffer from limits in the speed and scale of biomaterial production, high production overhead, and limited reproducibility. Both production methods are limited in their production sustainability, scale, turnaround, and resource consumption, highlighting a need for an alternative method for latex production.[13] To address this need, we engineered a polyisoprene synthesis pathway into *E. coli* for production of latex polymers. These polymers could then be constructed into membranes for building our bioballoon, and the ability to grow rubber-producing cultures from a single cell could provide a sustainable and renewable source of a complex building material in space.

With a resilient membrane capable of being produced in space, the balloon now requires novel atmospheric data collection techniques. To add functionality to our balloon, we characterized a cassette of chromoproteins for use as part of a bio-thermometer. In planetary exploration, monitoring temperature changes in the atmosphere is important for predicting weather patterns and atmospheric stability, which vary drastically with respect to altitude. This can help predict the weather that various rovers or exploration outposts would have to deal with without relying on expensive satellite equipment. Additionally, the vertical temperature profile of a certain region of a planet's atmosphere can be used to predict how easily a payload will fall through the atmosphere and land on the planet's surface.[14] On Earth, vertical temperature profiles can be obtained using electronic temperature sensors mounted on weather balloons. These balloons go up to different heights in the atmosphere and relay real-time measurements of the atmospheric

temperatures. While these electronics are easy to obtain and replace if they are broken on Earth, in the context of planetary exploration, they are irreplaceable and unreliable, as it would take years to send replacement parts. Thus, a precise biological temperature sensor must be developed in order to address these concerns. The biological temperature sensor presented herein can be expressed in a small culture of *E. coli* and is easily maintained and replaced.

Section I. Engineering *E. Coli* to produce cis-1,4 polyisoprenes(latex)

In order to form our bio-membrane, we sought to address the need for a faster and more economical alternative latex production system. To achieve our objective, we designed a transgenic organism capable of mimicking the natural rubber production process found in *H. brasiliensis*. Not only would a transgenic single cell organism allow for optimization of polymer synthesis, but it would also permit a high yield in polymer-producing cells. A scaled-up cell culture bioreactor would yield large volumes of both rubber-producing cells and cis-polyisoprene polymers with minimal growth media input. For these reasons, we were motivated to metabolically engineer *E. coli* for high-yield production of cis-polyisoprene compounds when supplied with basic cell growth media and nutrients.

Rubber (cis-1,4-polyisoprene) synthesis pathway

Although there are a variety of polyisoprene polymer forms, natural rubber is largely composed of cis-1,4-polyisoprene. To produce the cis-1,4-polyisoprene polymer, rubber trees such as *H. brasiliensis* employ cis-prenyltransferase enzymes (also commonly referred to as rubber transferases) to link individual isoprene monomers into a polymer. The primary monomer in a cis-1,4-polyisoprene chain is isopentenyl pyrophosphate (IPP), which has a more reactive isomer, dimethyl-allyl pyrophosphate (DMAPP). To initialize chain elongation, prenyltransferase enzymes begin with a single DMAPP molecule and iteratively add IPP onto the chain, allowing for extension of the polymer. However, to be enzymatically active, cis-prenyltransferase enzymes require a magnesium(II) supplement and small rubber particle protein (SRPP) coenzyme that serve as activators.[15] Without a magnesium (II) supplement and SRPPs, prenyltransferases are incapable of polymer chain extension. Therefore, to polymerize rubber chains, the key xenogeneic components our host organism will need include (1) prenyltransferases, (2) IPP substrate, (3) DMAPP substrate, (4) a magnesium(II) supplement (such as $MgSO_4$), and (5) small rubber particle protein coenzymes.

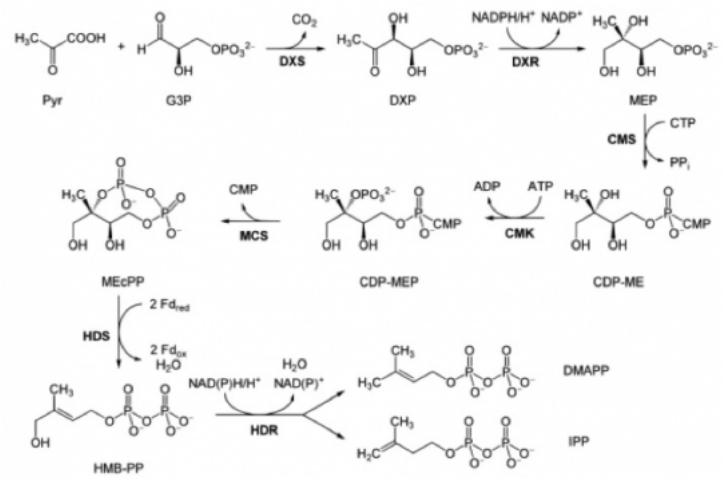


Figure 1.1: MEP/DOXP pathway. The rate limiting step is the first step, in which pyruvate and G3P are converted into DXP. Through constitutive expression of DXS we are able to accelerate the process and push the equilibrium of the reaction towards favoring the products, IPP and DMAPP.[20]

DXS synthase optimization

Although IPP and DMAPP could be supplied directly to the media, producing both compounds within the host organism from basic media is essential to space applications in order to reduce the mass which must be supplied from Earth. IPP and DMAPP are endogenous to many species, and the two common IPP biosynthesis pathways were identified: the mevalonate (MVA) pathway and methylerythritol phosphate (MEP/DOXP) pathway. While both pathways generate IPP and DMAPP from sugar, only the MEP/DOXP pathway is endogenous in E. coli.[16] Through a six step process, the MEP/DOXP pathway converts molecules of pyruvate and glyceraldehyde 3-phosphate (G3P) into IPP and DMAPP. While incorporating the MVA pathway into E. coli would further enhance IPP and DMAPP output, we opted for optimizing the MEP/DOXP pathway to minimize the step count from converting a substrate into IPP.[17, 18] Additionally, since G3P and pyruvate are products of glycolysis, providing a glucose supplement to E. coli would allow for G3P and pyruvate use by the MEP/DOXP pathway without increasing cell stress.[19]

