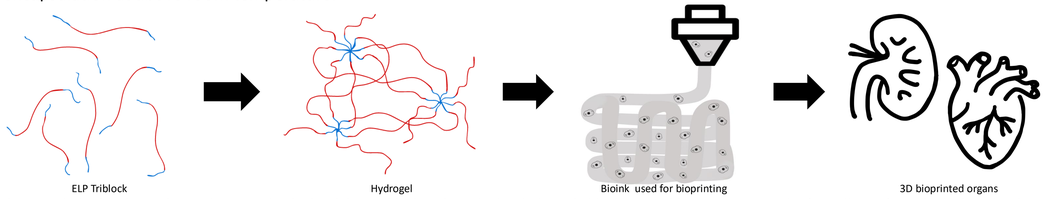
Synthesis and Thermo-Characterization of an ELP Diblock Intended for Use as a Hydrogel Bioink

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Introduction

The development of bioinks is crucial for advancing bioprinting. Despite various bioinks being explored or invented, challenges such as low mechanical strength, rapid gelation, and high viscosity are always persistent. This is why our research addresses the challenges by utilizing recombinant protein technology to engineer elastin-like polypeptides (ELPs) that form thermo-responsive hydrogels. The ideal bioink is a liquid at room temperature but a hydrogel at body temperature, which is why we designed and synthesized an ELP diblock that dimerizes to form a triblock ELP with alternating hydrophobic and hydrophilic segments, aiming for optimal self-assembly and cell encapsulation at above room temperature.



The intended triblock copolymer design with a hydrophilic midblock and two hydrophobic side blocks assembling into a network of micelles to form the hydrogel. When hydrogels are seeded with living cells they are called a bioink and can be printed through a nozzle to make living structures such as tissues or organs.

Protein Design:

Hydrophobic[1]:
(GFGVP
GIGVP
GIGVP
GIGVP
$GIGVP)_3$

SuperFolder Green Fluorescent Protein[3,4]:
- Visualization
- Optimal Expression

sfGFP

Hydrophilic[2]:
(GVGVP
GEGVP
$GVGVP)_5$

Cys/Ser

Serine
sfGFP
sfGFP
sfGFP
Serine

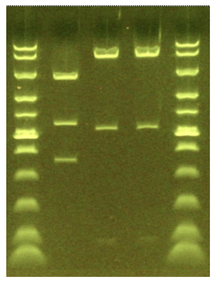
sfGFP

The design of the proteins produced in this project based on previous work of Adil Mistry [1]. Diblock assembled from previously prepared genes [2] with the cysteine will dimerize at the transition temperature forming the triblock which will form the micelles network, or the hydrogel, while diblocks with the serine will only form micelles.

Results

Plasmid Digestion

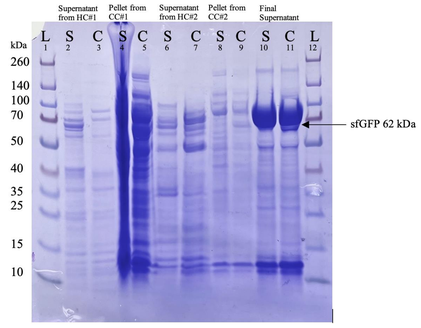
- After digestion, running a DNA gel electrophoresis helps view the number and size of the DNA bands as it separates the DNA fragments based on their length.
- The expected number of bands for each digest is **3 bands**, which can be observed on the gel in lanes 2, 3, and 4.
- The digestion appears to have **worked** to **completion**.



DNA gel electrophoresis after digestion. The two outer lanes contain the ladder.

Protein Purity

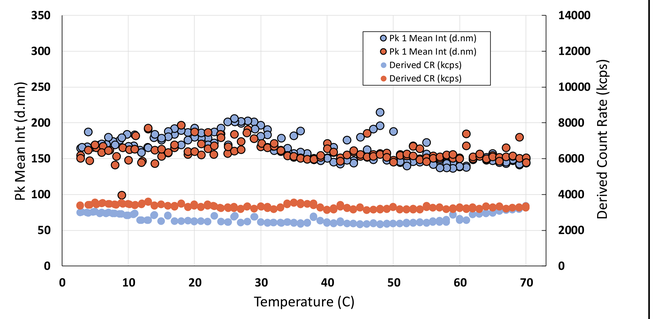
- The expected **molecular weight** for both proteins is approximately **62 kDa**, which can be observed on the gel in lanes 10-11 where a prominent band can be seen between markers 50-70 kDa.
- The **wastes** from the cycles were run to assess how much was **left behind** and by looking in lanes 6-9 we can visualize that insignificant amounts of our protein were left behind.



SDS-Page of the waste products during ITC and the final protein samples. "S" and "C" is for samples containing serine and cysteine, respectively. Lanes labeled "L" contain the ladder.

Particle Size and Transition Temperature

- **Characterization** of the protein containing the serine was done using a Malvern Zetasizer Nano-ZS with a 173 backscattering angle, through a temperature sweep from 3°C to 70°C and back, with a supply of desiccated air.
- The data **does not** show any signs of aggregation or phase separation between 3-70°C. We can assume that the transition temperature for the hydrophobic part is **lower** than 3°C and the micelles have already formed, and they remain stable in high temperatures. A **new hydrophobic block** needs to be designed.
- The stability in the particle size and count rate while warming up and cooling down might mean that the protein is **reversible**.
- The temperature is still not high enough to see the **hydrophilic block** coming out of the solution and aggregating which indicates its design is **successful**.