To increase flux through the MEP/DOXP pathway, 1-deoxy-D-xylulose 5-phosphate synthase (DXS) was overexpressed. Although all of the enzymes in the MEP/DOXP pathway could have been overexpressed, prior research determined DXS to be responsible for the rate limiting step.[15] To control for DXS expression, the DXS enzyme was codon optimized and reintroduced into E. coli under an IPTG-inducible promoter (DXS plasmid), allowing for regulated production of DXS. A supplement of IPTG would then activate expression of DXS, which in turn would expand the pathway bottleneck at the beginning of the MEP/DOXP pathway. Accelerating the process limiting chemical conversion of G3P and pyruvate to 1-Deoxy-D-xylulose 5-phosphate (DXP or DOXP) ultimately results in a greater output of IPP and DMAPP.[18]

To design an optimized DXS synthase, the sequence for the DXS gene was obtained from EcoGene (EG13612). For future protein purification and characterization, a FLAG, tetracysteine, and hexahistidine tag were also attached to the DXS gene. A double terminator, (BBa_B0010 and BBa_B0012) was also used to ensure appropriate transcriptional arrest.[20] The construct was synthesized in two parts, and Gibson assembly was used to insert them into linearized pSB1A3. The resulting construct was transformed into T7 express cells.

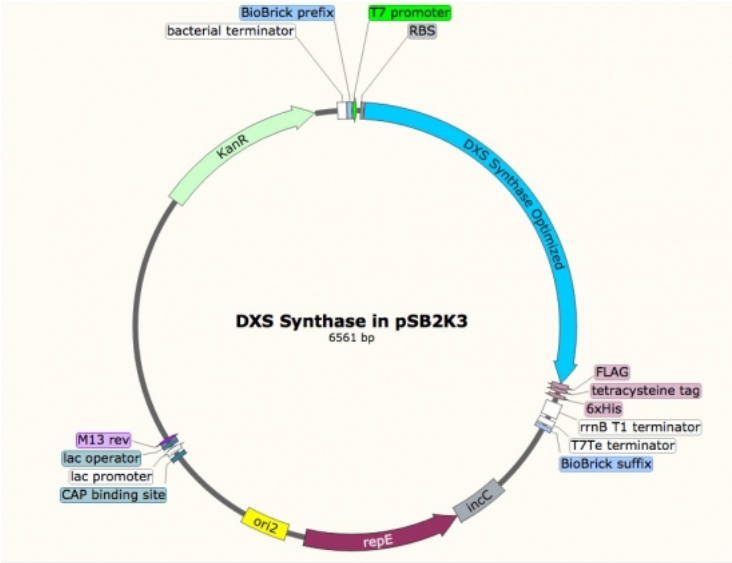


Figure 1.2: Plasmid map of DXS synthase vector. DXS synthase gene is linked to a T7 Elowitz IPTG inducible promoter that will allow for high expression of DXS.

Incorporating rubber synthesis into E. coli

While DXS synthase optimization can increase the amount of IPP and DMAPP substrate available for cis-1,4-polyisoprene formation, we also needed to incorporate the isoprene synthesis pathway from *H. brasiliensis* into *E. coli.* A second component was introduced into our system—the prenyltransferases and SRPP coenzyme needed for isoprene extension. We identified well-characterized cDNA sequences from *H. Brasiliensis* for prenyltransferases directly involved in natural rubber biosynthesis. Two enzymes were selected, HRT1 and HRT2 (Accession No. AB061234.2 and AB064661.2), which are two cis-prenyl chain elongating enzymes isolated from *Hevea* latex.[**22**] Found predominantly in fresh *Hevea* latex, these proteins synthesize new rubber molecules. Because either HRT1 or HRT2 could be used, both genes were incorporated into a plasmid under an IPTG-inducible promoter to allow for maximum expression of enzyme.

Since prenyltransferase requires an SRPP and various chemical cofactors for enzymatic functionality, we identified the most commonly expressed SRPP in *Hevea* latex as a potential cofactor for HRT1 or HRT2.[12, 23] However, because there are different classes of SRPPs that can play a variety of roles in *Hevea* latex production, SRPPs with high expression profiles were selected that had also been identified as rubber elongation factors.[24] Additionally, small rather than large SRPPs were targeted, since large rubber particle proteins would be difficult to produce in *E. coli* due to their size.[25] The gene of the single SRPP we selected (AF051317) was subsequently incorporated into the prenyltransferase cassette.[18] Since prenyltransferase activity depends on SRPP presence and gene order in an operon can influence expression level, the SRPP gene was inserted before the two prenyltransferases to account for the possibility that the first protein in the cassette would be highly expressed. This cassette was then linked to IPTG-inducible T7 Elowitz high-copy promoters to allow for regulated expression. All proteins were also tagged with a FLAG, tetracysteine, and hexahistidine tag to allow for protein purification assay. A double terminator, (BBa_B0010 and BBa_B0012) was also used to ensure appropriate transcriptional arrest (Figure 1.3).

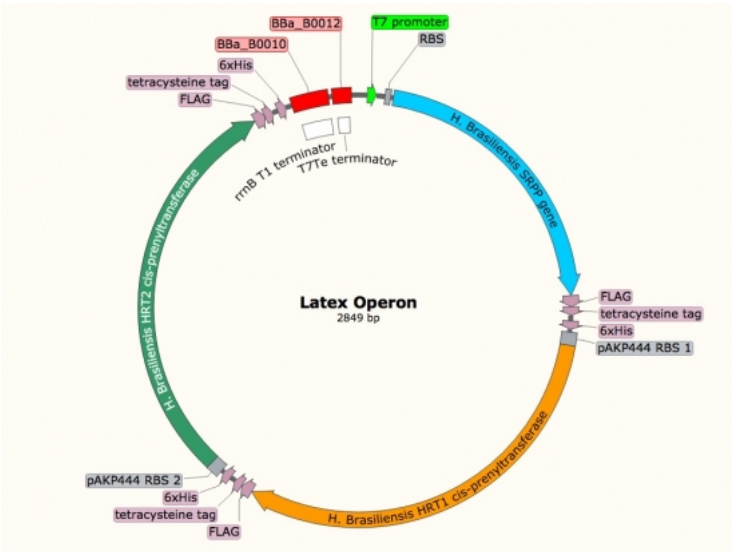


Figure 1.3: Construct containing prenyltransferases HRT1, HRT2, and SRPP cofactor. All components are driven by a T7 IPTG-inducible promoter, with unique ribosome binding sequences.

To produce our plasmid, SRPP, HRT1, HRT2 were synthesized on three different fragments. These fragments were then linked together via Gibson Assembly, which yielded a cassette of genes. This cassette was then incorporated into pSB1C3, which granted the construct chloramphenicol (Chl) resistance. The construct (the latex operon) was then transformed into T7 express cells.

Although our system was incorporated on two plasmids, all four components (HRT1, HRT2, SRPP, and DXS synthase) could be included on the same construct for ease of transfection. However under our present schema, transfection of *E. coli* with the DXS plasmid would allow for an organism that produces IPP and DMAPP in high volume which could be useful for alternative applications such as terpenoid synthesis. For full realization of high throughput IPP and cis 1,4-polyisoprene production in *E. coli*, all plasmids are to be transfected into the host to allow for high expression of all pathway elements. To verify all elements were property transfected, each plasmid had distinct selection markers.

An item of concern is the metabolic strain each of these plasmids will place upon *E. coli.* IPP is a byproduct of glycolysis and cellular respiration; enhanced conversion of pyruvate and G3P or acetyl-CoA to IPP will decrease the amount of substrate available to the host for ATP production. To address this concern, the entire system has been placed under the control of IPTG-inducible promoters, allowing for separate growth and production phases.

After *E. coli* cells have been transfected with both plasmids, a supplement of magnesium sulfate, IPTG, and glucose is sufficient to induce protein expression, activity, and cis-polyisoprene polymer synthesis. Once produced, HRT1 and HRT2 bind with SRPP and Mg(II) to become enzymatically activated. (Figure 1.4) DXS synthase accelerates the conversion of pyruvate and G3P to DXP. Downstream, the activated HRT1 and HRT2 complexes can extend IPP into polyisoprene via dephosphorization. (Figure 1.5)

In order to produce latex, the materials needed are transformation competent *E. coli* cells, LB growth media, aqueous $MgSO_4$ or another Magnesium (II) supplement, the two aforementioned plasmids for transfection and/or any variation, glucose solution and any standard cell transformation kit. Bioreactors can be used to culture large volumes of cells to maximize polyisoprene yield.

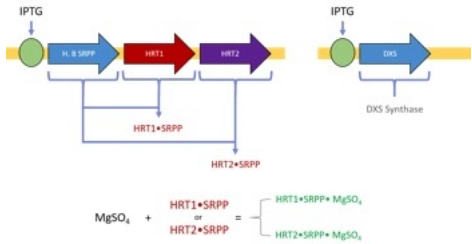


Figure 1.4: HRT1, HRT2, SRPP, and DXS expression is induced by IPTG presence. Once produced, HRT1 and HRT2 bind with SRPP and $MgSO_4$ to become enzymatically activated.

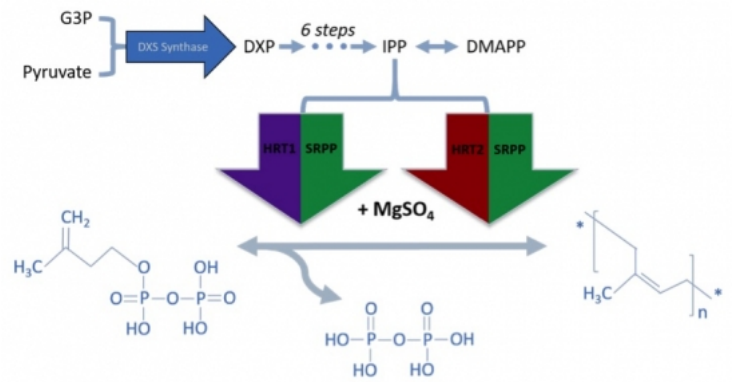


Figure 1.5: DXS synthase accelerates the conversion of pyruvate and G3P to DXP. Downstream, the activated HRT1 and HRT2 complexes can extend IPP (left) into polyisoprene (right) via dephosphorization.

Data/results

To induce latex production, IPTG (400 mM), glucose (1 M), and $MgSO_4$ (400 mM) were added directly to 350 ml liquid cultures growing at 37˚C at 1 µL/mL. After being kept overnight, a second round of IPTG, glucose, and $MgSO_4$ was added. Latex extraction was performed on 350 ml of our cells containing the plasmids for the Latex Operon and DXS Synthase. Cells were spun down and fractionated into the cell pellet, where our polymer product was contained endogenously, and LB media. In order to lyse the cells, chloroform was used to dissolve polyisoprenes in solution.[25, 26] The solution was agitated to ensure all cells were lysed, then filtered to yield chloroform and its solutes. Finally, methanol was added to precipitate the latex from the chloroform solution. The LB fraction was also subject to a similar procedure except the chloroform layer was obtained from the LB fraction by separation of the solvent layers.

After this procedure, 1.5 ml of a rubbery, off-white polymer from the methanol layer precipitated (Figures 1.6, 1.7). For a quick characterization test, the polymer was dried and burned, which resulted in a smell of burning rubber and a strong black smoke—typical of rubber fires. Additionally, our extract (when dried) is highly compressible and elastic. Further tests with mass spectrometry, acetone characterization, degradation, and physical stress tests will be needed to confirm the composition of the polymer. Questions we hope to answer with continued development are whether our product can be used as a rubber substitute in industrial applications, steps that could be taken to optimize for product yield, and whether our product could be tagged for secretion into cell culture media, mitigating the need for cell lysis for recovery of the product.