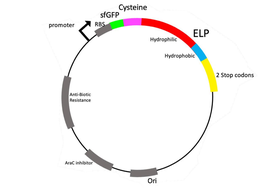


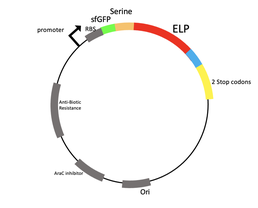
Measurements between 3° and 70°C at a 2.3 µM sfGFP-Serine. Red represents heating up to 70°C, and blue represents cooling down to 3°C.

Methods

Gene design

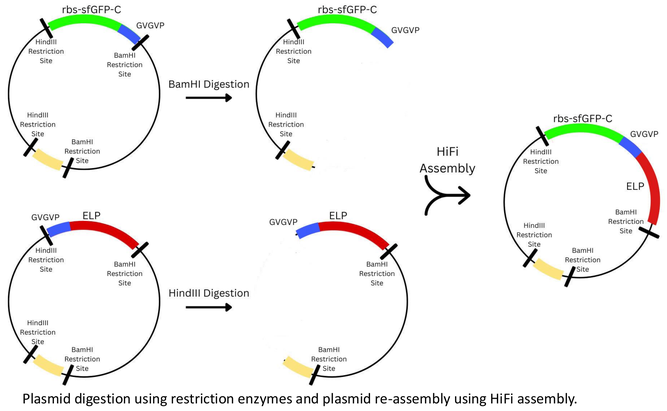
- sfGFP: for visualization and optimal expression
- Serine or Cysteine
- Hydrophilic block $(VEV)_5$
- Hydrophobic block $(FIIII)_3$
- Two stop codons
- AraC operon: for a tightly regulated expression
- Kanamycin anti-biotic resistance





Full map for both plasmids to be assembled.

Plasmid Digestion and Assembly



Plasmid digestion using restriction enzymes and plasmid re-assembly using HiFi assembly.

Digestion:
- **Restriction enzymes** are used to **cut** the circular DNA plasmids into linear DNA fragments.
- The restriction enzymes used in this process are BamHI and HindIII, as these can cut the DNA in a place where the remaining overhangs can be ligated back together in the HiFi assembly.

HiFi Assembly:
- HiFi assembly **connects** the overlapping DNA sequences. This allows digested plasmids to be reassembled into whole plasmids again.

Transformation and culturing

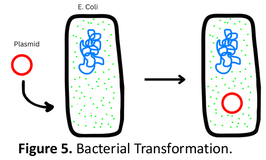
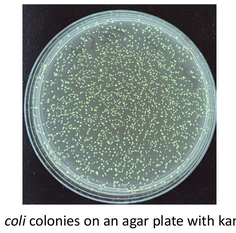


Figure 5. Bacterial Transformation.

Transformation:
- Each of the plasmids were transformed into *E. coli* (C3040) to **separate** the plasmids from each other since *E. coli* can only take up 1 plasmid at a time.

Plating:
- Because the plasmids contain a Kanamycin resistance, only cells with the correct plasmid will grow on the Kanamycin plate used.

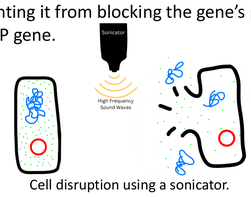
Culturing:
- A **single** colony can be picked off the plate for culturing and protein expression.



Many *E. coli* colonies on an agar plate with kanamycin.

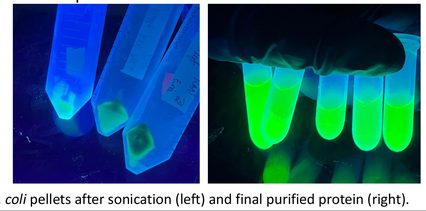
Expression and purification

Expression:
- Cultures of *E. coli* are **grown** and then induced to produce large quantities of ELP.
- Proteins will not be produced until the cells are **induced** to express using **arabinose** which binds to the AraC repressor protein, preventing it from blocking the gene's promoter to activate the ELP gene.



Cell disruption using a sonicator.

Purification:
- **Sonication** is used to burst the cellular membrane and release the ELPs.
- **Inverse temperature cycling** (ITC) is used to separate our ELPs from the other components by taking advantage of the transition temperature.



E. coli pellets after sonication (left) and final purified protein (right).

Future Work

- Future research will optimize the protein expression and purification processes to increase yield.
- Explore amino acid substitutions to refine the ELP's hydrophobic block further to achieve the desired transition temperature.
- In the long run, the performance of the hydrogel in 3D bioprinting of complex tissue structures will be tested, along with its rheological properties and long-term biocompatibility and stability.

References

1. Adil Mistry, Master's Thesis (2022).
2. Amin Boukzam, unpublished data.
3. sfGFP was obtained from Dr. Edward Turk
4. Pedelacq, J. D., & Cabantous, S. (2019). *Development and Applications of Superfolder and Split Fluorescent Protein Detection Systems in Biology*. International journal of molecular sciences, 20(14), 3479.

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