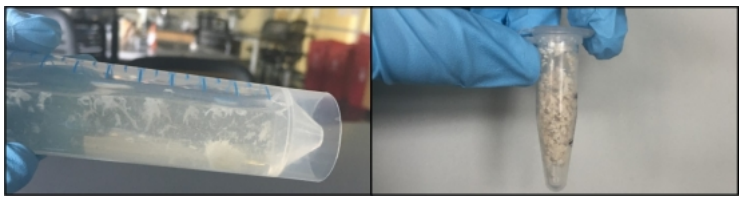


Figure 1.6 (left): A close-up of our manufactured latex: an off-white, rubbery substance precipitated in our methanol experiments. Figure 1.7 (right): A vial of polymer extract dissolved in methanol (70%). Note the white fibrous precipitate throughout the solution.

Materials and Methods (Latex Expression)

The purpose of the invention was to enable *E. coli* to produce isoprene polymers (the precursor and main component of synthetic and natural rubbers) using basic cell growth media and nutrients, such as glucose, magnesium, nitrogen compounds, etc.

Plasmid Assembly

Plasmids were assembled into the iGEM standard plasmid backbones pSB1C3 and pSB2K3. Gene inserts were synthesized by Integrated DNA Technologies (6024 Silver Creek Valley Road, San Jose, CA 95138 USA) as gBlocks, and assembled according to Gibson Assembly Protocol standards with NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs).

Cell Culturing

NEB 5-alpha and T7 Express Competent *E. coli* strains were acquired from New England Biolabs for use in all transfection experiments. Liquid cultures were grown in LB medium and incubated overnight at 37°C with agitation. IPTG was added after 18 hours of incubation to T7 cells transfected with the DXS synthase operon and the gene cassette containing both cis-prenyltransferases and SRPP to stimulate transcription. $MgSO_4$ was also added to a concentration of 0.5mM as an activator for both cis-prenyltransferases to engage in isoprene chain polymerization.

Protein Characterization

Cell lysate was run through an SDS-PAGE gel (using Sigma-Aldrich-08091, SDS Gel Preparation Kit) to confirm the production of both cis-prenyltransferases, SRPP, and DXS synthase. Additionally, cell lysate was run through a Lumio™ gel (using a Lumio™ Green Detection Kit) with luminescent ladders to confirm presence of DXS synthase and SRPP. Observed bands were then compared to the expected band size of all proteins to confirm results.

DNA Sequencing and Analysis

Liquid cultures of NEB 5-alpha cells containing the two latex production plasmids were run through a Qiagen Miniprep Kit to isolate nongenomic long double stranded DNA. Samples were then mixed with water and primers flanking the sites of each of the four inserted genes of interest according to ELIM premix DNA sequencing standards. Samples were then sent to ELIM Biopharmaceuticals (25495 Whitesell St, Hayward, CA 94545, USA) for sequencing. Chromatogram results were analyzed using the bioinformatics tool Geneious (Biomatters, 185 Clara St #101a, San Francisco, CA 94107, USA) for alignment with expected results.

Section II. Characterizing Chromogenic Proteins

Materials and Methods (Chromoprotein Characterization)

To create a biological temperature sensor, chromogenic proteins were obtained from the DNA2.0 (37950 Central Ct, Newark, CA 94560, USA) paintbox and from the iGEM registry. Chromogenic proteins (chromoproteins) are colored proteins that have been extracted from a variety of organisms such as the sea anemone, *Actinia equine* [30], or have been synthetically produced, as was the case for the chromoproteins from DNA2.0. Like all proteins, chromogenic proteins are sensitive to heat; however, their sensitivity to heat is coupled with a visible color loss or change. As the research herein confirms, different chromoproteins respond to different temperatures by either losing their color or changing to a different color.

Plasmid Assembly

To investigate the changes undergone by these chromoproteins when heated, coding sequences for AE Blue and the 12 DNA2.0 chromoproteins were cloned into pSB1C3 with Gibson Assembly. A FLAG, lumino and 6x histidine tag were added by flanking primer ends in order to extract the expressed protein using nickel column purification. The lumino tag is a specific six amino acid sequence that binds to LumioTM Green [32] which allows the fusion of the lumino tag and the chromoprotein to be detected on an SDS-Page Gel without having to run a staining protocol. The FLAG tag allows for anything after its sequence to be cleaved off of the protein when the extracted protein is incubated with enterokinase [33], in case the lumino or histidine tag interferes with the chromoprotein structure.

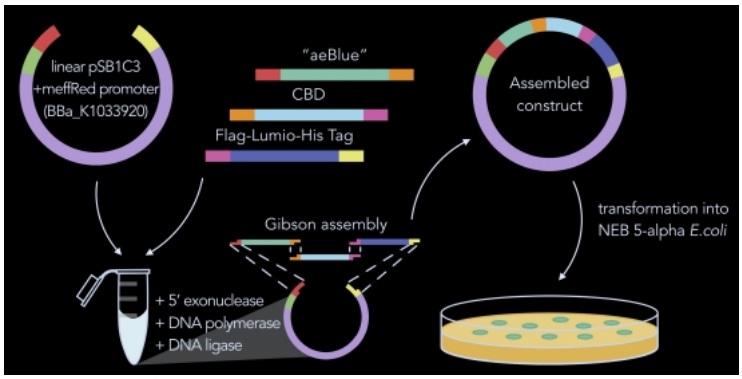


Figure 2.1: The Gibson Assembly Process for creating tagged chromoprotein constructs.

Additionally, a cellulose binding domain isolated from PCR was added to each of the chromoproteins. The cellulose binding domain sequence isolated from *Cellulomonas fimi* has been shown to bind irreversibly to cellulose. [34] In order to ensure that the addition of this larger protein did not interfere with the structure of the chromoproteins, both the chromoprotein without the cellulose binding domain and with the domain were experimented with.

Cell Culturing and Analysis

After performing a Gibson Assembly to place the DNA sequence for the DNA2.0 chromoprotein and the sequence for the Flag-Lumino-His Tag, all twelve of the DNA2.0 chromoproteins were color-verified via colony PCR using iGEM's VF and VR primers (Figure 2.2). Next, a large (250-500 mL) liquid culture for each of the cells expressing the chromoprotein then extracted the expressed protein using 3 mL HisPur Ni-NTA nickel columns (ThermoFisher). The extracted protein was then dialyzed and concentrated using microfiltration centrifuge tubes in order to perform heat tests.

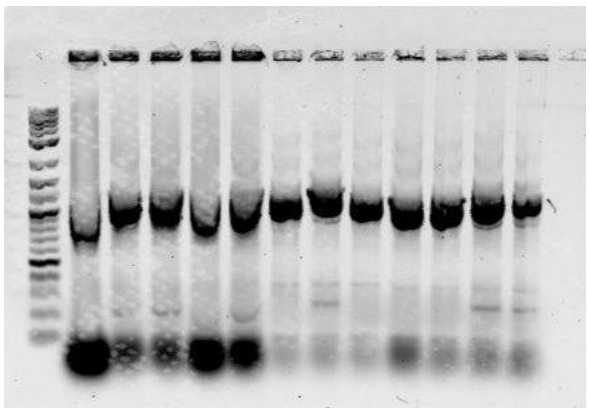


Figure 2.2: Gel run of our chromoproteins and Flag-Lumino His tag. Ladder(left), DonnerMagenta, VirginiaViolet, ScroogeOrange, LeorOrange, DreidelTeal, VixenPurple, SeraphinaPink, CupidPink, TinselPurple, PrancerPurple, MaccabeePurple, BlitzenBlue. As can be seen in the image, there is a strong band near the 1000 bp mark where we would expect to see it.

Cell Lysate Heat Tests

An assortment of 12 chromoproteins (PrancerPurple-PP, CupidPink-CP, TinselPurple-TP, VixenPurple-VP, MaccabeePurple-MP, SeraphinaPink-SP, LeorOrange-LO, ScroogeOrange-SO, BlitzenBlue-BB, DreidelTeal-DT, DonnerMagenta-DM, VirginiaViolet-VV) were tested in a closed system with no air interaction. 5 mL cultures of each chromoprotein were spun down and 30 µL of the pellet cells with 1 µL of 0.165 M EDTA were added. The PCR tubes were then heated for 5 min at a starting temperature of 40 °C and removed from the thermal cycler. A picture was taken before returning them to the thermal cycler for 5 more minutes at a temperature 5 °C higher than the previous. This process was repeated until 100 °C was reached. As can be seen in Figure 2.3, groups of chromoproteins lose their color at specific temperatures. Additionally, CupidPink and DonnerMagenta change from pink to purple at 70 °C and 80 °C respectively.

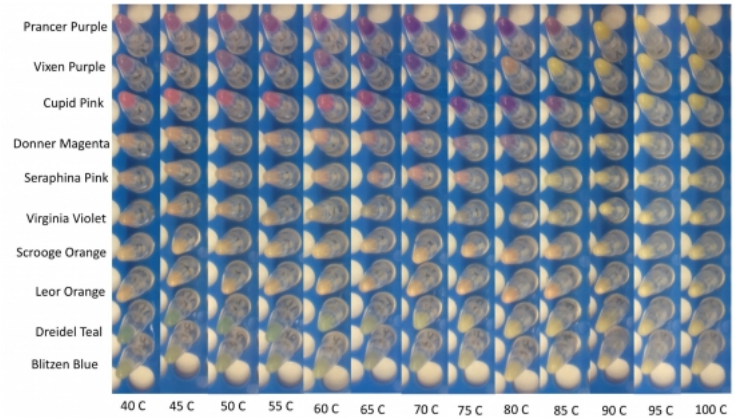
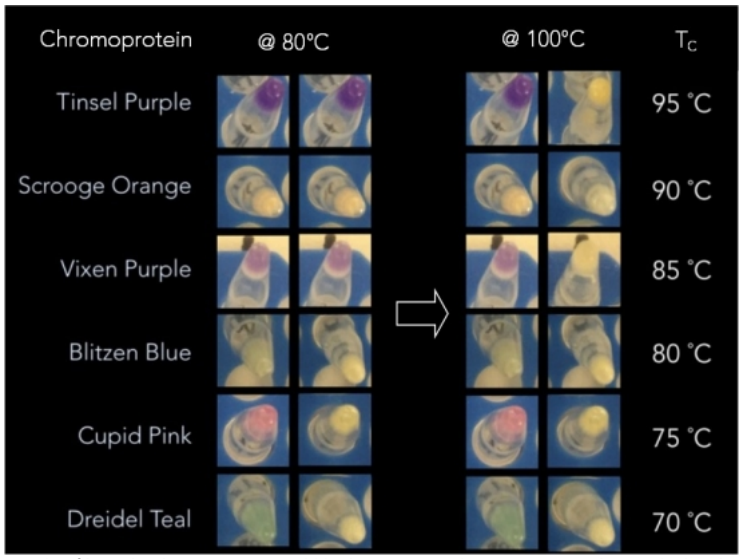


Figure 2.3: Here are all of the chromoproteins at a variety of temperatures. As the temperature gets higher they all lose their color at different temperatures.

Using the results of the experiment above, it was possible to build a thermometer from the select groups of chromoproteins that respond to different temperatures. Shown in Figures 2.4 and 2.5 is a prototype of how that would work. Until the temperature reaches the temperature indicated on the right, the color remains constant.



Figures 2.4 (left) and 2.5 (right): Here is an example of how the thermometer would look at 80 °C and at 100 °C. Note how only the chromoproteins with a critical temperature (T_C) on the interval 80-100 °C exhibited a color change.

These cell lysate heat tests were done in a closed environment, so further tests were done in an exposed air environment. This was done based on the idea that the observed color change was dependent on the interaction of water molecules with the barrel of the chromoproteins. Therefore, in the exposed air system, the water molecules would not be trapped with the chromoproteins and color change might be observed sooner. To test this, an apparatus was used that would heat a glass petri dish evenly and allow for a video to be taken of the cell lysate as it was being heated up.

Twelve chromoproteins that were expressing good color in our cultures were selected for this assay and can be seen in Figure 2.6 on the left. The glass petri dish above was heated to 85 °C which caused all of the chromoproteins to lose their color as seen in Figure 2.6 in the middle. After this, the glass petri dish was removed from heat and 20 µL of DI water were added to each of the chromoproteins (Figure 2.6, right). This was done on the theory that the chromoproteins had not denatured, but rather had lost their color because all of the water molecules which were supporting the structure of their barrel had been driven out by the heat.

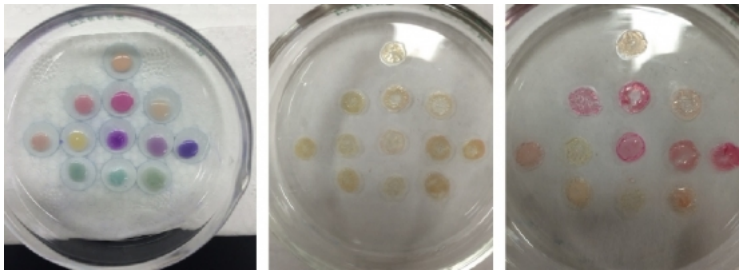


Figure 2.6: (left) 0 µL droplets of cell lysate taken from spun down 5 mL cultures of cells expressing chromoproteins were placed on a glass petri dish. Order of chromoproteins--top row: TinselPurple, second row: spisPink, CupidPink, asPink, third row: DonnerMagenta, scOrange, PrancerPurple, tsPurple, VixenPurple, fourth row: BlitzenBlue, DreidelTeal, AE Blue; (center) The glass petri dish above was heated to 85 °C which caused all of the chromoproteins to lose their color as is seen below; (right) Chromoproteins after being brought back to room temperature and rehydrated with 20 µL of DI water.

Hydration Assay

In order to investigate the role that water molecules play in the chromoproteins' color change observed in response to heat, a lyophilizer vacuum was utilized to sublime the water from the chromoproteins without heating them. The water vapor was then pumped out of the system. After an hour in the lyophilizer, the filter paper was removed from the chamber and photographed (Figure 2.7, center). Water was then added to to the right column of the filter paper which caused the color to return (Figure 2.7, right) as can be seen by comparing the top and bottom rows of this image.

Figure 2.7: (center) Two replicates of PrancerPurple (top) and ScroogeOrange (bottom) are shown after 1 hour of lyophilization; (right) After rehydration of the right hand column of chromoproteins.

Data/results

When testing the cell lysate of the chromoproteins in a closed system it was interestingly observed that if there were certain cultures that were not showing color on a particular day, low heat treatments performed in the closed system described above caused color to appear (Figure 2.8).

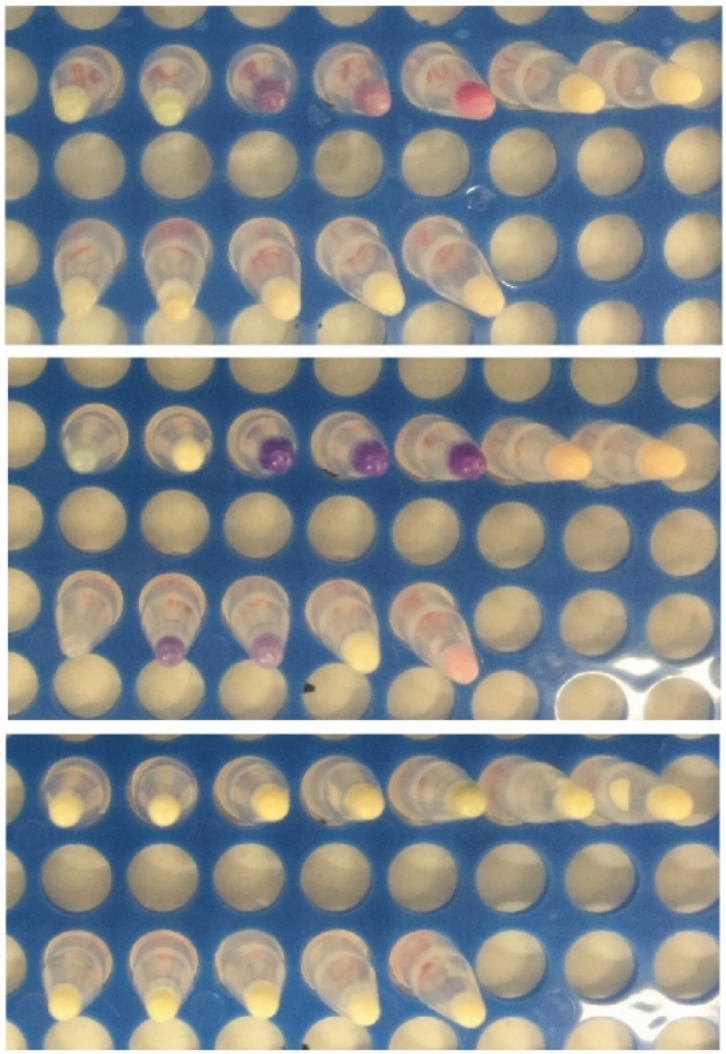


Figure 2.8: As can be seen in the top picture, the bottom row of five PCR tubes containing 30 µL of the cell pellet do not show any color. The chromoproteins in the bottom row are from DNA2.0: VirginiaViolet (left), MaccabeePurple, SeraphinaPink, TinselPurple, and DonnerMagenta. After these previously colorless cell pellets have been treated at 55 °C for 30 min, 60 °C for 30 min, and 60 °C for 30 min, color can be observed in the middle picture. This color then proceeds to disappear again as the temperature continues to increase and the color does not return after subsequent heat treatments as shown in the bottom picture.

As can be seen above in Figure 2.6, some of the chromoproteins regained color when water was added, but this color is different from the starting color. This could be due to the fact that the heating caused the chromoprotein structure to destabilize to a lower energy structure which causes it to have a different color when the water is added to support the barrel.

These results show that while color loss does occur when water is removed from the system, it does not have the same effect as heating the sample. In the heat testing experiments, PrancerPurple changed irreversibly to pink when heated whereas in the lyophilization experiment, it regains its color fully. This supports our hypothesis that the color changing in response to heat is dependent on both water loss and a structural change of the protein itself due to heat.

Similar experiments were done with the AE Blue-CBD fusion protein. With this protein, we saw that the blue color changed from blue to purple. This purple color immediately went away when the sample was removed from the chamber, suggesting that our hypothesis that the AE Blue chromoprotein's color changing capabilities are dependent on the presence of water inside the barrel. To further confirm this, we tested how the AE Blue-CBD fusion protein would respond to heat in a closed PCR tube. Since the PCR tube prevents water from being released from the system, the water molecules that support the barrel of the chromoprotein cannot be driven out when the tube is heated. Because of this, we no longer see the color change from purple to blue. Instead the chromoprotein turns a blue-green color, likely indicative of the protein simply denaturing in response to heat (Figure 2.9).

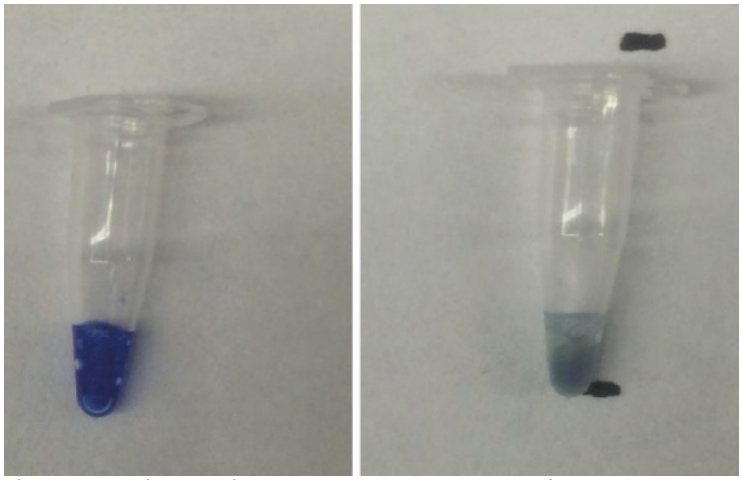


Figure 2.9: Left shows AE Blue-CBD protein before heat and left shows after the PCR tube was heated to 80 °C for 5 mins.

Results/interpretation

The basic structure of a chromogenic protein consists of a large beta barrel and a chromophore which is supported by the beta barrel. The chromophore interacts with light and gives the protein its distinctive color. Additionally, the shape of the beta barrel is supported by water molecules that interact with the side chains of the amino acids that the beta barrel comprises of. These water molecules are essential for the chromogenic protein to maintain its structure. In response to heat, our hypothesis is that these water molecules are released from the beta barrel, thus allowing the barrel to collapse and the chromophore to no longer be supported enough to produce color. [31]

The extracted 12 DNA2.0 chromoproteins with FLAG lumino his-tags were concentrated using microfiltration tubes then allowed to dry on cellulose sheets which had wax wells printed on them. These cellulose sheets with the dried chromoprotein were then placed in a preheated oven and the temperature was increased at five minute intervals until a temperature change was observed (Figure 2.10). This test shows a reversible color change for all 12 chromoproteins at lower temperatures than seen in cell lysate tests and also shows this heat testing can be done multiple times with consistent results. Because of the range of temperatures the chromoproteins lose color at there is the application for a paper base thermometer.

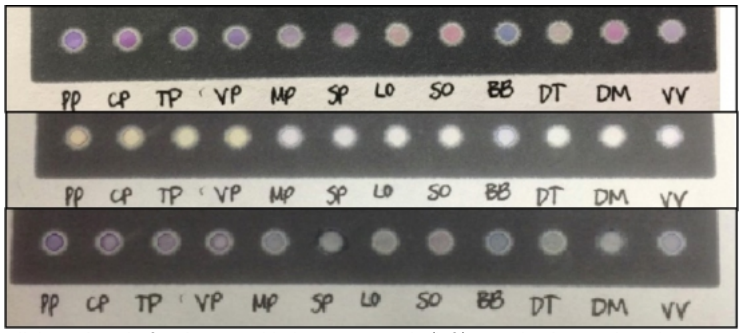


Figure 2.10: (top) 12 chromoproteins on the cellulose sheet before heat is added. PrancerPurple (left), CupidPink, TinselPurple, VixenPurple, MaccabeePurple, SeraphinaPink, LeorOrange, ScroogeOrange, BlitzenBlue, DreidelTeal, DonnerMagenta, VirginiaViolet; (middle) 12 chromoproteins after being heated to 60 °C. All lost color; (bottom) 12 chromoproteins after being rehydrated. Color regained.

Prototyping a biological thermometer

In the interest of moving towards a biologically based paper thermometer, a Gibson Assembly was performed to add a cellulose binding domain to the end of the chromoproteins. This was first done successfully with AE Blue from the iGEM registry. Once this construct was successfully transformed and expressed in bacteria, the protein was extracted and dialyzed. The concentrated protein was then pipetted onto cellulose sheets and underwent a series of heat tests, exhibiting reversible color properties.

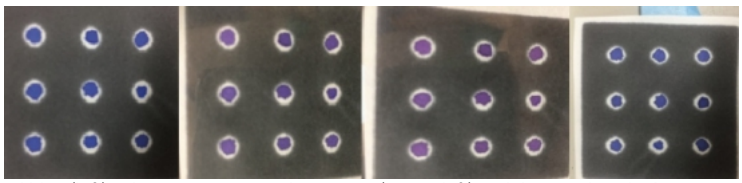


Figure 2.11: While this protein initially appeared blue (left), it begins to turn purple at 55 °C (center left) and this purple color becomes more pronounced as temperature is increased (center right). Interestingly, when allowed to cool back to room temperature, the blue color returned (right).

A N.A.S.A. sticker was then created using the AE blue fusion protein to coat a cellulose sheet as the blue background of the N.A.S.A. logo and added the lettering and red swoosh with stickers. This sticker was then fixed to a glass container using double sided tape (Figure 2.12, left). Hot water was added to the glass container and after 1 minute of exposure to heat, the AE blue-CBD fusion protein turned purple (Figure 2.12, center). Cold water was then added to the glass container and the AE blue-CBD fusion protein returned to its original blue color (Figure 2.12, right). This testing shows a reversible, color changing, temperature sensitive fusion protein and is the prototype of a biologically based paper thermometer.

Figure 2.12: (left) Before heat was added to our N.A.S.A. sticker made from AE Blue-CBD fusion protein; (center) After heat was added to our N.A.S.A. sticker; (right) After cold water was added to our N.A.S.A. sticker.

A cellulose binding domain was also added to the DNA2.0 chromoproteins using Gibson assembly. The sequence for these proteins were transformed into bacteria, expressed, extracted, and fixed to cellulose sheets. Figure 2.13 shows the 2 cellulose binding domain chromoproteins (PrancerPurple and LeorOrange) on cellulose sheets. After being allowed to set for three days, the binding capabilities of the cellulose binding domain was tested by washing the cellulose sheet the chromoproteins were bound to with a steady stream of water for 1 minute. After this washing, no color change was detected, indicating that the cellulose binding domain was effective at fixing the chromoproteins to the sheet (Figure 2.13).

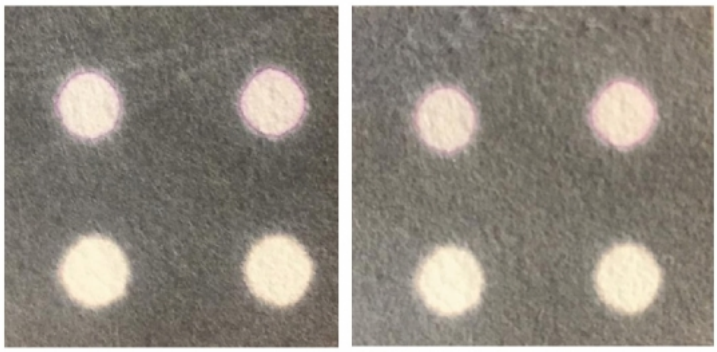


Figure 2.13: (left) Before cellulose binding domain testing. PrancerPurple-cellulose binding domain (top) and LeorOrange-cellulose binding domain (bottom); (right) After cellulose binding domain testing. PrancerPurple-cellulose binding domain (top) and LeorOrange-cellulose binding domain (bottom).

Conclusion

Our chromoprotein research not only characterized the way in which chromogenic proteins lose or change colors in response to heat, but also was expansive enough to identify a large enough span of chromogenic proteins that differentially respond to temperature. Using this differential response, a biological thermometer was constructed that was made up of six different chromogenic proteins. Each of the proteins in this set loses or changes color at a specific and distinct temperature. Lastly, the addition of a cellulose binding domain to these chromogenic proteins to allow them to bind to cellulose, allowing for the creation of a biologically-based paper thermometer. Furthermore, heat experiments that caused the emergence of color in cell cultures that were not expressing the expected color suggest that the culturing conditions for the expression of chromogenic proteins can be sensitive. Thus, if chromogenic proteins are being used as a marker on a target protein in a specific assay and the expected color is not being seen, this could be due to the chromogenic proteins themselves, not the lack of the target protein. With the knowledge presented above, false negatives might be avoided for these types of assays using chromogenic proteins as markers.

With regards to our balloon's membrane, through metabolic engineering we produced an organism capable of producing polyisoprenes in large yield. By optimizing the endogenous MEP/DOXP pathway in *E. coli* through overexpression of rate-limiting proteins and coupling it with expression of rubber transferases (prenyltransferases) used by *H. brasiliensis* for polyisoprene production, we obtained a rubber-like substance that exhibits the basic properties of latex.

Because our initial ignition and mechanical property tests are not indicative whether our product is chemically identical to cis 1,4 polyisoprene, additional chemical tests need to be performed. An H^1NMR and C^{13}NMR assay of our extract would allow for better characterization of the chemical properties of our extract. Comparison of H^1 NMR and C^{13} NMR peak placement with pure polyisoprene will be indicative of the similarities and differences in chemical composition of our product with actual synthetic rubber. Additionally, because our manipulation of the MEP/DOXP pathway and HRT1, HRT2 enzyme activity is not entirely optimized, future studies will include further optimization of the MEP/DOXP pathway and directed evolution of HRT1, HRT2 protein constructs to allow for faster production times. Furthermore, our yield is currently 1-2% by volume; this return could be further enhanced by adding an export tag to polyisoprene compounds that could be later cleaved. This would not only allow for more polyisoprene compounds to be dissolved in solution and easier to purify, but also prevent the host cell from using polyisoprenes as an energy source.

Figures/supplemental information

High resolution JPEG files for each figure referenced in the paper can be downloaded here: zipped figures (/files/posts/4625551815599982529/56ec91a5edfa27d326ec438a00488a36_Figures-20170123T214227Z.zip). Supplemental information on protocols and methodology can be found here: (http://2016.igem.org/Team:Stanford-Brown)http://2016.igem.org/Team:Stanford-Brown (http://2016.igem.org/Team:Stanford-Brown).

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Competing Interests

The authors have declared that no competing interests exist.

Ethics Statement

N/A

Data Availability

In alignment with the PLOS data sharing policy, all data, protocols, figures, field testing, videos, and documentation are fully available without restriction: (http://2016.igem.org/Team:Stanford-Brown)http://2016.igem.org/Team:Stanford-Brown (http://2016.igem.org/Team:Stanford-Brown).

Author Contributions

All authors listed meet the four conditions listed by the ICJME. Conceived and designed the experiments: GS CHP CG LR. Performed the experiments: CHP TCS TNS ER GS CG MB. Analyzed the data: GS CHP TNS TCS CG MB ER. Wrote the paper: MB TNS GS CHP TCS ER LR. Reviewed the paper: MB TNS ER GS CHP TCS CG LR.

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